COMPARATIVE ANALYSIS OF TWO ATTACHMENT VARIANTS OF BUTYRIVIBRIO FIBRISOLVENS

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CHAPTER 5

MORPHOLOGICAL EXAMINATION

5.1 Introduction

Together with the surface structure of the substratum (receptor), bacterial extracellular structures (ligands) are often involved in attachment to surfaces. Researcher studying on bacterial attachment in various environments have shown that the possible bacterial ligands involved in attachment processes are flagella, fimbriae, pili, extracellular polymers or attachment proteins (Fletcher 1980a, Ofek and Beachey 1980, Haahtela 1985, Macnab 1987, Vesper 1987, Quintero and Weiner 1995, Allison and Sutherland 1987, Miron *et al.* 2001). Nili (1995) used SEM to show that the S and L variants of *Butyrivibrio fibrisolvens* E14 used in this project do not have fimbriae or flagella. Therefore, extracellular polymer (EP) and attachment proteins are likely ligands that may be involved in attachment of the S variant to surfaces.

Since the cytoplasmic (Nili 1995) and total extracellular (this work) protein profiles showed no significant difference between S and L, it is likely that EP is the major determinant of attachment. Scanning electron microscopy (SEM) studies of various *B. fibrisolvens* strains showed extension of EP from the cell

surface (Cheng and Costerton 1977, Dibbayawan *et al.* 1985, Cheng *et al.* 1988, Hespell *et al.* 1993), and this has been suggested to mediate cell-cell attachment (Cheng *et al. 1988).* In the present chapter, the morphology of the S and L variants of *B. fibrisolvens* was examined using SEM.

The growth characteristic of the S variant seemed to be age-dependent, and was clumpy during early growth but dispersed throughout the liquid medium after longer incubation, similar to that of *B. fibrisolvens* OB156 (Beard et al 1995). Stages in the development of EP structure were therefore studied by examining the morphology of the two variants at various stages of growth.

In addition, the effect of carbon sources on S and L morphology were also studied. Therefore, the SEM preparations were carried out using S and L cultures grown on defined medium containing various carbon sources. Plate grown cells were used instead of liquid grown cultures, to enhance possible bacterium-surface interactions.

5.2 Results

5.2.1 Morphological examinations

The morphology of S and L variants at various stages of growth was examined using SEM. Both variants were grown on plates in defined medium containing maltose or starch. Cells were harvested at approximately early log, log, stationary or late stationary phases.

The results are shown in Figures 5.1 to 5.8. The numbering (1, 2, 3a and 3b) indicates possible stages in EP biosynthesis or steps in attachment. At early log, S cells seemed to initiate EP production (Figure 5.1) to mediate either cell-cell or cell-surface interactions (step 1). The EP then spread to neighbouring cells or surfaces (Figure 5.2) at late log phase (step 2). At early stationary phase (Figure 5.3 and 5.4) the EP had developed into a more complex structure. Figure 5.3 shows cell-cell interactions (step 3a) and Figure 5.4 shows cell-surface interactions (step 3b), from early stationary phase S cells.

Although EP production was less than S cells, the L cells also initiated EP synthesis (Figure 5.5) in early log phase. However, in log phase (Figure 5.6), the EP was not spread to other cells or surfaces (step 2), and immediately clumped, forming globular EP, as was seen for S cells at step 3a. At early stationary phase, cell-cell interactions (step 3a) were also observed (Figure 5.7), but the more complex structures (step 3b) seen with S cells were not observed (Figure 5.8).

At late stationary phase (Figure 5.9 and 5.10), most S and L cells were lysed. Almost no EP was attached to the cells (Figure 5.9), although a little was still seen associated with S cells (Figure 5.10).

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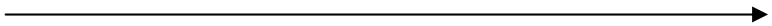

Figure 5.1 SEM of *B. fibrisolvens* E14 S at early log phase.

Showing the initial stage (1) of EP biosynthesis. Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early log phase and were prepared for SEM, number (1) indicates a possible step of attachment or EP biosynthesis (initiation). The magnification was up to 30,000 x.

Figure 5.2 SEM of *B. fibrisolvens* E14 S at log phase.

Showing polymer spreading (stage 2) to surface or neighbouring cells. Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM, number (2) indicates a possible step of attachment or EP biosynthesis (polymer spreading). The magnification was up to 30,000 x.

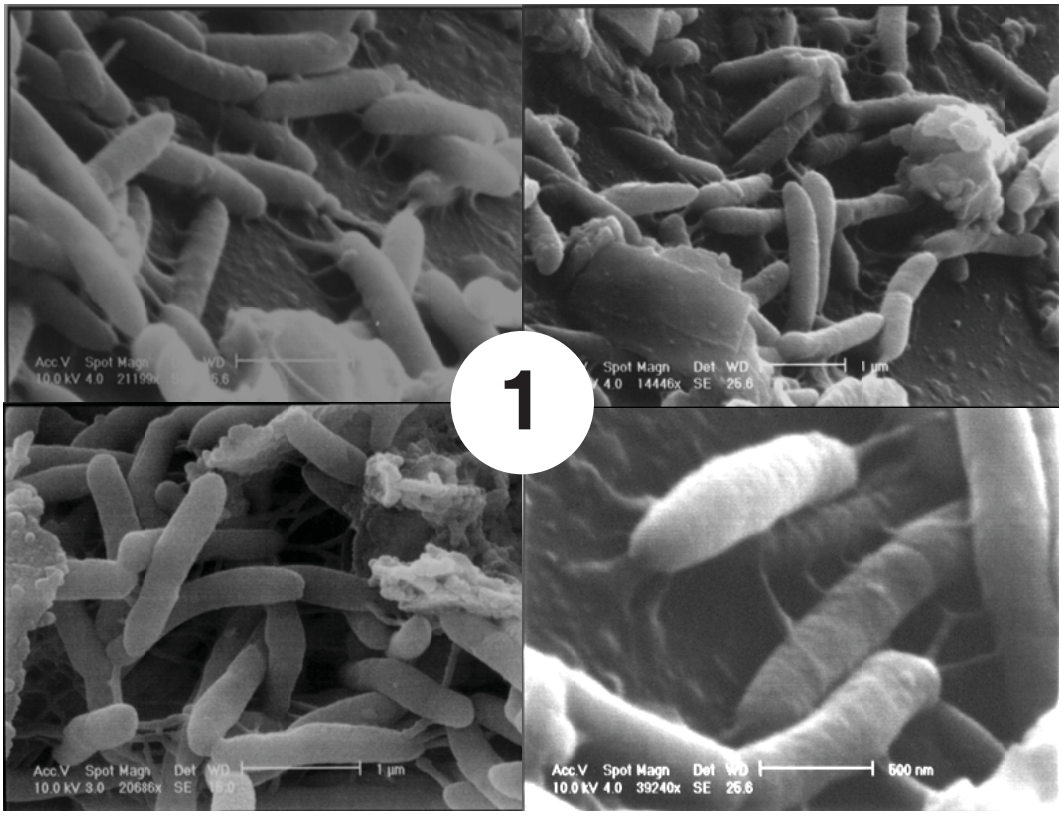

Figure 5.1 SEM of *B. fibrisolvens* E14 S at early log phase.

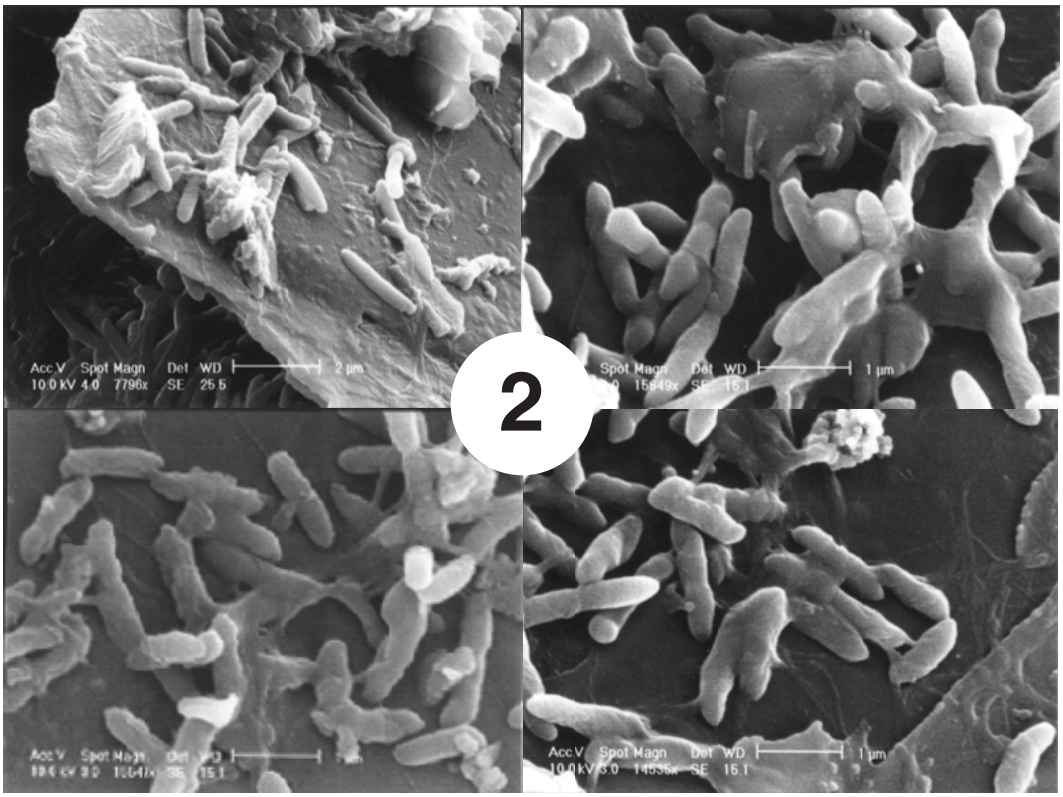

Figure 5.2 SEM of *B. fibrisolvens* E14 S at log phase.

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Figure 5.3 SEM of *B. fibrisolvens* E14 S at early stationary phase.

Showing cell-cell association (stage 3a). Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early stationary phase and were prepared for SEM, number (3a) indicates a possible step of attachment or EP biosynthesis (cell-cell interaction). The magnification was up to 30,000 x.

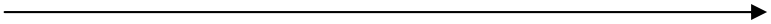

Figure 5.4 SEM of *B. fibrisolvens* E14 S at early stationary phase.

Showing cell-surface association (3b). Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early stationary phase and were prepared for SEM, number (3b) indicates a possible step of attachment or EP biosynthesis (cell-surface interaction). The magnification was up to 30,000 x.

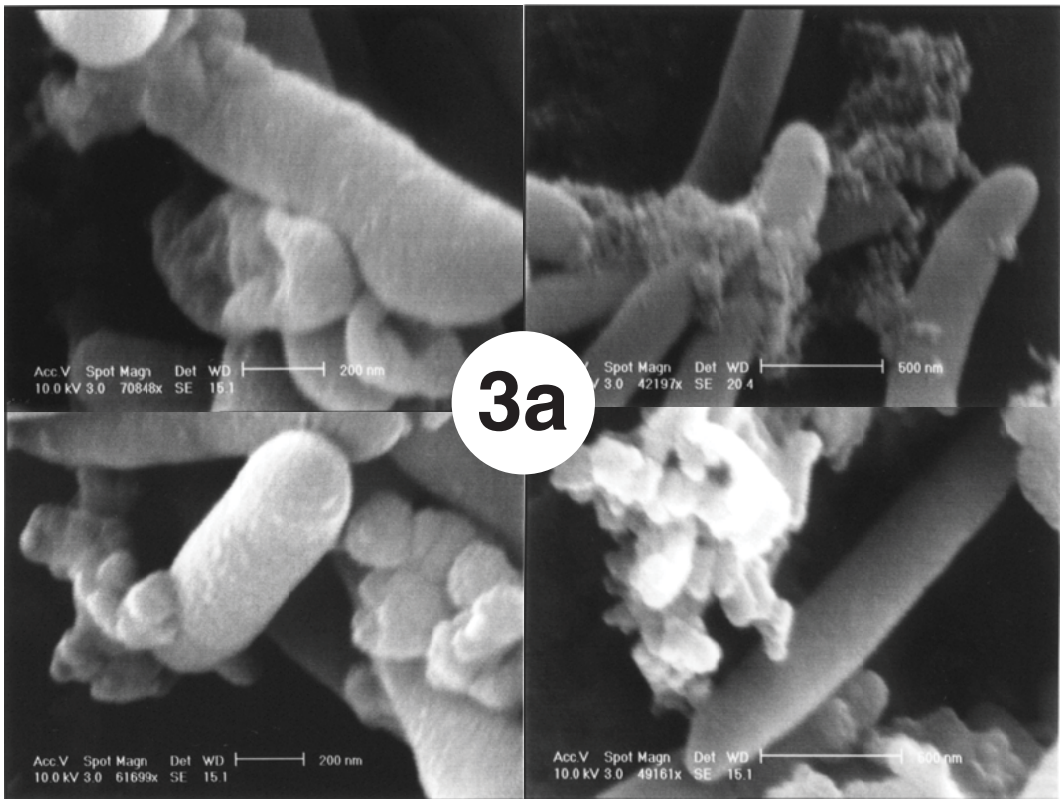

Figure 5.3 SEM of *B. fibrisolvens* E14 S at early stationary phase.

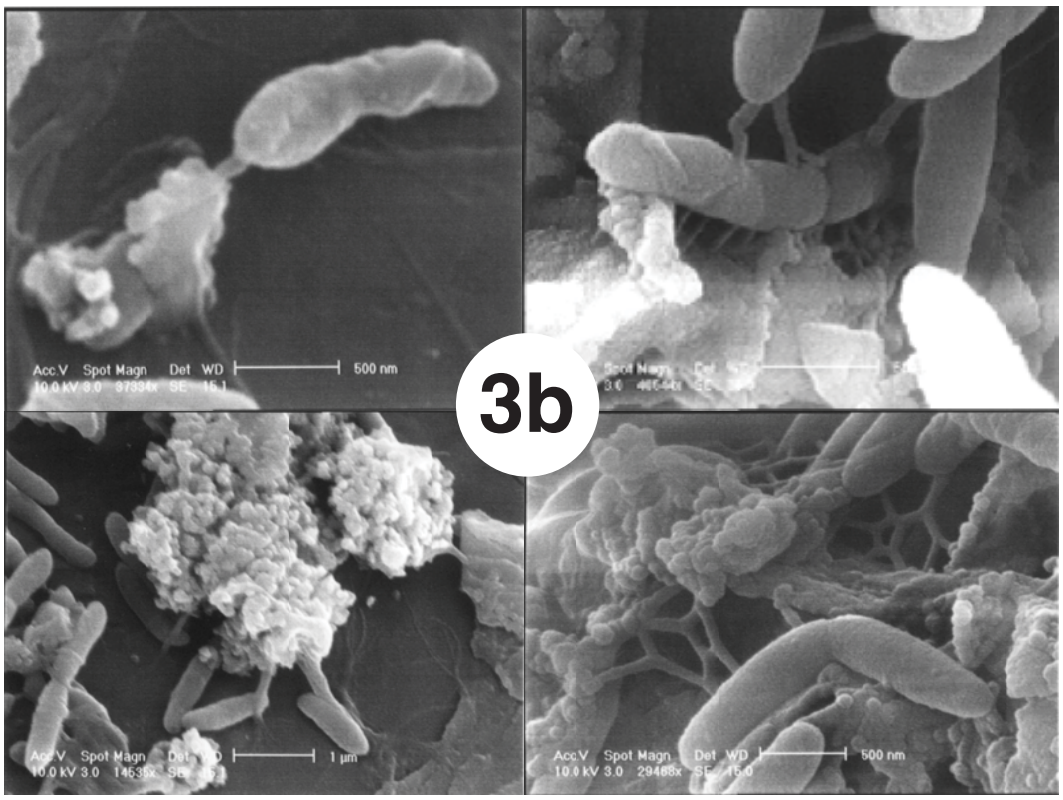

Figure 5.4 SEM of *B. fibrisolvens* E14 S at early stationary phase.

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Figure 5.5 SEM of *B. fibrisolvens* E14 L at early log phase.

Showing the initial stage (1) of EP biosynthesis. Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early log phase and were prepared for SEM, number (1) indicates a possible step of attachment or EP biosynthesis (initiation). The magnification was up to 30,000 x.

Figure 5.6 SEM of *B. fibrisolvens* E14 L at log phase.

Showing no polymer spreading (no stage 2). Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM, number (3a) indicates a possible step of attachment or EP biosynthesis (cell-cell interaction). L EP is in form 3a instead of 2 polymer spreading. The magnification was up to 30,000 x.

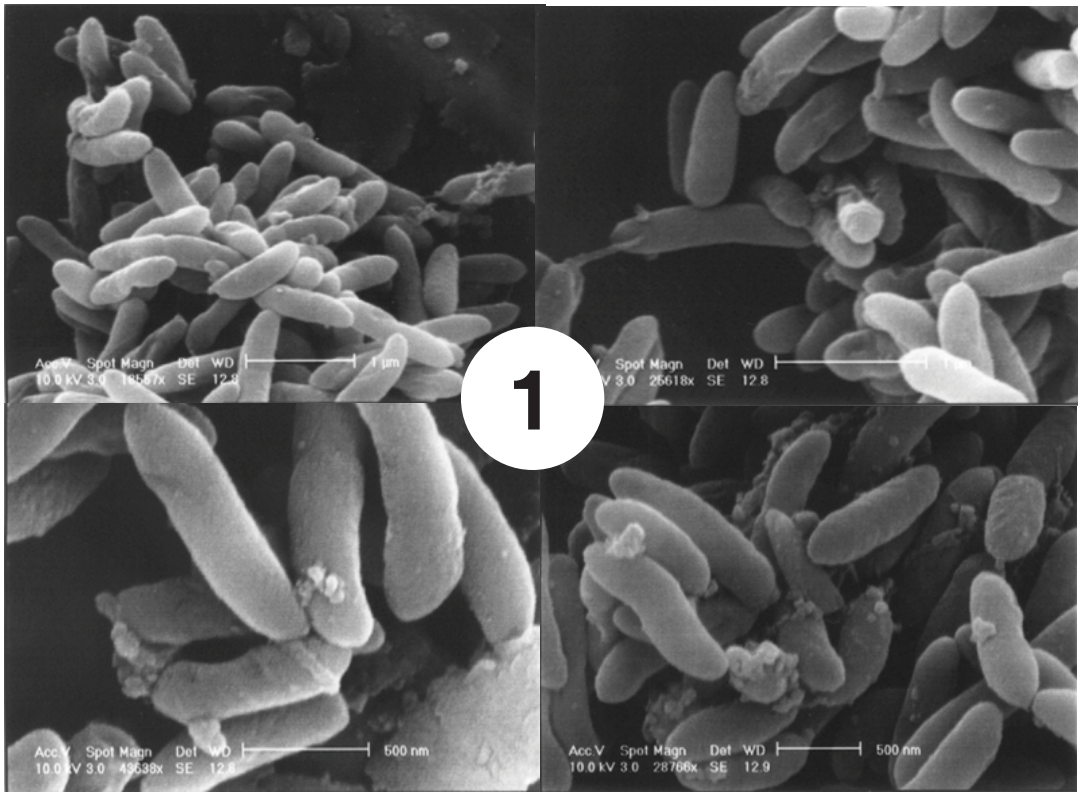

Figure 5.5 SEM of *B. fibrisolvens* E14 L at early log phase.

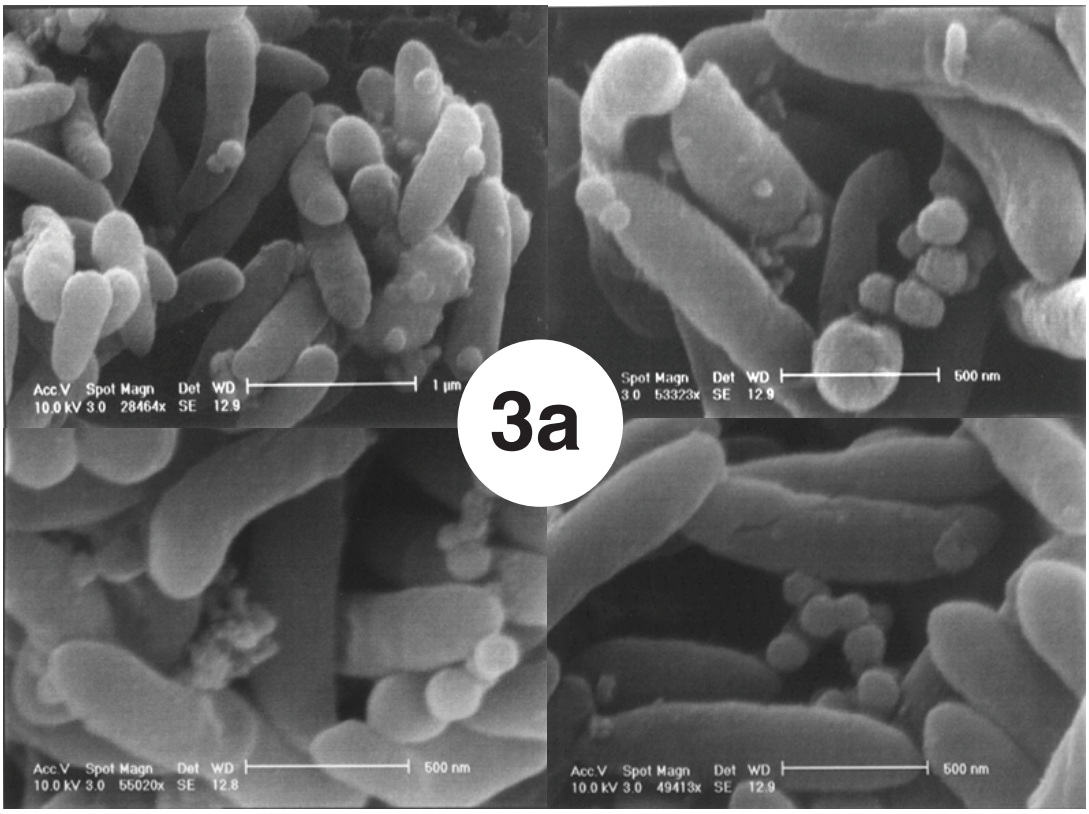

Figure 5.6 SEM of *B. fibrisolvens* E14 L at log phase.

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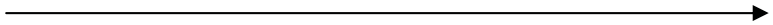

Figure 5.7 SEM of *B. fibrisolvens* E14 L at early stationary phase.

Showing cell-cell association (3a). Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early stationary phase and were prepared for SEM, number (3a) indicates a possible step of attachment or EP biosynthesis (cell-cell interaction). The magnification was up to 30,000 x.

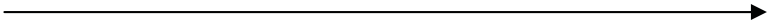

Figure 5.8 SEM of *B. fibrisolvens* E14 L at early stationary phase.

Showing no cell-surface association (no stage 3b). Cells were streaked onto defined medium containing 0.5 % (w/v) maltose or starch and were incubated anaerobically at 39^{o}C. Cells were harvested at early stationary phase and were prepared for SEM, number (3a) indicates a possible step of attachment or EP biosynthesis (cell-cell interaction). L EP is in form 3a instead of 3b (sell surface association). The magnification was up to 30,000 x.

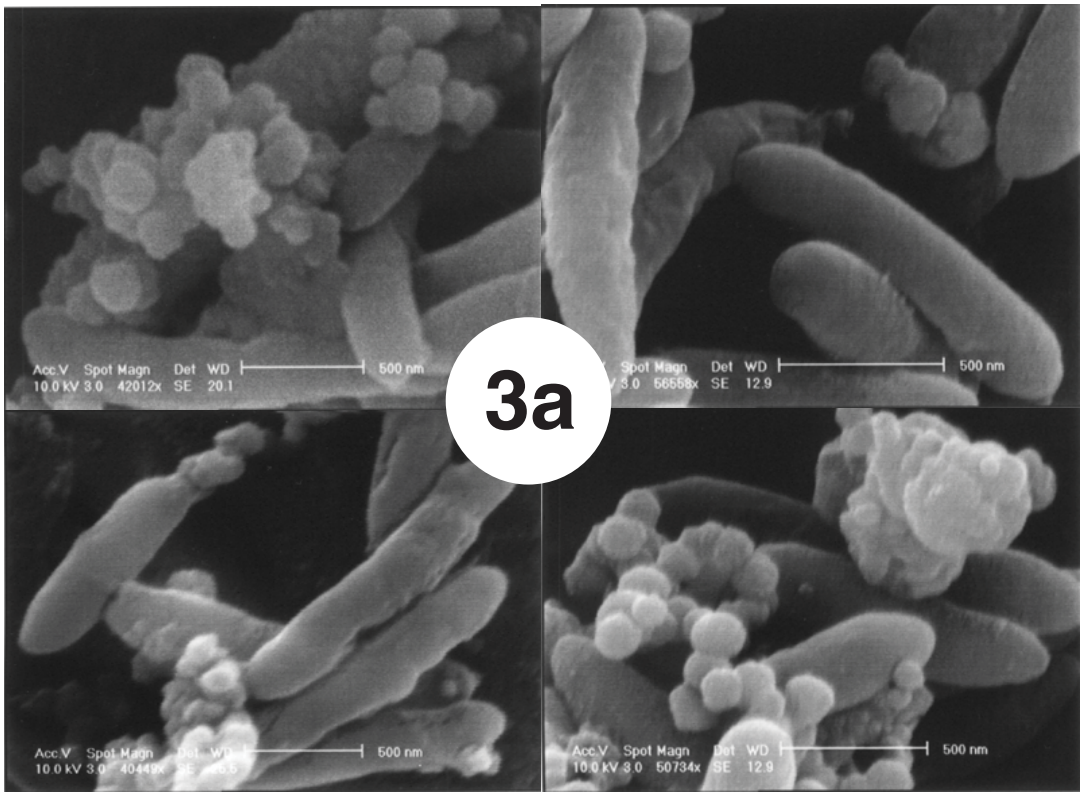

Figure 5.7 SEM of *B. fibrisolvens* E14 L at early stationary phase.

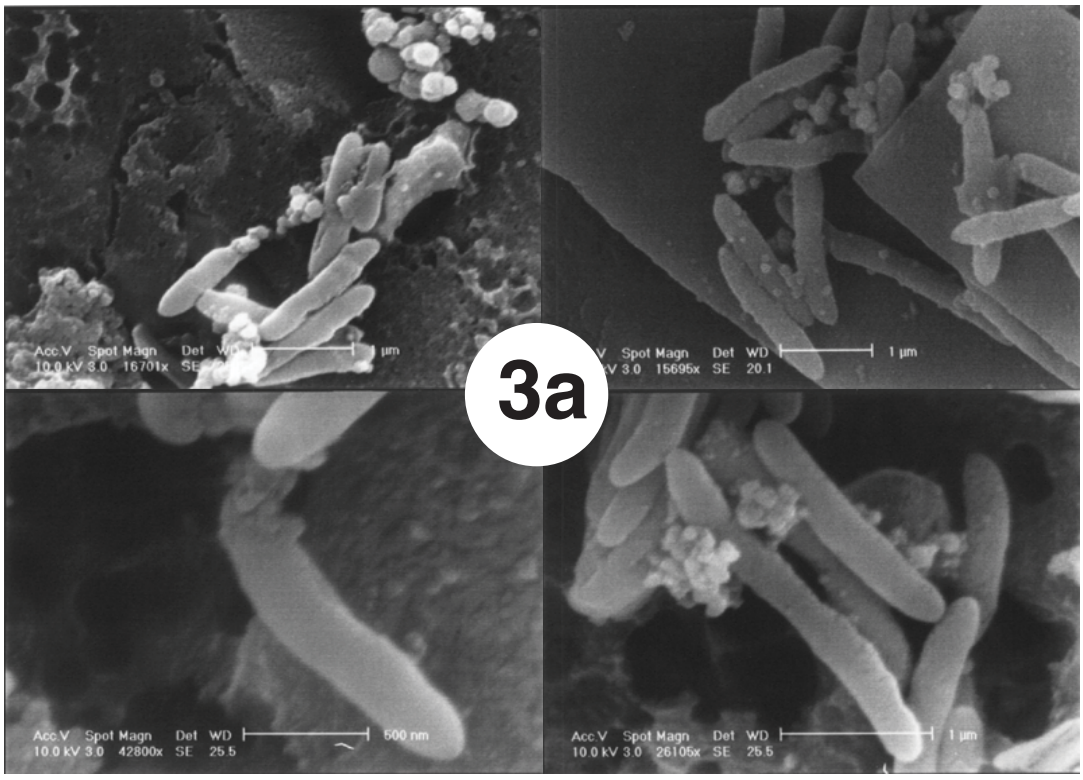

Figure 5.8 SEM of *B. fibrisolvens* E14 L at early stationary phase.

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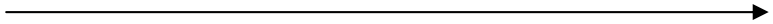


Figure 5.9 SEM of *B. fibrisolvens* E14 S at stationary phase.

Cells were streaked onto defined medium containing 0.5 % (w/v) starch and were incubated anaerobically at 39^{o}C. Cells were harvested at stationary phase and were prepared for SEM. The magnification was up to 30,000 x.

Figure 5.10 SEM of *B. fibrisolvens* E14 L at stationary phase.

Cells were streaked onto defined medium containing 0.5 % (w/v) starch and were incubated anaerobically at 39^{o}C. Cells were harvested at stationary phase and were prepared for SEM. The magnification was up to 30,000 x.

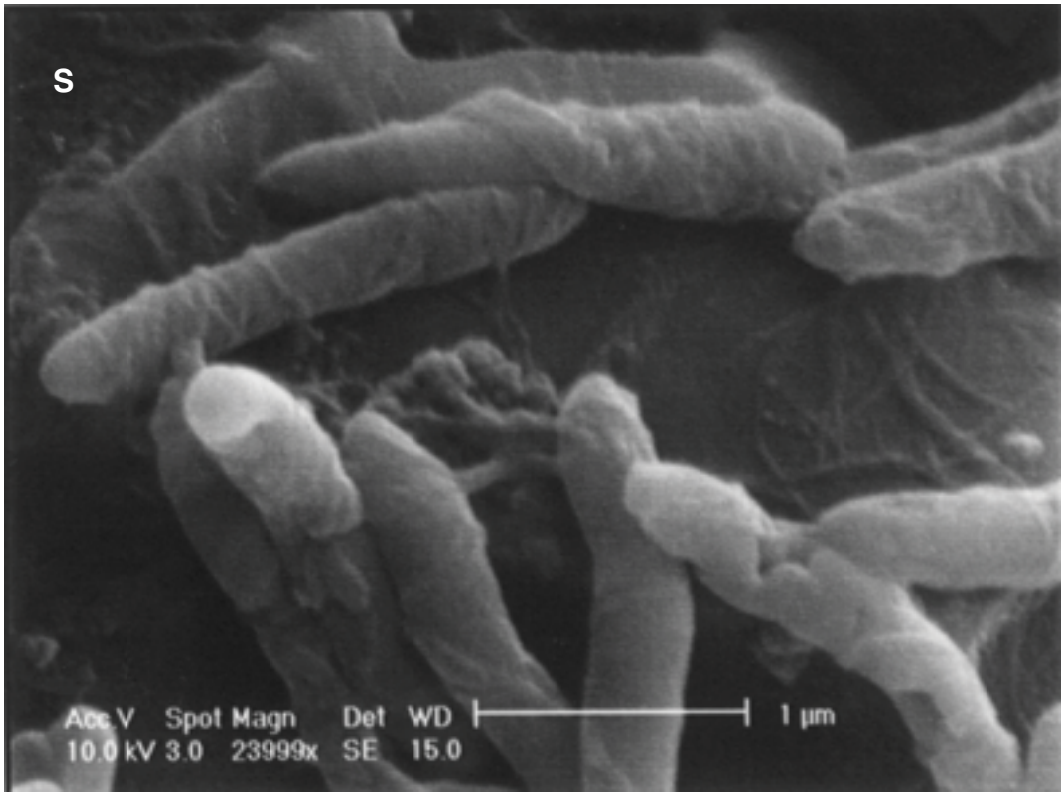


Figure 5.9 SEM of *B. fibrisolvens* E14 S at stationary phase.

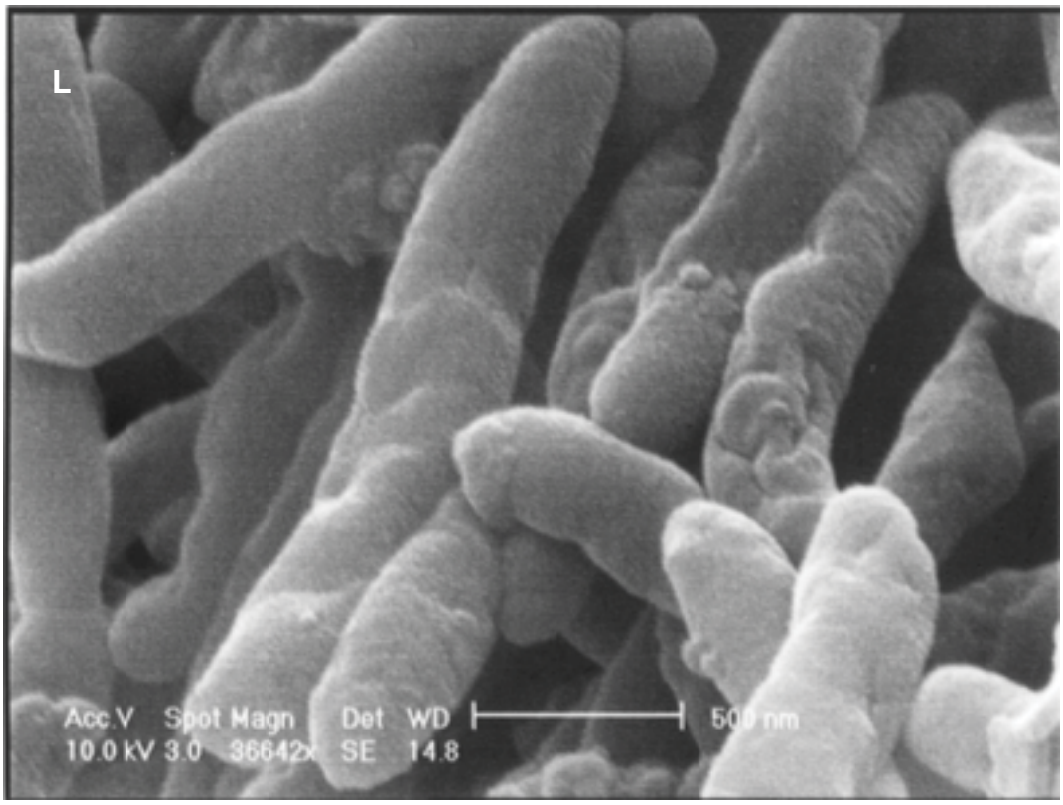


Figure 5.10 SEM of *B. fibrisolvens* E14 L at stationary phase.

5.2.2 Effect of carbon sources on morphology.

The effect of carbon source on the morphology of S and L variants was examined using SEM. Both variants were grown on plates of defined medium containing glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch. BHI grown cells were also included for comparison. Cells were harvested at approximately log phase and prepared for SEM, as described in the methods.

Most of the S variant EP was spread to surfaces, while that of L was mostly condensed, forming globular EP (Figure 5.11 to 5.18). Compared with cellobiose grown cells (Figure 5.12), more polymer spreading was observed when S cells were grown in glucose (Figure 5.11), sucrose (Figure 5.15), maltose (Figure 5.16) and starch (Figure 5.17). Besides inducing less polymer spreading in the S variant, cellobiose seemed to induce more globular EP in both S and L variants, compared to other carbon sources. The L variant mostly showed globular EP on all carbon sources. For complex carbon sources such as xylan (Figure 5.11) or crystalline cellulose (Figure 5.12), it was more difficult to compare, due to the difficulties in differentiating between EP and substrate polymers.

During the SEM studies it was noted that the globular EP seemed loosely attached to the cell and may be released to the medium at a later time (Figure 5.18).

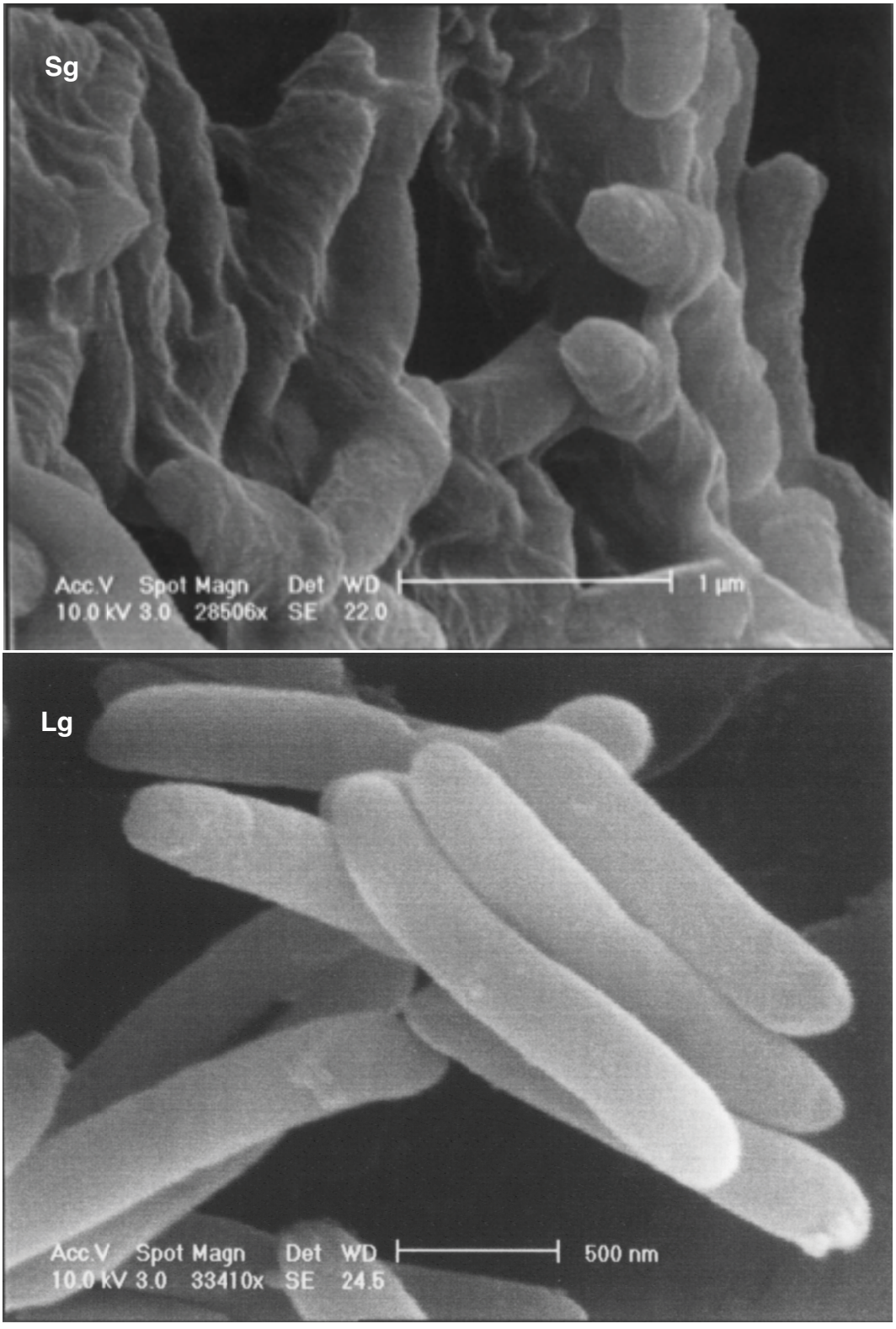


Figure 5.11 SEM of *B. fibrisolvens* E14 S and L grown in glucose.

Sg and Lg were *B. fibrisolvens* E14 variants S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) glucose and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 35,000 x.

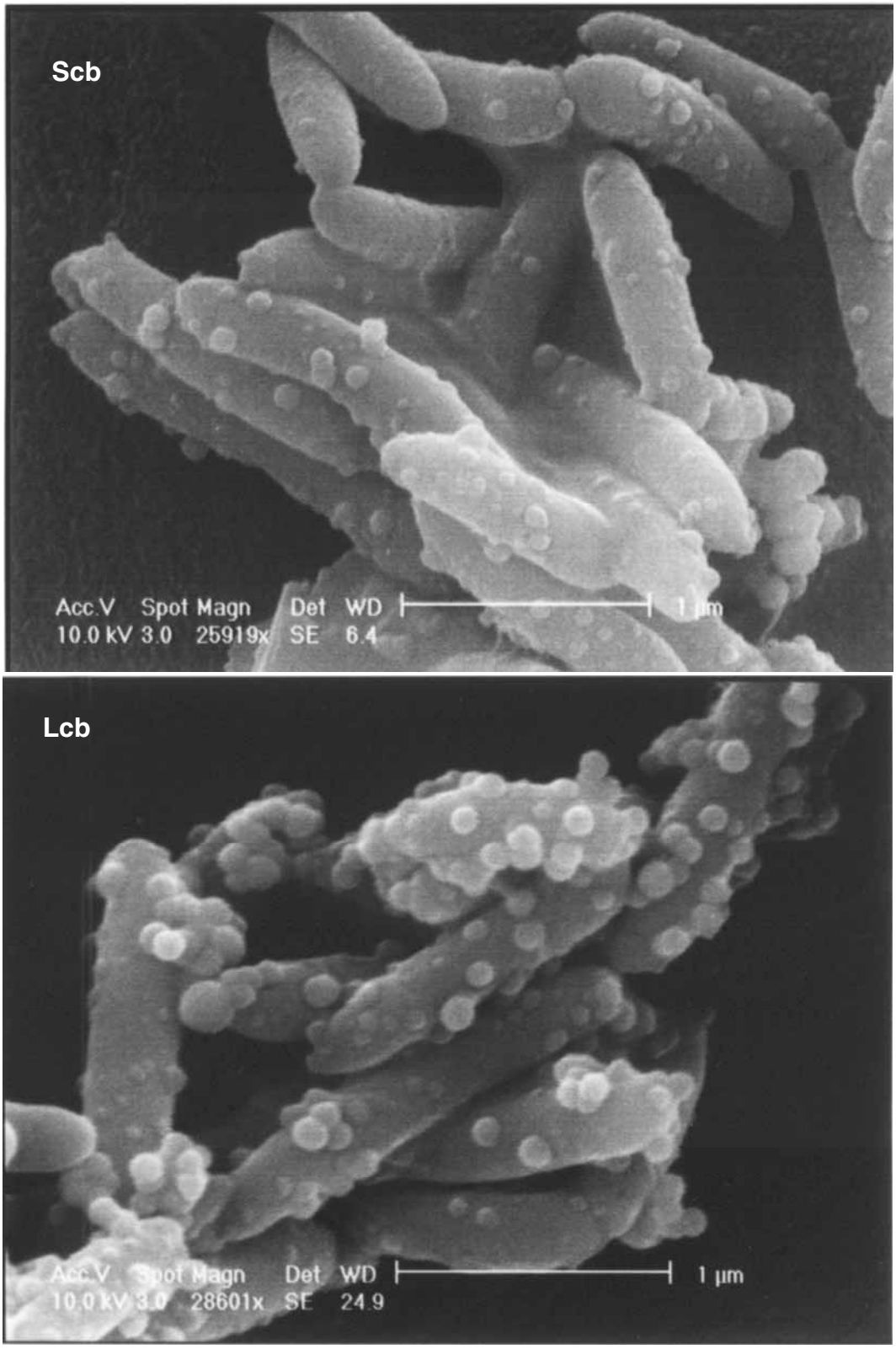


Figure 5.12 SEM of *B. fibrisolvens* E14 S and L grown on cellobiose.

Scb and Lcb are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) cellobiose and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 30,000 x.

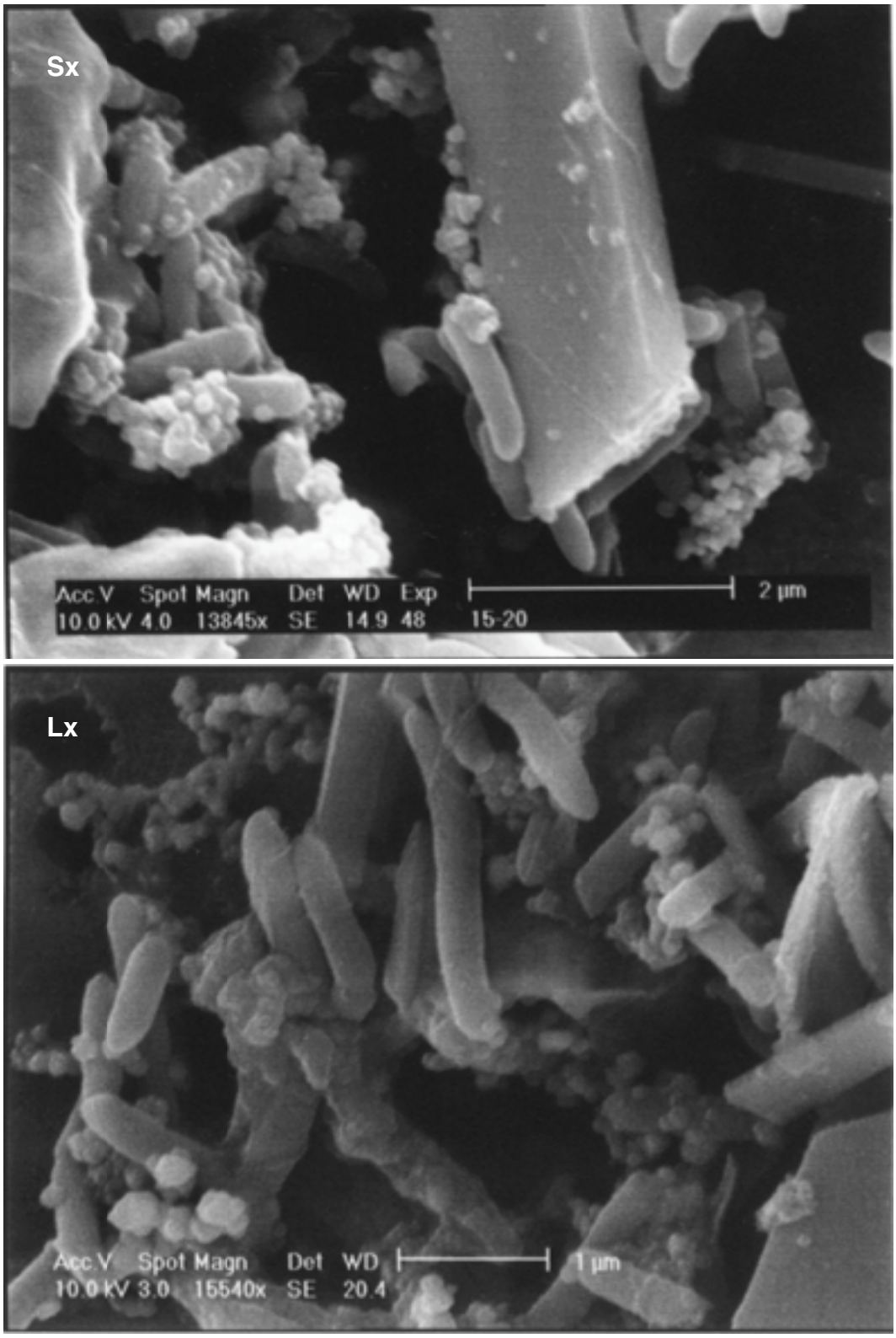


Figure 5.13 SEM of *B. fibrisolvens* E14 S and L grown on xylan.

Sx and Lx are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) xylan and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 16,000 x.

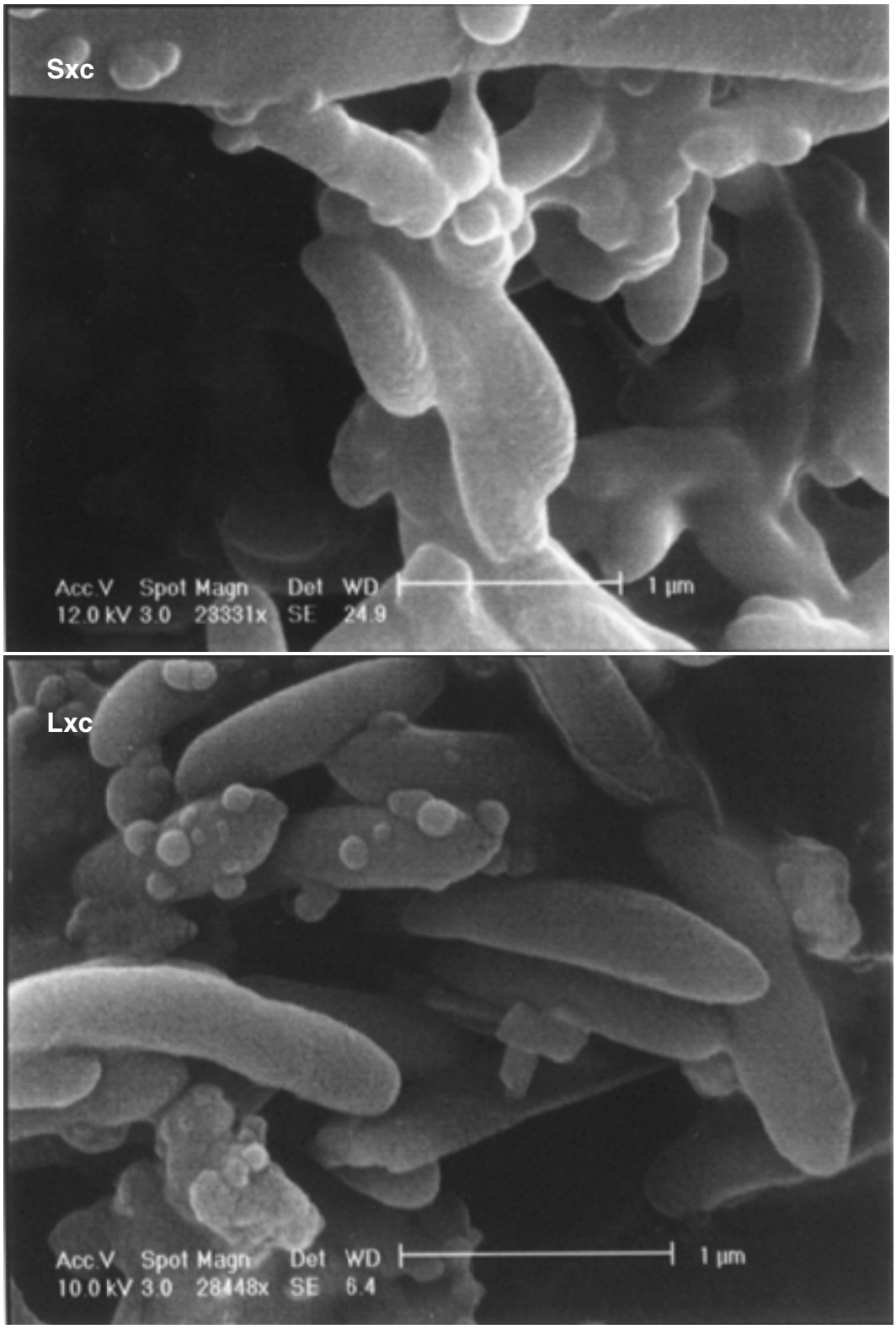


Figure 5.14 SEM of *B. fibrisolvens* E14 S and L grown on cellulose.

Sxc and Lxc are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) cellulose and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 30,000 x.

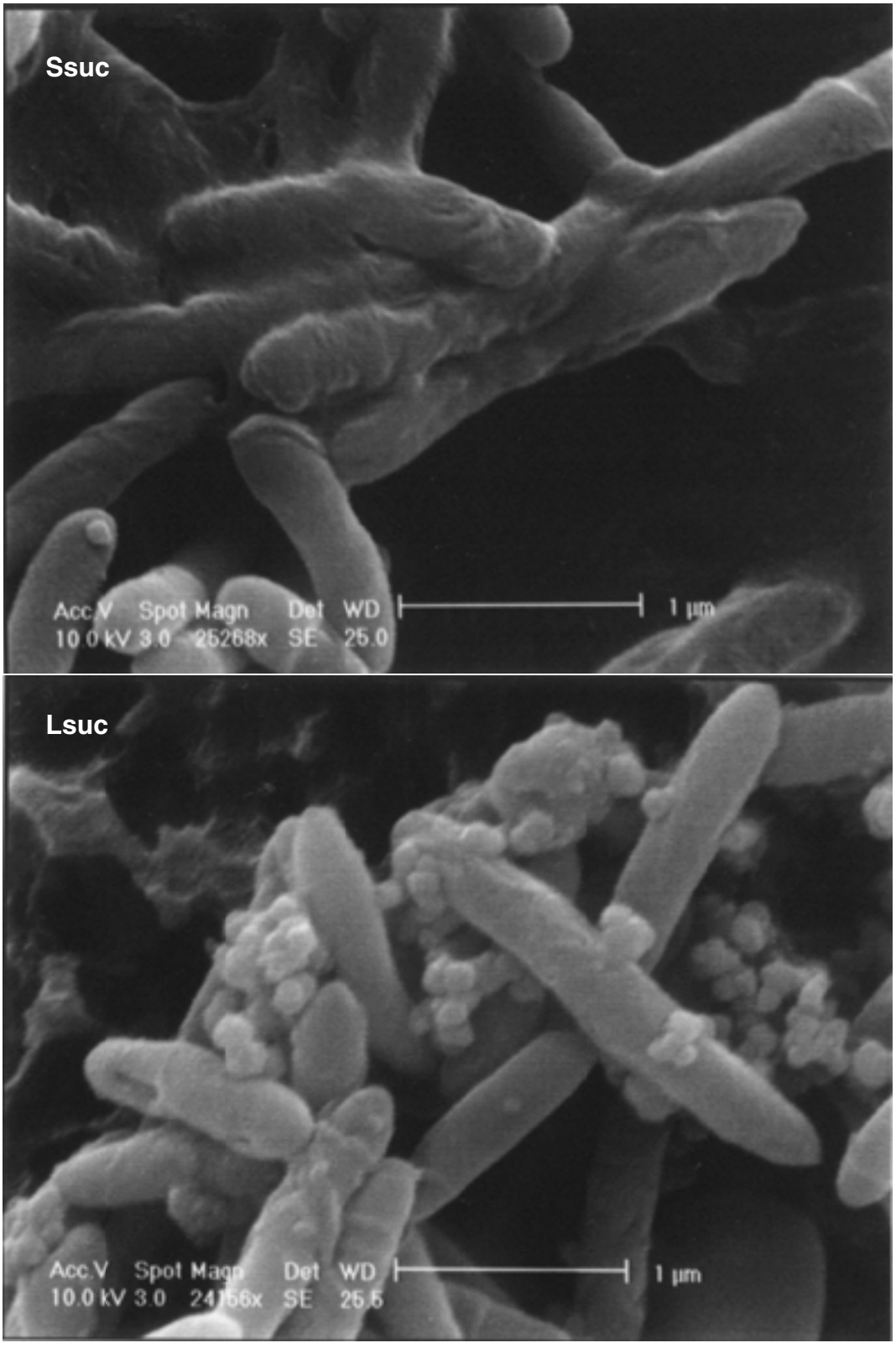


Figure 5.15 SEM of *B. fibrisolvens* E14 S and L grown on sucrose.

Ssuc and Lsuc are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) sucrose and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 30,000 x.

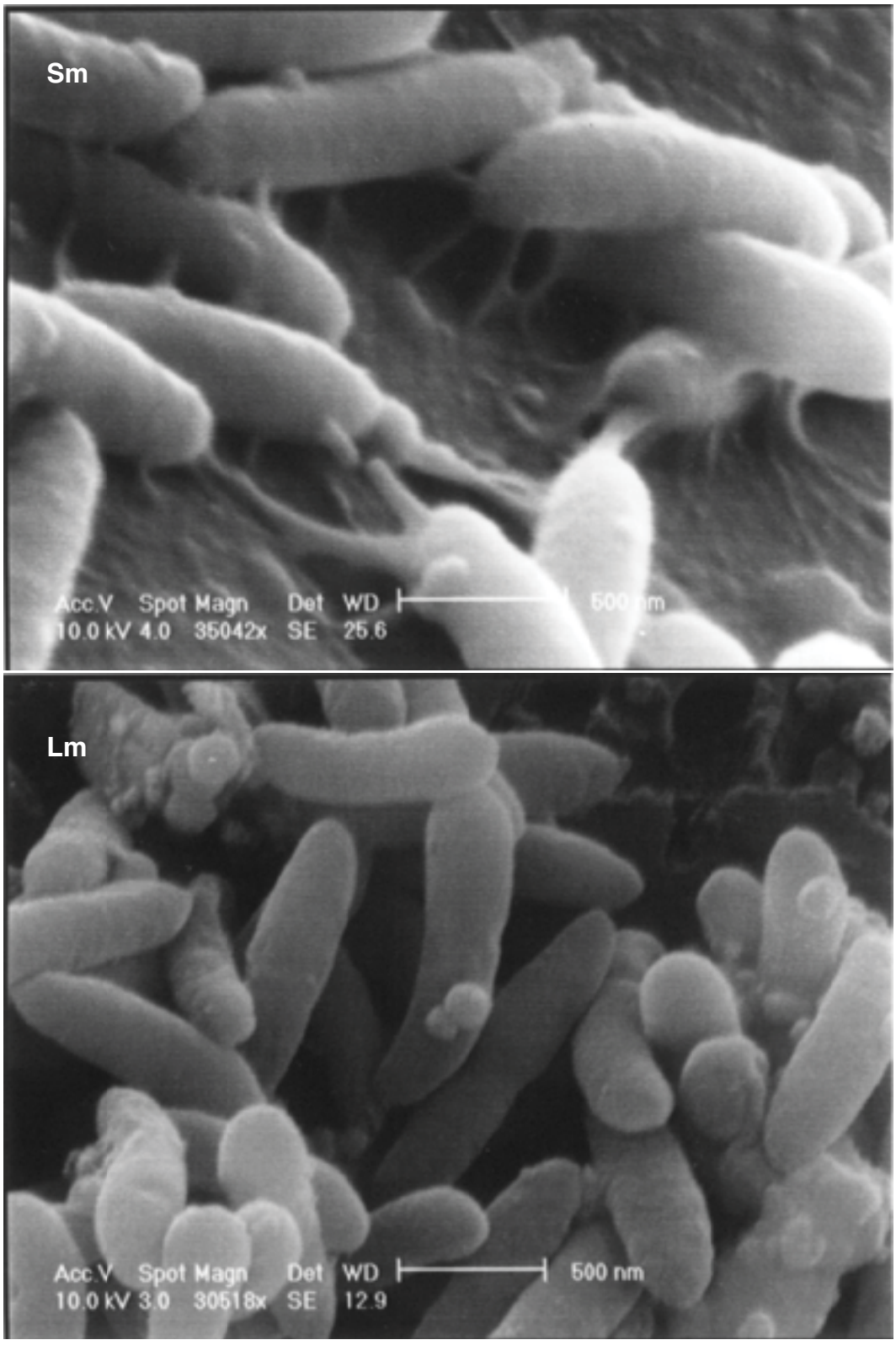


Figure 5.16 SEM of *B. fibrisolvens* E14 S and L grown on maltose.

Sm and Lm are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium containing 0.5 % (w/v) maltose and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 36,000 x.

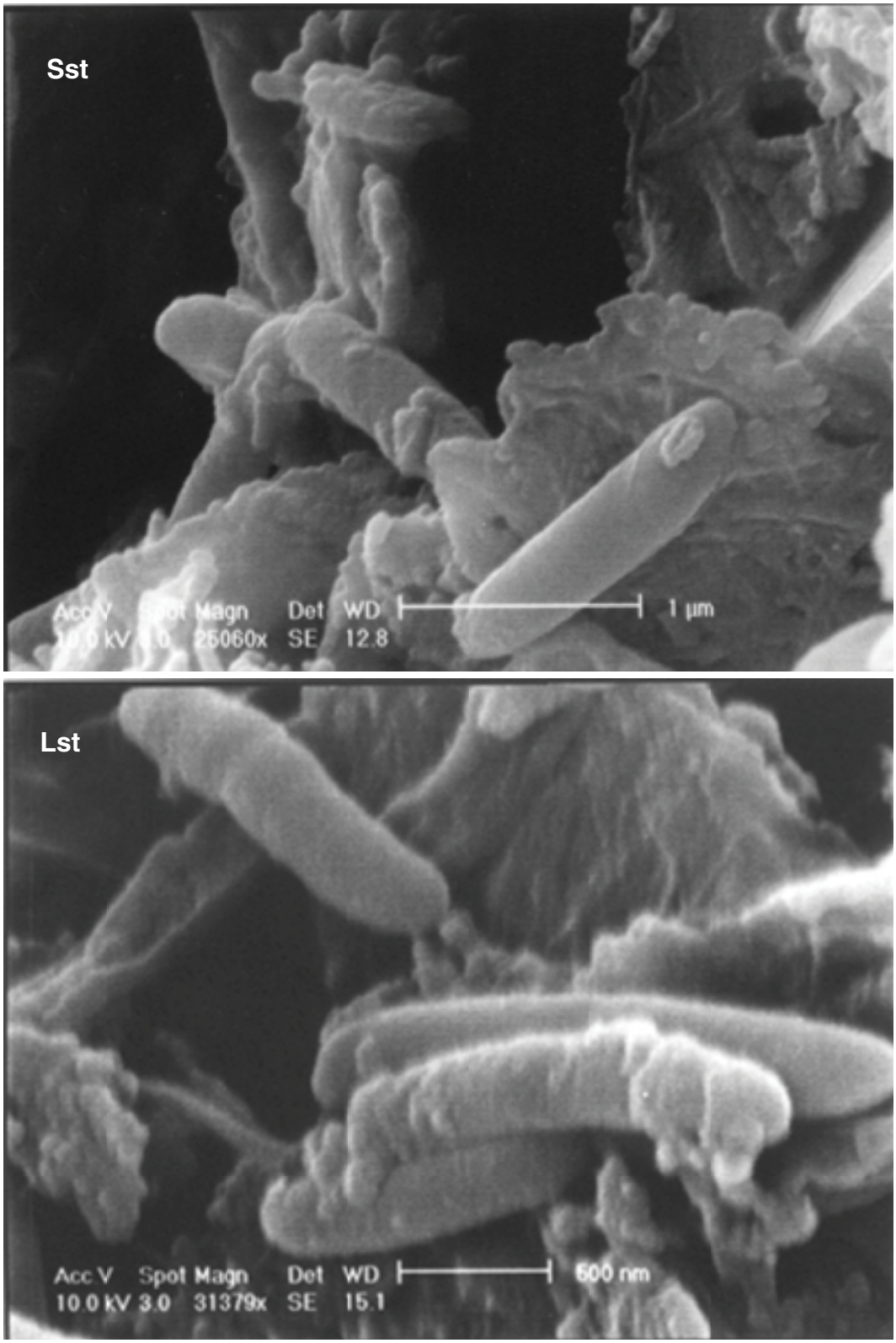


Figure 5.17 SEM of *B. fibrisolvens* E14 S and L grown on starch.

Sst and Lst are *B. fibrisolvens* E14 S and L, respectively. Cells were streaked onto defined medium) containing 0.5 % (w/v) starch and were incubated anaerobically at 39^{o}C. Cells were harvested at log phase and were prepared for SEM. The magnification was up to 32,000 x.

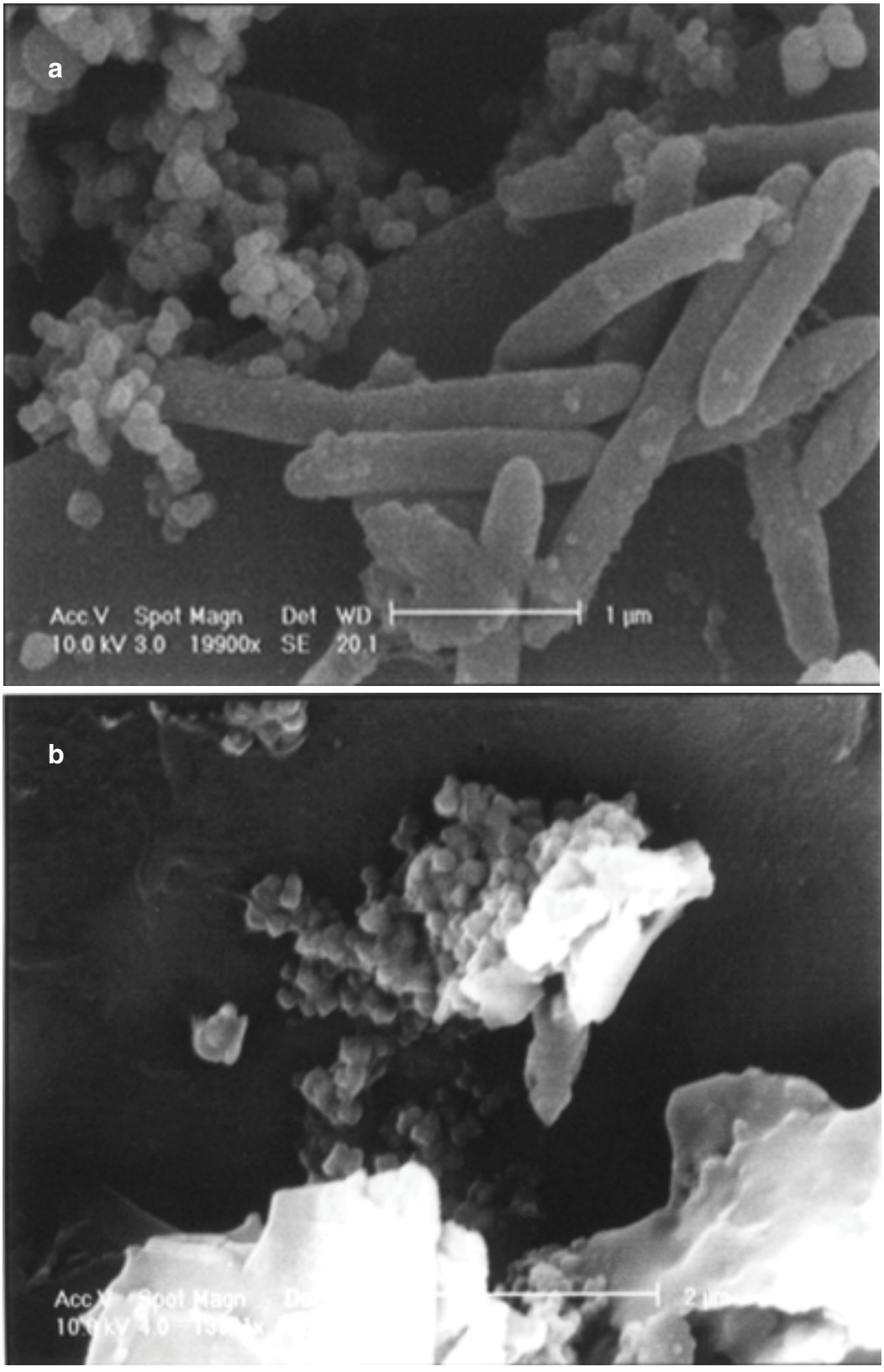


Figure 5.18 Secreted extracellular polymer (EP).

The electron micrograph was of starch-grown *B. fibrisolvens* E14 S cells at early stationary (a) or late stationary (b) phase. The magnification was up to 20,000 x.

5.3 Discussion

Based on the SEM results, possible steps in the extracellular display of EP are outlined in Figure 5.19. At step 1 (early log phase), S cells initiate the production of EP to mediate cell-cell or cell-surface attachment. The polymer then develops and spreads to neighbouring cells or surfaces (step 2, log phase). A more complex polymer network develops in either step 3a (cell to cell association) or 3b (cell to surface association) during late log to early stationary phase. It is not known whether the complex structure is due to a self-association process driven by thermodynamic stability considerations or simply reflecting an accumulation of excess EP. The complex polymer is mostly secreted (some may still be cell associated) to the environment (Figure 5.9 and 5.10) during early and late stationary phases. The fact that EP for attachment was secreted at the end of growth phase may be part of the final step in EP biosynthesis or as a cellular response to reduce the energy drain in maintaining EP synthesis, due to the lack of nutrient availability.

The L cell initially displays EP similarly. However, the L cells EP seems unable to spread, and it immediately clumped (forming globular EP) without undergoing step 2, or step 3 b (cell-surface association). These two steps (2 and 3b) seemed to be mostly missing from the L cells; only steps 1 and 3a were seen.

A similar case has been reported for marine *Pseudomonas* (Fletcher 1980a), where the EP of unattached cells was found to condense, forming globular EP. The EP was unable to develop a fine extracellular structural network as those in attached cells (Figure 5.20) and the globular EP from the unattached cells seemed

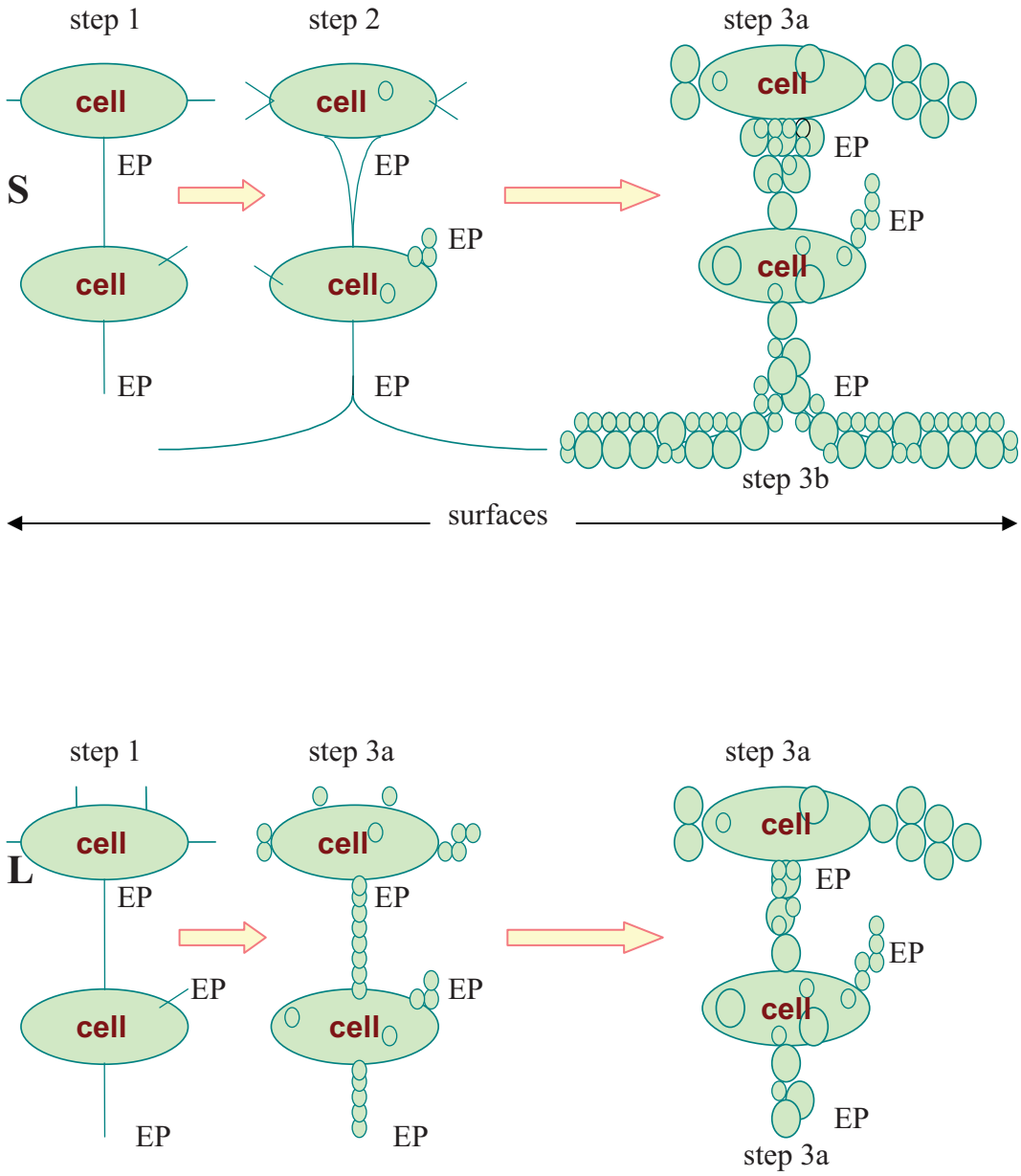


Figure 5.19 Possible steps in EP biosynthesis and attachment of *B. fibrisolvens* E14 S and L cells to a surface.

S and L represent *B. fibrisolvens* E14 S and L cells. The numbering (1, 2, 3a, 3b) indicates possible steps in the surface display of EP. Steps 2 and 3b were mostly missing in the EP display of L cells, and L cells seemed unable to spread EP to neighbouring cells or surfaces.

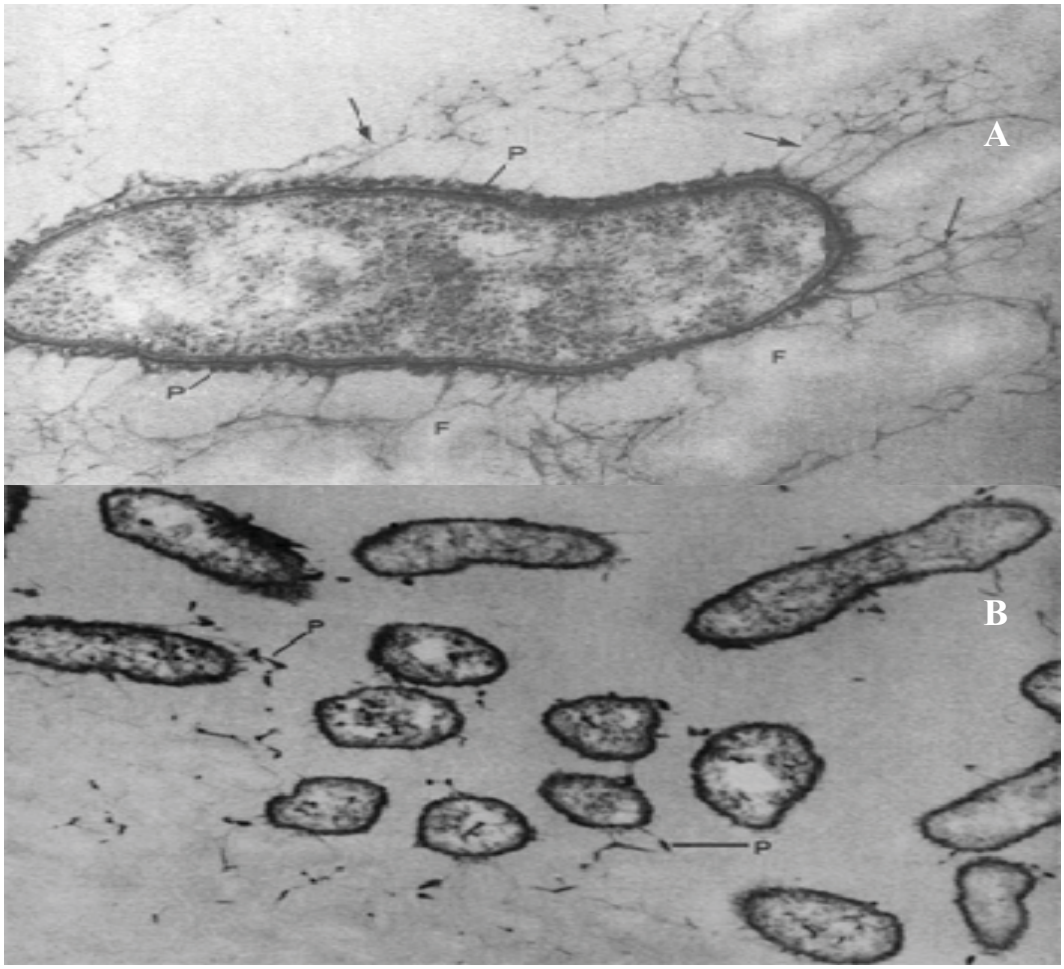


Figure 5.20 Extracellular polymers of attached and unattached cells (Fletcher 1980a)

A and B were attached and unattached (mutant) cells of marine *Pseudomonas*, respectively.

not to mediate attachment to surfaces. It has been suggested that globular EP ('knobs') may mediate cell-cell association of *B. fibrisolvens* strain C3 (Cheng and Costerton 1977) (Figure 5.21). However, since in the current study, the L cells also produced globular EP, did not attach to surfaces and were separable at all stages, it is unlikely that attachment of S cells is mediated by globular EP alone. The structures that mediate cell-cell association (Figure 5.21) may also be different from the EP 'knobs' as described by Cheng and Costerton (1977), and

further investigation is required to determine whether S cells were attaching to each other through these knob-like structures (stage 3a) or whether they existed side by side without any interaction.

Figure 5.21 Cell-cell association of *B. fibrisolvens* C3 (Cheng and Costerton 1977).

Arrow indicates structures that may mediate cell-cell interactions. The structures may or may not be the same as the globular structures around the cells.

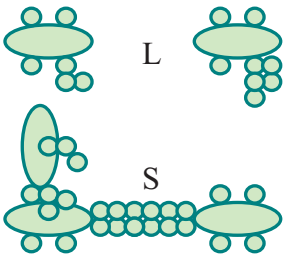
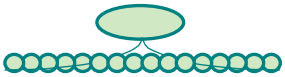
EP biosynthesis of various microorganisms is known to involve various steps such as regulation, precursor synthesis, polymerization and export (Kranenburg *et al.* 1997). Polymerization and export are often very complex and culture age dependant (Fletcher 1977, Ofek and Beachey 1980, Kranenburg *et al.* 1997). Attachment in most adherent bacteria is also time-dependant (Costerton *et al.* 1985, Fletcher 1991). S cells may assemble EP in several distinct steps (1, 2, 3a and 3b) and be culture-age dependant, and this may relate to stages in attachment to surfaces. The behaviour of S cells during early stages of growth may

correspond to steps 1 and 2 while during stationary phase may correspond to steps 3a or 3b. Culture age-dependent attachment of S cells is similar to that reported for the marine *Pseudomonas*, where the greatest tendency to attach to surfaces was during log phase and progressively declined during stationary phase and cell death (Fletcher 1977, 1980a). The structure of the EP may be at least partly responsible for the decline in attachment; as the bacteria attached to the surface grow, excess secreted EP may simply diffuse away from the cells. Late EP may also be compositionally different from early EP and therefore functionally less efficient in attachment. The bacteria could then be desorbed from the surface (Zvyagintsev *et al.* 1977, Fletcher 1980b). Time-dependent desorption has been demonstrated for *Bacillus mycoides* and *Serratia marcescens* from various substrata (Zvyagintsev *et al.*1977, Fetcher 1980).

The differences in EP structural morphology between S and L cells are illustrated in Table 5.1. During the experiment, it was noted that there were two forms of stage 3a that were difficult to differentiate, regardless of whether the structures were involved in cell-cell association or just secreted and accumulating around the cell but not mediating cell attachment. These were seen in both cell types, but in L cells, very few were seen to form connections between cells. Although these observations need further research, the complex globular structures (3a and 3b) seemed to be part of a defined EP structure rather than simply an accumulation of excess cellular waste. For bacteria in culture, nutrient uptake must come directly from the medium, and in EP surrounded cells, by transport through the EP (Costerton *et al*. 1981). It is possible that the EP might also provide a storage function for fermentable carbohydrate. However, *B. fibrisolvens* EPS appears

stable even after cell lysis and death (Stack and Ericsson 1988, Stack and Weisleder 1990), and there is little indication of fermentation of the EP. In most adherent bacteria, attachment is a time-dependant process, accompanied by the production primarily of EPS (Costerton *et al.* 1985, Fletcher 1991). Whether the structure of the secreted EP has any significance is not clear, but there was clear differences, for example when cellobiose was used, the EP was

Table 5.1 Differences in morphology between *B. fibrisolvens* E14 variants S and L.

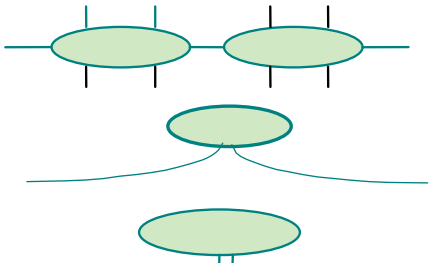
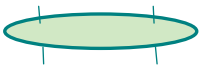
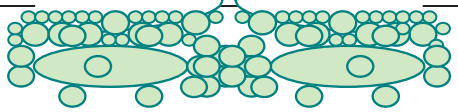
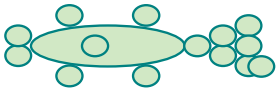
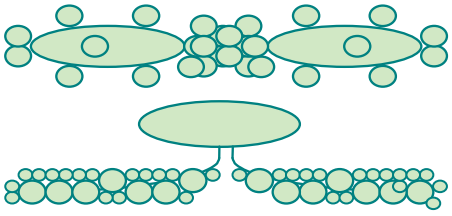
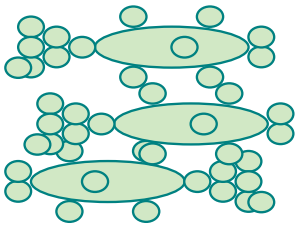
Form / stage		S	L	Description
1		+	+	more in S (early stage)
2		+	-	none/very little in L (cell-surface association)
3a		+ +	+ +	present in both S and L, but more in L (globular EP around the cell) present in both S and L, but more in S (cell-cell association)
3b		+	-	none/very little in L (cell-surface association)

B. fibrisolvens S and L, respectively. + or – indicates EP present or not present (or very little). Numbering (1, 2, 3a and 3b) reflects possible stages of EP display.

predominantly of a globular nature rather than the more evenly distributed EP associated with other substrates. It is possible that this simply reflects the amount of EP produced rather than a specific function of the polymer. This is perhaps

reinforced by the results of experiments that showed little differences across various carbon sources, with the exception of cellulose and cellobiose that induced less EP and of a more globular nature (Table 5.2).

Table 5.2 Effect of carbon sources on the morphology of *B. fibrisolvens* E14 S and L.

Carbon sources	Cell form induced as observed by SEM	
	S variant	L variant
glucose, maltose, sucrose, or starch		
cellobiose		
Xylan or cellulose		

Diagramatic representation of EP structures associated with *B. fibrisolvens* E14 S and L after growth on various substrates.

An alternate hypothesis is that the structure of the EP affects the strength of attachment of the bacterium to its substrates. Unfortunately, a method to measure the strength of attachment is not yet available. This could be used to determine whether attachment was stronger or weaker when more globular EP was produced, such as in the presence of cellobiose. In most cases, attachment is

described in terms of the number of attached cells rather than the strength of attachment of the organism to a specific surface (Ofek and Beachey1980; Fletcher 1980a, 1985, 1991; Rasmussen *et al*. 1989; Fujino *et al.* 1996; Dufrêne *et al*. 1996).

The interaction of bacterial ligands with surface receptors is comparable to antigen-antibody or plant lectin-sugar interactions (Ofek and Beachey 1980), and therefore the specificity can be demonstrated by blocking the interaction, ligand or receptor. This can be done with a large excess of the native ligand or receptor. However, in most cases, neither the ligand nor receptor have been isolated or identified, therefore experiments are usually performed using their analogues or haptens either identical to or resembling the native ligand or receptor (Ofek and Beachey 1980). Some receptors of microbial attachment to epithelial cells, such as L-fucose for *Vibrio cholera*, D-mannose for *E. coli*, sialic acid for *Mycoplasma sp.*, β-galactosyl residues for *E. coli* K-88, or N-acetyl-D-galactosamine for *L. buccalis* have been identified (Collier 1980, Kondo *et al.* 1976, Ofek and Beachey 1980, Fletcher 1991). These receptors may be useful to further study the attachment of *B. fibrisolvens* E14 S to surfaces, and may show clearer results than the common plant sugars used here. It may also be helpful to test attachment inhibitors/activators such as proteolytic enzymes, albumin, concanavalin A, lectins, neuraminidase, sodium metaperiodate, specific antibodies or other common substances that have been used to study microbial attachment to surfaces (Ofek and Beachey 1980, Fletcher 1991).

Based on glucose content, it has previously been reported that both S and L cells produced EPS, but higher levels were produced by the S cells (Nili 1995). Through SEM studies, it was also noted that S cells produced more EP than L cells, although it was still unclear whether the EP composition was only EPS or mixed with other components. This is examined separately in Chapter 6 (characterization of EP).

In conclusion, both S and L cells developed EP, but lack of polymer spreading was observed in the L cells. Two forms of EP were observed, globular and dispersed. Glucose, sucrose, maltose and starch seemed to induce more polymer dispersion in S cells, compared to cellobiose, and S cells produced more EP than L cells.