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BIOCHEMICAL CHARACTERIZATION OF THE BACILLUS SUBTILIS
MACROFIBER CELL SURFACE

by

Uttam Chand Surana

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1987
As members of the Final Examination Committee, we certify that we have read
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entitled Biochemical Characterization of the
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SIGNED: Uttam Swang
To the memory of my brother, Shikhar.
ACKNOWLEDGEMENT

The long and at times dry years of graduate school are familiar to everyone who has lived them. However, these times are constantly made interesting and stimulating by people one is surrounded. It has been a privilege to be associated with individuals from whom I have learned a great deal.

I gratefully acknowledge the guidance and support provided by Dr. Neil H. Mendelson throughout the course of this investigation. His readiness for frank discussions with insight and exceptional sense of humor is greatly appreciated.

Drs John Spizizen, Nobuyoshi Shimizu, Carol Dickmann, William Grimes and John Rupley served as members of my graduate committee. I am thankful for their timely help and advice.

I wish to thank my long time research advisor Dr. Anadi Chatterjee and colleagues Anuradha Lohia, Amit Mukherjee, Debabrata Raychaudhuri, Debopam Chakrabarty, Gautam Banerjee and Sailen Barik for sharing the times at the Bose Institute, Calcutta, India.

During my stay in the United States I have been blessed by the company of Deborah Rankert, Margaret Briehl, Dr. Dennis Ray, Jane and Didier Favre, Alan Wolfe, Telsa Mittelmeier and Dr. Dan Betinec with all of whom I have spent a great deal of time that was frequently punctuated with discussions (heated at times) about issues academic or otherwise.
Angelo Longo and Sam Rua deserve special thanks for their friendly assistance in the use of amino acid analyzer for the analysis of cell wall hydrolysates.

Finally, my sincere thanks to my family in India for years of encouragement and support without which everything would have been so much more difficult.
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ABSTRACT

Cell walls of *Bacillus subtilis* macrofibers have been biochemically analyzed to determine the contribution of various surface polymers in the twist regulation. Helix hand inversion was induced by a variation in either the growth temperature or the nutritional composition of the culture medium. Initial experiments had demonstrated a fivefold difference in the sensitivity of right- and left-handed forms to muramidases indicating modifications of peptidoglycan as a possible mechanism underlying inversion. An examination of lysozyme susceptibility of purified cell walls and whole cells derived from the two structural forms, however, exhibited no significant difference suggesting loss of the relevant component(s), perhaps biomechanical in nature, during disintegration of macrofibers.

The effect of various twist modulators such as trypsin, ammonium sulfate and D-alanine on the development of helical twist in both switchable and "fixed" mutants were studied. The interaction matrices have established D-alanine as the most potent of right-factors. Intestinal alkaline phosphatase is reported as a newly discovered antagonist to the development of leftward twist. Heat inactivation and protein purification experiments strongly indicated that twist modulation was due to the phosphatase activity rather than minor protease contaminants.
The chemical composition of cell walls purified from right- and left-handed structures was determined. No twist correlated differences in the overall content of peptidoglycan, teichoic acid and teichuronic acid constituents were detected. Evidence is presented for the absence of correlation between the extent of ester-linked alanine substitution and twist state. These findings suggest that gross changes in wall composition is perhaps not the mechanism for hand inversion. From profiles of the wall associated proteins, a 200 Kdal band has been identified whose presence is strongly correlated with the development of leftward twist. This polypeptide was found to be highly sensitive to trypsin; a property it shares with a previously proposed left-twist protein. Preliminary evidence for isolation of left-hand specific polyclonal antibodies is also presented.

FJ7, a switchable mutant, was successfully transformed with a plasmid containing the *Streptococcus* transposon Tn917. A small bank of insertional mutants has been constructed for the isolation of mutants impaired in heix hand inversion.
CHAPTER 1

INTRODUCTION

In procaryotes, the cell surface participates in the regulation of important functions such as growth, division, and morphogenesis. Throughout the cell cycle, envelope biosynthesis, surface extension and septum formation must be co-ordinated with other cellular activities such as DNA replication and genome segregation if a successful division is to be achieved. Any departure from this co-ordination usually results in highly abnormal cells (Slater and Schaechter, 1974).

The determination and maintenance of bacterial morphology are perhaps the two most conspicuous roles of the cell wall. The loss of characteristic cell shape upon removal of the wall is in itself strong evidence that wall polymers are involved in shape maintenance. However, for elucidation of the principles that govern shape determination, an understanding of the organizational and dynamic aspect of the cell surface is essential. Lyt- mutants of Bacillus subtilis which grow as multistrand helices (macrofibers) are well suited to study these parameters (Mendelson 1976, 1978). It has been proposed that twist states of a given macrofiber represent the conformational states of the main stress-bearing surface polymer, peptidoglycan (Mendelson, Favre and Thwaites, 1984). Hence, a change in the helical twist, induced by genetic or environmental factors, is a manifestation of
the structural reorganization within the cell surface. These alterations, although too minute to be easily discernible in bacterial cells due to small size, are revealed in macrofibers as clearly perceptible changes in the helical orientation. Therefore, the macrofiber system represents an amplified model of a single cell in context of the cell surface related phenomenon and provides a unique experimental approach to the study of structural dynamics of wall polymers.

The chemical composition and topology of the cell envelope biosynthesis are of primary importance in bacterial morphogenesis. Equally significant are the structural features such as glycan chain length, the extent of cross-linking, and electromechanical properties. Whereas some of these features are influenced by the cellular content of autolytic enzymes, others stem from the biochemical makeup of the cell wall which undergoes substantial variation in response to growth environment. Since the structural dynamics of the Gram-positive cell surface is directly dependent on the nature of the wall polymers, a general description of their composition and related properties is pertinent.

The Gram-positive Cell Wall

The cell envelope of Gram-positive bacteria has a complex polymeric composition. Its thickness ranges from 20 to 50 μm in most organisms with peptidoglycan constituting 50-90% of its weight (Rogers, 1981). The remainder consists of negatively charged teichoic and
teichuronic acids, neutral polysaccharides and noncovalently associated polypeptides.

Composition and Structure

Peptidoglycan, the major stress bearing polymer, is composed of glycan strands substituted with small peptides. In _B. subtilis_ (a Gram-positive organism) the glycan part is a string of alternating residues of N-acetylg glucosamine and its 3-0-D lactyl derivative, muramic acid, linked via a β-1-4 glycosidic bond. The lactyl moiety of muramic acid bears a short peptide tail (L-ala:D-glu:meso-dap:D-ala) that cross-links two parallel glycan strands. The cross linkages involve the carboxyl group of D-alanine of one peptide and the free amino group of meso-diaminopimelic acid of the other. This interlaced architectural scheme is what makes murein a "bag shaped molecule" of cellular dimensions (Weidel and Pelzer, 1964).

The basic structure of peptidoglycan is subject to considerable chemical modifications in response to the conditions and mode of growth. Amidation of free carboxyl groups of the peptide moiety is fairly common. For example, the α-carboxyl group of D-glutamic acid and the D-center of diaminopimelic acid are amidated in _Staphylococcus aureus_ and _B. subtilis_ (Schleifer and Kandler, 1972). Although the physiological consequences of these changes are not yet understood, some other chemical modifications are known to drastically alter the sensitivity of muropeptide to lysozyme. In this category, O-acetylation of muramic acid residues in _S. aureus_ and _Lactobacillus acidophilus_ (Tipper et al., 1965; Coyette and Ghuysen, 1970) and the
occurrence of nonsubstituted N-acetylglucosamine in *Bacillus cereus* have been most studied (Araki et al., 1972). The susceptibility to muramidases is also affected by the presence of excess free amino groups in peptidoglycan.

Glycan chain length and the extent of cross-linking, the two important parameters of Gram-positive walls that contribute to its structural integrity, have also been found to vary considerably. The cross-linking index ranges from 20% in *E. coli* to 93% in *S. aureus*. In *B. subtilis* spores, 55% of the muramic acid residues form a lactam involving the lactyl moiety and the free amino group at the C-2 position (Wrath and Strominger, 1972). An obvious consequence of this modification is a significant reduction in the cross-bridge formation and in turn, an altered sensitivity to lysozyme. It has been claimed that a change in both glycan chain length and the degree of cross-linking accompanies the morphological shift from rods to cocci in *Arthrobacter crystallopoites* (Krulwich and Ensign, 1968; Krulwich and Pate, 1971), although no such correlations were detected in the rod mutants of *B. subtilis* (Taylor, Ward and Rogers, 1979).

Despite fairly detailed knowledge about the biochemical nature of peptidoglycan, there is little agreement about its three-dimensional conformation. Based on infrared spectroscopy and density measurements it was suggested that the structure of glycans is similar to that of chitin (Formanek et al., 1976). X-ray diffraction analysis, however, does not support the parallel arrangement of the glycan strand as required by the chitin model (Labischinski et al., 1979). Tipper (1970) has suggested a configuration in which the disaccharide units
are almost at right angle to each other along the central axis, enabling them to form cross-links in either plane. Whatever the structure, these models tend to perceive the cell wall as a rigid entity. Considering the dynamic nature of growth and division, the cell envelope will have to be flexible enough to allow surface extension and other relevant modifications. These features and the ability of peptidoglycan to change volume in response to ionic conditions were first included in a model proposed by Oldmixon et al. (1974). This model, in its low density form, allows sufficient elasticity for the changes that must occur during active growth. The "true" structural nature of murein, however, still remains unknown.

In addition to peptidoglycan, the wall of Gram-positive organisms contain negatively charged polymers called teichoic acids. In *B. subtilis* 168, the wall teichoic acids are chains of 15-20 glycerol phosphates molecules strung together by phosphodiester bonds. One end of the chain is secured at C-6 of muramic acid via a mannose containing linkage unit (Shunji, 1984) whereas the other remains free. Moreover, the C-2 position of glycerol residues are substituted with either glucose or D-alanine. The extent of substitution is dependent on the growth conditions and the type of strain.

The abundance of equally spaced negative charges on teichoic acids hinted that they may act as the binding sites for divalent cations. Heptinstall et al. (1970) were able to show that the concentration of wall-bound magnesium was drastically affected by the presence or absence of anionic polymers. Hence, one of the potential
functions of teichoic acids may be to act as a scavenger of essential ions. In *S. pneumoniae*, the lytic enzyme N-acetyl-muramyl-L-alanine amidase requires choline containing teichoic acid for its degradative action. Replacement of the choline moiety with ethanolamine renders the cell walls highly resistant to amidase, implying that the autolysins have strong affinity for teichoic acids, and that the anionic polymers may be involved in the regulation of lytic activity in Gram-positive organisms. These implications, however, have not been fully substantiated.

When *B. subtilis* cells are grown in a phosphate-limited medium, the teichoic acids in the wall are replaced by another anionic polymer called teichuronic acid which is, under normal circumstances, present in small quantity (Ellwood and Tempest, 1972). The latter is composed of alternating residues of N-acetylgalactosamine and glucuronic acid. The mutants of *Micrococcus luteus* that grow as regular packets of separation suppressed cells were shown to lack teichuronic acid (Yamada, Hirose and Matsuhashi, 1975). Similarly, a novobiocin resistant mutant of *Bacillus licheniformis* which grew as long chains was found to be devoid of the same polymer indicating its involvement in cell separation (Robson and Baddiley, 1977).

Besides the components mentioned above, the envelope of Gram-positive bacteria contains a number of polypeptides, most of which are noncovalently associated. Some of the biosynthetic and remodeling enzymes, such as transpeptidase and autolysins, would be expected to reside in the walls. While investigating the DNA-cell wall association
in *B. subtilis*, Doyle et al. (1980) identified a 48K protein present on the outermost surface of the cell wall. The cellular function of this protein, however, is unknown. Other examples of wall associated proteins include protein A of *S. aureus* and proteins M, T and R of group A *Streptococcus* (Sjoquist et al., 1972; Salton, 1953) which contribute to the antigenic properties of cell envelope in these organisms. Whether the surface bound polypeptides influence the glycan strand conformation remains to be determined.

**Physical Properties**

In addition to various chemical modifications discussed above, cell walls show considerable physical flexibility in terms of expansion and contraction. These properties have been investigated in some detail by Knowles (1971) and Marquis and his coworkers (1968, 1973). The change in dextran impermeable volume (DIV) was the index used for the measurement of elasticity. In the experiments involving whole cells and isolated walls of *S. aureus* and *Bacillus megaterium*, it was observed that the DIV was greatly affected by alterations in pH, ionic strength and the extent of ester-linked D-alanine substitution. These effects were found to be compatible with the charge status of wall constituents. However, similar variations were observed following osmotic pressure changes in whole cells (Ou and Marquis, 1970). Hence, both the electrostatic forces and osmotic pressure contribute to the overall physical properties of the cell wall which are thought to be of considerable importance in shape determination and maintenance (Rogers, 1979).
Biosynthesis and Assembly

The basic framework of peptidoglycan synthesis (Tipper and Wright, 1979) can be divided into three stages: 1) formation of the disaccharide pentapeptide, 2) transfer of this precursor to a lipid carrier and 3) incorporation of the newly synthesized subunits into the pre-existing wall. The initial reactions involve formation of two UDP-linked amino sugars, N-acetylglucosamine and N-acetylmuramic acid. The amino acids (L-ala, D-glu, meso-dap and two residues of D-ala in B. subtilis and E. coli), with the exception of D-alanine, are then added to the lactyl moiety of muramic acid by specific ligases. The D-ala dipeptide is formed in reactions catalyzed by a racemase and a ligase, and then transferred to the muramyl-tripeptide. These steps are important control points in regulation of the extent of cross-linking.

In the second stage, UDP linked muramyl-pentapeptide is accepted by the lipid carrier, a C55 undecaprenyl phosphate. The ultimate building block, disaccharide pentapeptide, is synthesized by linking N-acetylglucosamine to the nonreducing end of muramic acid while it is still attached to the carrier (Anderson et al., 1966). Finally, the nascent peptidoglycan subunits are translocated across the cytoplasmic membrane and bonded to the pre-existing glycan strands. There are two possible mechanisms for incorporating the newly synthesized precursor, namely, formation of either a glycosidic bond (transglycosylation) or a peptide bond (transpeptidation, the reaction inhibited by B-lactam antibiotics). Whether the former occurs prior to the latter or vice versa remains debatable (Rogers, Perkins and Ward, 1980).
In *B. subtilis* 168, CDP-glycerol is the only substrate needed for the teichoic acid synthesis. The newly made subunits are linked to the already existing teichoic acid chains in the walls (Burger and Glaser, 1964). The direction of extension was found to be similar to that of glycogen i.e. by addition of nascent units to the reducing termini (Kennedy and Shaw, 1968). Glucosylation of the free hydroxyl group of glycerol is catalyzed by glucosyl transferase (Brooks, 1971).

The co-ordination between peptidoglycan and teichoic acid syntheses was first reported by Mauck and Glaser (1972). Using the phosphate limiting conditions described by Ellwood and Tempest (1969) these authors demonstrated that in *B. subtilis* 168, teichoic acids were linked to the newly synthesized peptidoglycan instead of the pre-existing material. When the experiment was repeated in the presence of penicillin, peptidoglycan without cross-links continued to be made; however, no attachment of accessory polymers was detected. These and other (Wyke and Ward, 1975) observations suggested that linking of the acidic polymers requires concomitant cross-bridge formation. This co-assembly scheme, possibly significant in determining the glycan orientation, seems to be strain specific since in *B. subtilis* W23 anionic polymers were transferred exclusively to the pre-existing murein (Wyke and Ward, 1977).

**Effect of Environment on Cell Wall Composition**

Although the primary structure of cell wall is genetically determined, changes in its composition in response to varying growth conditions are well documented (Schleifer, Hammes and Kandler, 1976).
As briefly mentioned earlier, an interesting regulatory circuit involving teichoic acids has been uncovered in *B. subtilis* by Ellwood and Tempest (1972). Growth in phosphate-limited medium resulted in the disappearance of teichoic acid with a concomitant increase in the teichuronic acid content. The rate of replacement, faster than a simple dilution of the pre-existing material, indicates that some sort of control is operative. There is evidence for the relative stability of biosynthetic enzymes, UDP-glucose dehydrogenase and CDP-glycerol pyrophosphorylase, being the main regulatory element. Although the physiological role of phosphate-induced polymer switching is not clear, the anionic polymers must be involved in some critical cellular function since mutants completely devoid of these surface components have never been isolated.

The amino acid composition and pH of the growth medium are among other factors that bring about changes in the chemical makeup of walls in many Gram-positive organisms (Schleifer, Hammes and Kandler, 1976; Ellwood and Tempest, 1972). In some instances, modifications of cell wall composition is reflected in altered sensitivity of mucopeptide to lysozyme. Magnesium-limited cells, for example, lyse at a considerably slower rate relative to phosphate-deprived cells. This difference cannot be attributed to the presence of teichuronic acid in the latter since ammonium-starved cells which contain a full complement of teichoic acid lyse at a faster rate. It has been suggested that the differences in the susceptibility to muramidase are due to alterations in mucopeptide composition (Ellwood and Tempest, 1972).
Autolytic Enzymes

N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase are the predominant lytic enzymes found in many Gram-positive organisms. The former hydrolyses the glycan strands liberating the reducing groups of glucosamine residues, whereas amidase cleaves the bond between the glycan and the peptide tail. Very little is known about the cell surface distribution of autolysins except in *S. faecalis* in which most of the lytic activity is concentrated near the septal region (Higgins, Pooley and Shockman, 1970; Shockman, Daneo-Moore and Higgins, 1974).

Mutants of *B. subtilis* that have lost the bulk of their autolysins grow as separation-suppressed filaments and show a reduced rate of cell wall turnover (Pooley, 1976; Fein and Rogers, 1976; Chatterjee et al., 1976). Since both cell separation and turnover require controlled hydrolysis of insoluble wall material, the above findings appear to implicate autolysins in these cellular functions. That the correlation between turnover and lytic enzymes may not be as strong, has been shown by Vitkovic et al. (1984). Also, some mutants of *L. acidophilus*, although retaining <2% of the lytic activity, undergo a normal rate of turnover (Boothby et al., 1973). However, in the absence of knowledge about the minimum amount of lytic enzymes needed for cell wall extension and turnover, no definite conclusion can be drawn. Equally uncertain is the role of hydrolytic enzymes in the generation of acceptor sites for the newly synthesized peptidoglycan
subunits (Shungu, Cornett and Shockman, 1979; Fein and Rogers, 1976; Rogers, Perkins and Ward, 1980).

Lytic-deficient mutants of *B. subtilis* are generally devoid of flagella and can easily be isolated as strains that are resistant to flagella-adsorbing phages. Furthermore, based on a recent genetic analysis, Pooley and Karamata (1984) have suggested that mutations previously designated as *lyt-1*, *lyt-2* and *lyt-15* may be under the regulation of *fla* genes. These observations define a critical role for autolysins in flagellation.

**Shape Determination and Surface Topology**

Bacterial cells display a variety of simple forms, most common being rods, spheres and helices. How the various shapes are attained from similar surface composition has remained a long standing question despite a fairly detailed knowledge about the chemical makeup of the cell envelope. The physical and biochemical properties of surface polymers must interact in a not yet understood manner to give a cell its characteristic form. *Arthrobacter crystallopoites* and the rod mutants of *B. subtilis* have been subjects of much investigation in this context. The former undergoes a "natural" morphological variation in that it transforms from rod to coccal shape when grown in glucose-limited condition (Luscombe and Gray, 1971). Krulwich et al. (1967a) have shown the glycan chains in spheres to be significantly shorter compared to those in rods. The elevated level of lytic activity in coccal form supports this claim.
Differences in the dextran impermeable volume and amino acid composition of cross-bridges have also been noted in the two morphological forms (Krulwich et al., 1967b). The change in shape of rod$^+$ mutants of B. subtilis, however, is not accompanied by the differences demonstrated in Arthrobacter.

The RodA (tag-l) mutant undergoes a derepression of peptidoglycan synthesis at the restrictive temperature leading to a reduction in the teichoic acid to murein ratio (Rogers, McConnell and Hughes, 1971; Boylan et al., 1972). The situation is somewhat similar in a mutant that grows as spheres upon phosphate starvation (Forsberg et al., 1973; Forsberg and Rogers, 1974). The cells are unable to synthesize negatively charged polymers under these circumstances. The correlation between altered ratio of surface polymers and the change in shape suggests that the charge status of cell wall may affect its topology at the time of co-assembly, ultimately leading to a morphological alteration. The rodB mutant, however, is capable of attaining coccal form without any apparent change in the teichoic acid content (Rogers et al., 1971). Investigations in E. coli have indicated that the shape determining function is governed by penicillin binding proteins (PBP$s$). Mecillinam, a B-lactam antibiotic that preferentially binds to PBP2, causes this normally rod shaped organism to grow as spherical cells (Spratt and Pardee, 1975; Spratt, 1975). In addition, some of the spherical mutants that are resistant to mecillinam seem to have lost their functional PBP2 (Spratt, 1977). In light of these facts the rodB mutation may affect a protein normally involved in
length extension (Mendelson and Reeve, 1973; Rogers, Perkins and Ward, 1980). The loss of this activity compounded with continued wall formation from the septal region will eventually give rise to oval or round shaped cells.

Clearly, there is evidence, though ambiguous, of biochemical modifications leading to morphological changes. Exactly how cell-shapes are transformed from one to another, requires precise knowledge of the topology of envelope biosynthesis. In this regard, many efforts have been made to determine whether the mode of surface extension is of dispersive or zonal nature. The experimental strategies have included pattern of flagella distribution, phage attachment (Archibald and Coapes, 1976; Archibald, 1976), immunofluorescence (Cole and Hahn, 1962; Hughes and Stokes, 1971), and radioactive labelling (DeChastellier et al., 1975a, b). *S. faecalis* (Higgins, 1976) and *B. licheniformis* (Hughes and stokes, 1971) provide two examples in which the wall polymers are synthesized in discrete zones. Based on immunofluorescence and computer simulation studies Higgins and Shockman (1976) have proposed a model according to which most of the cell wall synthesis in *S. faecalis* occurs in the septal region of the dividing cells. The wall polymers are fed out from this region to form the peripheral cell surface.

The situation in rod shaped organisms, such as *Bacillus*, is more complex. The mechanism of pole formation in these organisms may be similar to the one described for *Streptococcus*. However, the polar region accounts for only 15% of the whole surface. Attempts to
identify discrete growth zones along the cylindrical portion were complicated by a high rate of turnover. Early studies utilizing a phosphoglucomutase deficient mutant to reduce the effects of rapid turnover suggested diffuse intercalation as the mechanism for surface growth in bacilli (Doyle et al., 1977). Pooley (1976), however, has pointed out that the newly incorporated wall undergoes an upwelling process and spreads as it rises towards the outermost boundary causing the observed randomization of otherwise discrete growth zones. To test this hypothesis, this author and his colleagues labeled a strain of B. subtilis, reduced in turnover, with radioactive glucosamine. The tagged wall polymers were then chased through the surface; subsequent autoradiography demonstrated non-Poisson distribution of grains suggesting zonal insertion of the newly synthesized subunits (Pooley, 1979).

In the context of surface topology, questions of a different sort have been raised by isolation of lyt− mutant of B. subtilis which grow as helical structures (Mendelson, 1976).

**Helical Macrofiber System in Bacillus subtilis**

Lyt− mutants of B. subtilis grow as multistrand helical structures of macroscopic dimensions (hence macrofibers) in unaerated culture. These structures were first reported by Mendelson (1976, 1978). Since then, other examples have been published including *rodA lyt* and *rodB lyt* double mutants (Rogers and Thurman, 1978). The
forms resulting from the treatment with chlorpromizin (Tilby, 1977), although helical, are different from the one described by Mendelson.

Deficiency of autolysins leading to suppression of cell separation is a primary condition for macrofiber formation. In the initial phase of growth, the cells form long filaments undergoing "writhing" motions. These motions are nonrandom, perhaps caused by the surface-stress resulting from blocking of cell rotation during filamentous growth (Mendelson, 1982) or simply due to the unsynchronous nature of wall extension (Thwaites, personal communication), or both. An increase in the intensity of writhing owing to continued growth leads the structures to an inevitable self-contact. This further accentuates inhibition of rotation, causing accumulation of stress that eventually forces the filaments into their first helical fold. This process is reiterated until a multistrand helical form, incapable of undergoing any more folding, is attained. The entire scheme of macrofiber formation is considerably sensitive to shear forces. Hence, propagation and maintenance of macrofibers requires static conditions and a large surface area for adequate aeration.

Although the helix phenotype of a given strain is defined by the range of twist it can achieve, the individual structures, produced in a particular set of growth conditions, may be characterized by three basic parameters: helix hand, static twist and the folding threshold (Mendelson, 1982b). A macrofiber, in which the uppermost cellular filaments define an incident angle of $0^\circ$-$90^\circ$ with its transverse axis is considered right-handed (RH). The filaments of a left-handed (LH)
structure, on the other hand, will make an incident angle in the range of 90° to 180°. The static twist, simply put, is a measure of the "tightness" of a structure. Quantitatively, it is a function of the surface helix angle (\( \alpha \)) and the macrofiber diameter (2R): 2\( \pi R N = \tan \alpha \) (Mendelson, Favre and Thwaites, 1984). The third parameter, folding threshold, refers to "...an amount of energy required to force a macrofiber structure into an effective fold...". Since, initially, the energy that drives helical folds is generated from length extension, the folding threshold can also be defined as the average length at which steady state fibers fold.

Although it is genetically determined, the static twist of a given strain undergoes drastic phenotypic changes in response to varying growth conditions. FJ7, a strain whose helical properties are well characterized, grows as a right-handed helix at 20°C in TB medium supplemented with MgSO₄ but forms LH structures when cultured at 48°C. Upon transfer from 20°C to 48°C, a macrofiber fragment first undergoes a state of disorganization and then, with continued growth, organizes itself in a LH orientation. This phenomenon is referred to as temperature-induced helix hand inversion (Mendelson and Karamata, 1982). The inversion response, however, is not restricted to induction by temperature changes; it is also triggered by changes in the nutrient status of the growth medium. For example, some strains that form RH structures in TB grow as LH macrofibers in Sl and T, both of which are nutritionally inferior media than TB. The twist can further be affected by addition of ions such as magnesium and ammonium sulfate.
The extent of change in twist values was found to be a function of ion concentration.

What does the static twist of a macrofiber represent? According to Mendelson (1982a) the force that ultimately drives the helical folds arises during length extension. Underlying this proposition are two important assumptions: 1) the glycan chains are aligned in a helical orientation on the cell surface and 2) the cell wall, being a covalently closed network, accumulates stress due to addition of the nascent polymers. Under normal circumstances (i.e. single cell culture) the strain may be relieved by division and separation of daughter cells. In separation suppressed cells, however, the stress, accentuated by blocking of rotation, accumulates and is eventually borne out only by "snapping" of long filaments into helical folds. Thus, the static twist seems to manifest the glycan orientation and the stress murein must bear during active growth. Changes in the values of twist under various growth conditions, then, represent the alterations in the alignment of wall polymers in response to environmental factors. Such minute surface events, almost impossible to visualize in a single cell, are easily discernible in macroscopic helices. Hence, macrofibers may serve as a suitable model system to study the cell surface dynamics in greater detail (Mendelson, 1982b).

Some experimental evidence has already been gathered in support of the contention stated above. Treatment of macrofibers with lysozyme induces relaxation motions i.e. rotational movements of the structures around their longitudinal axes (Mendelson, Briehl and Favre, 1982).
Since this enzyme specifically hydrolyses glycosidic bonds in mureopeptide, induction of these movements suggest that "...helical shape is a stress-condition and that peptidoglycan is involved in locking the stressed cell surface into the deformed shape." The clockwise direction of relaxation motions, irrespective of helix hand, further indicates right-handedness being the relaxed state as well as the inherent helicity of peptidoglycan. The fact that the presence of D-alanine caused LH structures produced in S1 to grow as RH helices and that 48°C grown LH macrofibers were at least five fold more sensitive to lysozyme compared to their 20°C-RH counterpart suggest direct involvement of cell wall in the maintenance of helicity.

The production of helical structures of various twists and a complete inversion of helix hand by varying growth temperature was mentioned earlier. It has been observed that upon transfer from 20°C to 39-48°C macrofiber fragments must grow for a certain length of time (inversion lag) at higher temperature before the inversion process begins. Using the strains FJ7 and SDS118, Favre et al. (1985) demonstrated that the inversion lag shortens as the difference between 20°C and the temperature at which the LH structure are being produced increases. Since 48°C-macrofibers are of higher left twist relative to the ones grown at 39°C, the above finding could mean that the newly synthesized wall of higher twist has greater effect on the orientation of pre-existing RH material. Hence, the apparent static twist after the lag is a result of competition between the new and the old wall. The kinetics of right to left inversion does not seem to be a
ramification of growth since the doubling time within the range of 39-48°C does not vary significantly (Favre, Thwaites and Mendelson, 1985). The inversion in the reverse direction (LH to RH), however, shows asymmetry in that the lag time is not influenced by the twist value of the newly inserted RH wall material. Although the meaning of this asymmetry is not yet clear, according to the authors it indicates "differences between right- and left-handed structures themselves".

The minimum time a macrofiber fragment could spend at 48°C in order to undergo inversion was found to be 3 minutes. In a pulse experiment, FJ7 macrofibers grown at 20°C were incubated at 48°C for 3 minutes and transferred back to 20°C. The RH structures underwent a transient inversion at low temperature prior to attaining its characteristic right-handed twist. No such inversion was observed if chloramphenicol was present during the high temperature pulse. This experiment suggested dependence of the inversion process on some protein factor(s) whose synthesis is temperature inducible (Favre, Karamata and Mendelson, 1985). The inability of FJ7 macrofibers to change helix hand at 48°C when grown in the presence of a low concentration of trypsin supports this notion. The amount of protease used in these experiments did not seem to affect the overall growth rate.

Wolfe (1985) has discussed some of the disadvantages of using the temperature-inducible system to study the kinetics of helix hand inversion. Since growth at elevated temperature is significantly accelerated and may bring about alterations in the wall composition
unrelated to twist determination, a clear interpretation of results is difficult. This author followed an experimental regime similar to the one described above except that the nutritional up- or down-shift, was used to induce changes in static twist instead of temperature. The results were found to be qualitatively similar to those described for the temperature inducible system indicating a fundamental similarity of the helix hand inversion processes. The requirement of a protease sensitive factor(s) for the development of leftward twist in the nutrition regime confirms the previous findings and suggests that this protein, henceforth called the left-twist protein, is LH orientation specific.

It is clear from the foregoing discussion that the cell wall polymers can exist in a myriad of twist states. Although it is tempting to envision the helix-hand inversion process as being governed by a genetic switch that determines orientation of surface polymers merely through its on/off status, the fact that none of the macrofiber producing strains studied so far exhibit a "fixed" static twist value makes an on/off mechanism unlikely. Even though some mutants are "locked" in a particular helix hand, they still display a spectrum of twist values albeit within the same helix hand (left or right). On the basis of these observations Wolfe (1985) proposed that it is more appropriate to consider the wall conformation-controlling element as a "twist-rheostat" which, once in operation, could regulate the "strength" of helix hand. No suggestions regarding the molecular meaning of this analogy were made.
Rather little is known about the genetic regulation of macrofiber formation. Saxe and Mendelson (1984a) have identified two genes, fibA and fibB, involved in macrofiber production. fibA causes a short-lived division suppression and thus results in poorer structures whereas fibB governs a prolonged suppression and leads to highly organized macrofibers. Neither of the mutations, however, were implicated in the helix hand determination. In another study these authors isolated an SPO-l resistant mutant of C6D, a strain that grows as left-handed structures at 20°C (Saxe and Mendelson, 1985b). The phase resistant lesion mapped in the gtaC region and affected macrofiber formation. This gene is known to code for a phosphoglucomutase, an enzyme responsible for glucosylation of teichoic acids. Although gtaA and gtaB mutations lead to the same phenotypic defect in teichoic acid, these mutations do not alter the macrofiber morphology in any detectable way. The reason why gtaC alone affects the fiber production is not clear at present.

lyt-1, lyt-2 and lyt-15 were mentioned earlier in connection with flagellation. Mutations in any of these genes affect the cellular content of autolytic enzymes and result in suppression of cell separation, a condition conducive to macrofiber formation. Recently, a strain of B. subtilis carrying Tn917, a Streptococcus transposon that confers erythromycin resistance, has been isolated that grows as multistrand helical structures (Streips, personal communication). Originally this strain was found in a collection of insertion mutants containing a copy of the transposon at random locations around the chromosome. The site of insertion has not yet been mapped.
Rationale and Nature of This Investigation

If the observed conformational states indeed reflect the orientations wall polymers can assume, a knowledge of the principles underlying twist regulation should provide a deeper understanding of the dynamic nature of the Gram-positive cell wall. Although kinetic and physiological aspects of the inversion process have been studied in some detail, the biochemical basis of it has not been investigated.

The five fold higher sensitivity of LH macrofibers to lysozyme suggested that the modifications which make LH helices a better substrate must arise during the inversion process if the LH structures were produced from RH macrofibers. Since susceptibility to muramidase has been attributed to changes in the chemical identity of wall polymers, it seemed reasonable to assume that a biochemical analysis of the cell envelope may provide clues to the mechanism of hand inversion.

Cell walls from the strains exhibiting various ranges of twist were purified and analyzed for overall composition. Molar ratios of the components constituting peptidoglycan, teichoic acid and teichuronic acid were determined using appropriate chemical and enzymatic assays. Similar analyses were conducted on cells grown in the presence of factors affecting conformational states in order to identify surface components relevant to helix hand inversion. Since the degree of cross-linking and glycan chain length significantly influence the structural integrity of peptidoglycan (Rogers, Perkins and Ward, 1980), these parameters may be of importance in determining the glycan orientation. The former was measured utilizing the
classical procedure involving DNP-derivatization of purified walls. However, keeping in view the variable nature of available detection methods, no attempts were made to estimate the chain length.

Polypeptides are important constituents of the Gram-positive walls. The experiments by Favre et al. (1985) and Wolfe (1985) have established the necessity for a surface protein(s) in both temperature- and nutrition-induced inversions to left handedness. Thus a characterization of the wall associated polypeptides was undertaken to identify the putative left-twist protein (LTP). Accessibility of this protein to trypsin digestion in intact cells provided an important criteria for its identification. These investigations were combined with physiological studies in which the effects of the interaction between various twist-influencing agents on the development of conformational states were examined.

An alternate and effective way to detect helix-hand specific surface modifications is to raise antibodies with specific affinity for the macrofiber surface of a particular twist state. It was hoped that, once available, these antibodies could be utilized to study surface migration during the inversion process. In the first attempt, rabbits were immunized with LH and RH structural forms of FJ7. The antisera were tested against macrofibers using immunofluorescence techniques. Encouraged by the initial results, efforts were made to raise twist specific monoclonal antibodies. Finally, insertional mutagenesis with a Streptococcus transposon has been used for isolation of mutants pertinent to the study of twist regulation.
In summary, this project was to focus on characterization of the macrofiber cell surface from a biochemical standpoint in order to comprehend the dynamic nature of the Gram-positive cell wall in greater detail.
CHAPTER 2

MATERIALS AND METHODS

The *B. subtilis* 168 strains used in this investigation and their helix phenotypes are listed in Table 1.

**Media and Growth Conditions**

All strains were regularly maintained on the solid TBAB medium which consisted of tryptose blood agar base (Difco Laboratory, Detroit, Mich.), 33.0 g/l and Bacto-agar (Difco), 5.0 g/l. For liquid cultures, the cells were grown in TB broth which was prepared by dissolving tryptose (Difco), 10.0 g; sodium chloride, 5.0 g and beef extract (Difco), 3.0 g in one liter of deionized water.

The medium for spheroplast formation (SMMP/BSA) was made by mixing equal volumes of 2XSMM (+BSA) and 4XPAB. 2XSMM is composed of sucrose, 342.3 g/l; maleic acid, 4.64 g/l; and MgCl₂.6H₂O, 8.15 g/l. For 4XPAB, 70 grams of Antibiotic media #3 (Difco) was dissolved in one liter of deionized water. The two media were steam sterilized individually and then filter sterilized BSA (2% final concentration) was added to the former.

Spheroplasts were regenerated on the plates containing DMB medium consisting of the following components: Bacto-agar, 8.0 g/250 ml 1M sodium succinate, 500 ml, pH 7.3; 5% casein hydrolysate, 100 ml; 5% yeast extract, 10 ml; K₂HPO₄ and KH₂PO₄ , 3.5 and 1.5 g/100 ml,
Table 1. *B. subtilis* strains used in this study.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>HELIX PHENOTYPE</th>
<th>SOURCE</th>
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<td></td>
<td></td>
<td>20°C 48°C</td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trp thy</td>
<td>NA NA</td>
<td>N. Mendelson</td>
</tr>
<tr>
<td>FJ7</td>
<td>metC lyt-2</td>
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<td>FJ7US1</td>
<td>metC lyt-2 Em Cm R R</td>
<td>RH LH</td>
<td>Constructed for use in this study</td>
</tr>
</tbody>
</table>

- **a** Plasmid mediated resistance to antibiotics: erythromycin, 1 μg/ml; chloramphenicol, 15 μg/ml.
- **b** Helix phenotype of macrofibers grown in TB. The medium was supplemented with 20mM magnesium sulfate for growth of FJ7 and FJ7US1 at 20°C. RH, right-handed; LH, left-handed
- **c** Not applicable.
respectively; 50% glucose, 10 ml; 1 M MgCl₂, 20 ml and 2% BSA, 2 ml. The constituents were autoclaved separately and then mixed in appropriate proportions. BSA was sterilized by filtration through a Millipore filter (0.45 μm pore size, Millipore Corp.). This medium was supplemented with 20 μg/ml of appropriate amino acids to meet the auxotrophic requirements. Antibiotics, erythromycin and chloramphenicol, were added if selection for resistance was desired. The cells used for spheroplast formation were grown in #416 medium composed of tryptone, 20.0 g/l; yeast extract, 10 g/l and NaCl, 10 g/l.

The effects of ions were studied in a medium designated T which was prepared by exclusion of NaCl and beef extract from TB broth. The macrofiber producing strains were propagated as helical structures in TB or T and were subcultured daily by transferring small fragments of "young" fibers to fresh medium.

**Determination of Helix Hand and Helical Twist**

The method for determining helix hand has been previously described by Mendelson (1978). During the normal course of length extension, the end-loop in a helical structure undergoes rotational movements, the direction of which can be used for a rapid estimation of the handedness. For the purpose of determining their helical orientation, macrofibers were directly observed under a binocular dissecting-microscope. A structure with the loop turning in a clockwise fashion when viewed from its tail end was considered right-handed. The direction of rotation in a LH macrofiber, on the other hand, would be
counterclockwise. For closer examination, wet mounts were observed under a phase contrast microscope. If the helically wrapped strands of a structure crossed the plane with their lower ends on the left and higher ends towards the right while focusing on the uppermost layer, it was regarded as being right-handed. Once again, the cellular filaments of LH macrofibers exhibited exactly the opposite orientation.

The static twist (N), measured as number of turns per mm, can be calculated using the mathematical equation, $2\pi RN = \tan \alpha$, where $\alpha$ and $2R$ are the helix angle and macrofiber diameter, respectively (Mendelson, Favre and Thwaites, 1984). This equation assumes that the filaments in a given structure are closely and uniformly packed and thus may be used to obtain fairly accurate results for macrofibers of high twist values. With decreasing uniformity and twist, a precise measurement of the diameter and helix angle is difficult and the twist values become highly variable. In most experiments, differences in the overall organization were large and therefore, quantitation of static twist was not deemed necessary. Instead, three representative structures from a culture, growing in a given set of conditions, were examined under a phase contrast microscope and a twist value was assigned as judged by the quality of their organization. As shown in Figure 1a, the qualitative scale used for this assignment spans from -6 to +6 with 0 (disorganized state with zero twist) as its center. The positive and negative signs represent the right- and left-handedness, respectively, of the macrofibers under observation. The structural organizations corresponding to various twist values are shown in Figure 1b.
Figure la. A scale for qualitative estimation of helical twist.

The central vertical line, designated DO, corresponds to 0 twist. The regions to the left and right of this line represent left and right handed domains, respectively, of the twist spectrum with LH\textsubscript{max} and RH\textsubscript{max} as extreme boundaries. Whereas - and + signs refer to RH and LH conformations, respectively, the numbers indicate the degree of twist estimated qualitatively by microscopic examination. LH, left handed; RH, right handed and DO, disorganized.
Figure 1a. A scale for qualitative estimation of helical twist.
Figure 1b. Phase contrast micrographs of macrofibers with structural organization representative of various twist values within the RH domain of the twist scale.

The structures were produced by growing F77 in T supplemented with varying amounts of ammonium sulfate and D-alanine. Macrofibers with similar helical organization were used to characterize the LH domain. (i) D3; (ii) RH (+1); (iii) RH (+2); (iv) RH (+3); (v) RH (+4); (vi) RH (+5); (vii) RH (+6). D3, disorganized; RH, right-handed; LH, left-handed.
Effect of Twist-influencing Factors

The presence of D-alanine, trypsin and ammonium sulfate in the growth medium are known to affect the attainment of final conformational state in a number of strains including FJ7 (Wolfe, 1985; Mendelson and Favre, 1987). To investigate the manner in which these factors compete to shape the eventual organization, two combinations were tested; D-alanine (sigma) vs ammonium ions and trypsin (299 unit per mg; Worthington) vs ammonium sulfate. T broth, to which various concentrations of these agents were added, was seeded with small fragments of macrofibers produced in T without any supplements. The fragments were allowed to regenerate into complete helical structures at 20°C for 18 hours before they were microscopically examined. The static twist was qualitatively estimated as described above.

The effect of intestinal alkaline phosphatases and proteases on the development of helical twist was determined in a similar manner. TB medium (pre-warmed at 48°C) containing various concentrations of these proteins were inoculated with small fragments of RH macrofibers (grown in TB at 20°C) and incubated at 48°C for three hours. No enzyme was added to the control cultures. At the end of incubation, the resultant structures were assigned twist values as before.

Isolation and Purification of Cell Walls

*B. subtilis* 168 was grown as a batch culture at 20°C and 48°C in medium (T or TB) supplemented with thymine and tryptophan (20 µg/ml each). When the cells reached mid to late exponential phase
(OD=0.6 at 660 nm), they were harvested by centrifugation at 7,000 revolutions per minute (rpm) at 4°C for 20 min. The macrofiber producing strains, FJ7, PS6µB and 8-4A were grown as static cultures. A flask containing 7 liters of suitable medium was seeded with small fragments of macrofibers. The seed had been prepared by breaking five to seven middle-sized fibers into small pieces with a sterile toothpick. To provide large surface area, the inoculated medium was dispensed equally into 14 aluminum trays (47X35 cm) which were then incubated at either 20°C (for 18 hours) or 48°C (for 4 hours). At the end of the incubation period, the culture was pumped directly into two pre-chilled, inverted flasks whose mouths were fitted with small funnels. The macrofibers were allowed to gravity-sediment into the funnels for four hours at 4°C. Whereupon, they were collected and pelleted by centrifugation at 10,000 rpm for 20 min.

The procedure for isolation and purification of cell walls was that of Fein and Rogers (1976). Briefly, the harvested cells were washed twice with cold saline, resuspended in 50 ml of 4% sodium dodecyl sulfate (SDS) and incubated at 100°C for 20 min. The cell suspension was washed with saline by centrifugation at 15°C to remove all traces of SDS. The pellet was resuspended in sodium phosphate buffer (0.05 M, pH 7.0) and cells were disrupted by sonication (Sonifier, W 350; Branson Sonic Power Co.) until 80-90% breakage (monitored using a phase contrast microscope) was achieved. Low speed centrifugation (2000 rpm for 5 min) was used to pellet unbroken cells
and the supernatant was treated with DNase (250 µg/ml; Sigma) and RNase (1 mg/ml; Sigma) at 4°C for 60 min. To rid the walls of membrane components, the crude preparation was first subjected to trypsin digestion (500 µg/ml) in Tris-HCl buffer (50 mM, pH 8.0) for 4 hours at 37°C. The walls were then washed several times, resuspended in deionized water and extracted with 40% phenol (final concentration) at 0°C for 30 min. Finally, the emulsion was centrifuged at 12,000 rpm for 20 min. The milky white layer from the interface was carefully collected and washed several times with cold deionized water to free it from residual phenol. The purified walls were lyophilized and stored at -20°C until further use.

**Lysozyme Sensitivity of Purified Cell Walls**

To compare the susceptibility of mucoprotein from various strains to muramidase, purified walls (500-800 µg/ml) were suspended in phosphate buffer (100 mM, pH 7.0) and the initial optical density (450 nm) was adjusted to a value within 0.50 to 0.60. The suspensions were warmed to 37°C before egg-white lysozyme (crystallized, dialyzed and lyophilized; Sigma) was added to a final concentration of 5 µg/ml. The incubation was continued at 37°C with mechanical shaking to keep the cell wall preparations suspended. The decrease in turbidity, i.e. dissolution of murein, was measured at 450 nm as a function of time using optically matched cuvettes of one cm path length until the optical density fell below 10% of the original value.
Methods for the biochemical analysis of murein have previously been described by Fein and Rogers (1976). 2.5 mg of purified cell walls were hydrolyzed with 6 N, 4 N and 2 N hydrochloric acid at 100°C for 16, 4 and 2 hours, respectively. HCl was removed by resuspending the hydrolyzed preparations in deionized water and drying over NaOH pellets under vacuum several times. The residues were dissolved in water and stored at -20°C.

Estimation of Amino Acids and Amino Sugars

6 N and 4 N samples were analyzed for amino acids and amino sugars, respectively, with a Beckman automated amino acid analyzer (model 121). 100 ul hydrolysates were diluted to 2.5 ml with citrate buffer (350 mM, pH 2.2) and applied to the anion exchange columns. The separation of glucosamine and galactosamine on the short column was achieved by 350 mM/210 mM buffer (pH 5.25) regime. All other components were separated on the long column utilizing 3.25-4.25 pH scheme. A representative elution profile is shown in Figure 2. The area under the peaks was integrated with a Spectro Physics 4000 central processor. To monitor the fluctuations introduced by the analyzer, arginine and valine were used as internal standards in every run and the variations were adjusted for in the final calculations.

For estimation of ester-linked D-alanine substitutions on teichoic acid, 2.5 mg of purified walls were suspended in 500 ul of 100 mM ammonium hydroxide and incubated at room temperature for 6 hours. Since ester bonds are alkali labile, this treatment results in release
Figure 2. A representative elution profile of various components in a cell wall hydrolysate chromatographed on an amino acid analyzer.

4N or 6N hydrolysates were analyzed on a Beckman amino acid analyzer under appropriate conditions (see Material and Methods for details). The numbers are indicative of the retention time (RT) for each constituent. Glucosamine, 342; galactosamine, 374; muramic acid, 1148; glutamic acid, 1190; alanine, 1498 and diaminopimelic acid, 1948. Arginine (RT, 970) and valine (RT, 1844) were used as internal standards.
of the ester-bonded D-alanine residues. After completion of the incubation period, samples were centrifuged in an Eppendorf microfuge for 6 min. The supernatant was collected, washed in vacuo and resuspended in water. The pellet was washed once by centrifugation and hydrolyzed with 6 N HCl for 16 hours. Alanine content of both pellet and supernatant was measured on the analyzer as outlined above. The extent of substitution was expressed as the percent-fraction of total alanine released in the supernatant.

Enzymatic and Chemical Assays

2 N hydrolysates were assayed for glycerol, glucose, phosphate and glucuronic acid. Since glycerol is usually present as both free and phosphate-linked forms in hydrolyzed wall preparations, it is essential to convert the latter into free glycerol molecules for an accurate measurement. A small sample of 2 N hydrolysate was made to 0.05 M with NH₄HCO₃ and digested with alkaline phosphatase (1.75 units, Sigma) at room temperature for 18 hours. The samples were boiled for 5 min and centrifuged in a microfuge (Brinkman Instrument Co, model-5414) for 2 min to remove the heat inactivated enzyme. Glycerol was estimated in the supernatant using glycerokinase (EC 2.7.1.30) and glycerol dehydrogenase (EC 1.1.1.6) as described by Wieland (1963).

A method involving reactions catalyzed by glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7.) was employed to measure glucose content in wall hydrolysates (Sigma Technical Bulletin No.510). For total phosphate determination, samples of hydrolyzed walls were mixed with 10% Mg(NO₃)₂ (in ethanol) and the mixture was evaporated to
dryness over a strong flame. The ashing process converted organically bound phosphate to inorganic form. The amount of the latter was determined by its reaction with ascorbic-molybdate solution (Ames and Dubin, 1960). Glucuronic acid in various samples was estimated according to the procedure of Disch (1947).

The Degree of Cross-Linking

The extent of peptide cross-bridge formation in murein was determined by the classical method of Ghysen, Tipper and Strominger (1965). Lyophilized walls (2 mg) were suspended in 300 μl of 1% K₂B₄O₇. To this, 30 μl of FDNB reagent (2,4-dinitrofluorobenzene, 130 ul in 10 ml of 100% ethanol) was added and the suspension was incubated at 60°C for 30 min. After acidification with concentrated HCl (150 μl), the mixture was extracted three times with 300 μl of ether to remove DNP derivatives of any free amino acids. The aqueous layer containing the DNP-derivatized cell wall was then hydrolyzed for 6 hours at 95°C and again extracted with ether. DNP-diaminopimelic acid (DNP-dap) was recovered from the aqueous layer by extraction with equal volume of water saturated n-butanol. The samples were dried in vacuo and redissolved in n-butanol. Various components were resolved on thin layer chromatography plates which were developed in benzyl alcohol: chloroform:methanol:water:NH₄OH (30:30:30:6:2). The spot corresponding to DNP-dap was scraped, vigorously mixed with 10 mM ammonia:methanol (1:1) and centrifuged to eliminate silica G. The optical density of the supernatants was measured at 360 nm. A control consisting of wall amino acids was treated in an identical manner. The extent of cross-
linking was deduced from the fraction of total diaminopimelic acid residues accessible to derivatization by FDNB.

Estimation of Enzyme Activities

The assay for alkaline phosphatase was that of Garen and Levinthal (1960). One ml of p-nitrophenylphosphate was mixed with 2 ml of Tris-HCl (1.5 M, pH 8.0) and incubated in a spectrophotometer (Spectronic 700, Bousch & Lomb) for 4-5 min for temperature equilibration. To this solution, 100 µl of the enzyme preparation was added and the increase in absorbance (at 410 nm) was monitored using an automatic recorder (Bousch & Lomb, model-D5116 6F). The enzyme activity, expressed in units/mg protein, was calculated from the linear portion of the curve. One unit liberates one micromole of p-nitrophenol/min at 25°C, pH 8.

For measurement of protease activity, 1 ml of 0.5% Azocoll (Calbiochem) suspension in 100 mM potassium phosphate buffer (pH 7.0) was mixed with 100 µl of the test solution. The suspension was incubated at 48°C. After 60 min, the particulate fraction was removed by centrifugation in a microfuge (9000xg for 5 min) and optical density of the supernatant was determined at 520 nm. The enzyme activity was expressed as trypsin equivalent, defined as the amount of trypsin in nanograms required to match the absorbance caused by 1 µg of the test enzyme. Total protein was measured using the standard method of Lowry et al. (1951).
Isolation of Cell Wall Associated Proteins and Determination of Their Protease-Sensitivity

The method adopted for isolation has been described by Brown (1972). Cells, grown either as batch culture or macrofibers, were harvested as before. After two washes with cold saline (0.9% NaCl), the wet weight of the pellet was determined. The cells were resuspended in Tris-HCl (50 mM, pH 8) containing 5 M LiCl at a concentration of 150-200 mg cell mass/ml. Since *B. subtilis* walls are known to secrete proteases, phenylmethylsulfonyl fluoride (Calbiochem) was added to a final concentration of 3 mM to prevent any proteolysis. The extraction mixture was incubated at 0°C for 60 min with intermittent agitation before it was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected and extensively dialyzed against deionized water at 4°C to remove lithium chloride. Finally, the protein solution was lyophilized, dissolved in 0.5-1 ml of distilled water and stored at -20°C.

There is evidence for involvement of a trypsin sensitive wall-associated polypeptide(s) in the attainment of leftward orientation. To identify such a component, 50 µl of LiCl extract (350 µg protein), made from cells grown in conditions conducive to the formation of LH macrofibers, was mixed with 10 µl of trypsin (0.1 µg/ml final concentration). The digestion mixture was incubated at 37°C in a shaker bath. At various time intervals, 8 µl samples were withdrawn and the protein profile was analyzed by gel electrophoresis (details of the method is described below).
Enzyme Inactivation

Some intestinal alkaline phosphatases (AP) have been shown to interfere with the development of leftward twist (henceforth referred to as its biological activity) in FJ7. That this effect is indeed due to AP's enzyme activity, rather than being a result of its molecular charges or contaminating proteases, was demonstrated by inactivation experiments. 50 mg of sheep intestinal phosphatase was treated with 8 M urea and 10% β-mercaptoethanol for 45 min at 25°C. The inactive enzyme was dialyzed extensively against deionized water at 4°C. The control consisted of a solution containing only the denaturing agents dialyzed in parallel with the inactive enzyme.

For heat inactivation, a 10 mg/ml solution of AP was incubated at 60°C in a water bath. At various time intervals samples were withdrawn and assayed for AP, protease and biological activities as described.

Protein Purification

To purify the alkaline phosphatase (AP) activity away from protease contaminants, 15 mg of dog intestinal AP (4 units/ml, purchased from Sigma) was applied to a hydroxylapatite column (1.5 X 31 cm, Bio-Rad) equilibrated with phosphate buffer (10 mM, pH 8.0) at 20°C. The column was first eluted with the same buffer and 4 ml fractions collected until no further 280 nm absorbing material could be detected in the eluent. This first peak was designated UNF-1. Elution was continued with a linear gradient of NaCl (10 mM to 300 mM) in phosphate buffer (10mM, pH 8.0) which resulted in appearance of a peak,
CF-1, at approximately 100 mM NaCl. Since UNF-1 contained most of the initial AP activity, it was further fractionated on a DEAE-cellulose (DE-52, Whatman) column (0.5 x 11 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C. The elution was performed with a step gradient of NaCl (concentrations: 0.05 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M) using the initial buffer. One ml fractions were collected and protein was detected by measuring optical density (OD) at 280 nm. Fractions from each peak were pooled separately and dialyzed extensively against deionized water at 4°C. AP, protease and biological activities in every peak were determined as described earlier. Although the protein recovery was variable, the elution profiles were highly reproducible under the stated conditions.

Isolation of Plasmid DNA

The most commonly used method for plasmid isolation is the alkaline extraction procedure (Birnboim and Doly, 1979; Rodriguez and Tait, 1983) that yields plasmid preparations virtually free of contaminating chromosomal DNA. Although a fraction of it becomes single stranded, plasmid DNA isolated using this regime is suitable for both transformation and restriction enzyme analysis.

Rapid Isolation (Miniscreen)

Cells from 1.5 ml overnight culture grown in TB supplemented with appropriate antibiotics were harvested by centrifugation in an Eppendorf microfuge for 1 min. The pellet was washed once with 1 ml of SET buffer (20% sucrose; 50 mM Tris, pH 7.6; 50 mM EDTA) and resus-
pended in 150 µl of the same buffer. To this suspension was added 20 µl of RNase buffer (pancreatic RNase A, 10 mg/ml in 0.1 M Na-acetate, 0.3 mM EDTA, pH 4.8, pre-heated to 80°C for 10 min) and 50 µl of lysozyme (in TEN buffer consisting of 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 mM NaCl). The mixture was briefly vortexed prior to incubation at room temperature for 15 min, whereupon 350 µl of lytic-mix (1% SDS, 0.2 N NaOH) was added. Spheroplasts were lysed by gentle mixing and the tubes were kept on ice. To neutralize the clear lysate 250 µl of Na-acetate buffer (3.0 M, pH 4.8) was added after 10 min and incubation on ice was continued for an additional 60 min. The protein-DNA clot formed during this period was removed by centrifugation in a microfuge for 6 min at 4°C. An equal amount of isopropanol was added to the supernatant and plasmid DNA was recovered by centrifugation in a microfuge for 10 min. The DNA pellet was washed with 70% ethanol, dried in a vacuum desiccator and finally dissolved in 10 µl of deionized water.

Large-scale Preparation

Large quantities of plasmid needed for transformation were prepared from 500 ml of overnight culture grown in conditions described above. Cells were harvested and washed with 30 ml of TEN buffer by centrifugation (6500 rpm for 10 min) at 4°C. The pellet was resuspended in 10 ml of SET buffer and treated with 2 ml of lysozyme (10 mg/ml) at 37°C. After spheroplast formation (>90%), the volume was adjusted to 30 ml with SET buffer and 65 ml of lytic mixture was added. The suspension was gently swirled and incubated first at 50°C for 30 min and then kept on ice bath for 25 min. Incubation was continued for an
additional 40 min after which it was neutralized with acetate buffer (3 M, pH 4.8). The chromosomal DNA clot was removed by centrifugation at 6500 rpm for 20 min. The supernatant was carefully filtered through a clean Kimwipe and treated with 0.2 ml of RNase buffer at 40°C for 20 min. An equal volume of chloroform:buffer-saturated phenol (1:1) was added to the filtrate, swirled vigorously and centrifuged at 6,500 rpm at 4°C for 10 min to separate the aqueous and organic phase. The upper layer which contained most of the plasmid DNA was collected and re-extracted until it turned clear. The DNA was precipitated overnight at -20°C in two volumes of ice-cold ethanol and recovered by centrifugation at 10,000 rpm at 4°C for 20 min. The air dried pellet was dissolved in 1 ml of TEN buffer.

Restriction Endonuclease Digestion

To confirm the presence of the desired plasmid in cells a "miniscreen" preparation was subjected to digestion by restriction endonucleases (purchased from United States Biochemical corporation, Cleveland, Ohio). The following components, mixed in a sterile microfuge tube, constituted the reaction mixture: 3-5 µg of plasmid DNA in 10 µl of deionized water, 4 µl of an appropriate restriction endonuclease (4 units), 2 µl of 10X reaction buffer and water to make the final volume to 20 µl. The digestion mix was incubated at 37°C for 2 hours after which the reaction was terminated by addition of 0.5 M EDTA (pH 7.5) to a final concentration of 10 mM. The resultant fragment patterns were analyzed by gel electrophoresis. λCI857Sam7 DNA,
digested with *HindIII* was regularly used for sizing of restriction fragments. The following endonucleases and buffer systems (IX) were utilized in the current investigation:

**BamHI:** 25mM Tris-HCl (pH 7.8), 100 mM NaCl, 10 mM MgCl₂, 100 µg/ml BSA, 1 mM dithiothreitol.

**EcoRI:** BamHI buffer.

**HindIII:** 25 mM Tris-HCl (pH 7.8), 50 mM NaCl, 10 mM MgCl₂, 100 µg/ml BSA, 1 mM dithiothreitol.

**Spheroplast Transformation and Regeneration**

pTV32ts, a 15.6 kb plasmid containing Tn 917, was introduced in FJ7 by the spheroplast transformation method (Spizizen, 1981). The recipient cells were grown in 10 ml of #416 medium supplemented with erythromycin (1 µg/ml) and chloramphenicol (15 µg/ml) at 30°C for 18 hours. 0.02 ml culture was withdrawn for viable count and the remainder was centrifuged at 3,000 rpm in GLC-1 (Sorvall) for 15 min at room temperature. The pellet was resuspended in 2 ml SMMP/BSA. 0.5 ml of a lysozyme stock solution (50 mg/ml in SMMP) was added to the suspension which was then incubated at 37°C with gentle shaking. Spheroplasts were pelleted, washed once with 5 ml SMMP by centrifugation as before and resuspended in 2 ml of SMMP/BSA.

For transformation, 0.5 ml of spheroplast preparation was mixed with 1.5 ml of polyethylene glycol-1000 (40%), 5-10 µg/ml of DNA and equal volume of 2X SMM. After two minutes, the suspension was diluted with 5 ml SMMP/BSA prior to centrifugation at 3,000 rpm for 10 min.
The pellet, suspended in 1 ml of SMMP/BSA, was gently shaken at 33°C for 90 min before it was appropriately diluted and plated on DMB (regeneration medium) plates containing erythromycin and chloramphenicol (1 µg/ml and 15 µg/ml, respectively). The spheroplasts were allowed to regenerate at 33°C for 48 hours after which they were isolated as single clones for further phenotypic characterization.

**Insertional Mutagenesis**

In *B. subtilis*, the methods available for obtaining random insertions of a transposon in the host chromosome (Youngman et al., 1983; Vandeyar and Zahler, 1986; Streips, 1986) are essentially variations of a common theme. A plasmid, pTV32ts in the current investigation, with temperature sensitive replicon carrying the transposon is introduced into a strain of interest. The cells are then challenged with appropriate antibiotic at restrictive temperature; generally, the survivors are insertional mutants.

FJ7US1, a derivative of FJ7 harbouring pTV32ts, was used for insertional mutagenesis. The cells were grown in 20 ml of TB supplemented with chloramphenicol (5 µg/ml) and erythromycin (1 µg/ml) at 30°C. After 18 hours, the culture was diluted (100 times) into 2 liters of pre-warmed (48°C) TB containing erythromycin (1 µg/ml) and growth was continued at 48°C for 24 hours. Since FJ7US1, being a lyt mutant, grows as long filaments, spheroplast formation prior to marker selection was necessary to avoid isolation of clones with mixed genotype. Hence, 5 ml of this culture was withdrawn and treated with lysozyme as previously described. Spheroplasts were regenerated on DMB.
plates supplemented with erythromycin (1 μg/ml) at 48°C. The erythromycin resistant clones that exhibited sensitivity to chloramphenicol were considered insertional mutants.

For the selection of mutants impaired in temperature induced hand inversion, insertional clones were grown at 20°C in conditions conducive to macrofiber formation. Fragments of these structures were seeded in TB at 48°C and allowed to grow for 3 hours. The mutants that retained RH orientation at high temperature were to be regarded as "fixed" RH mutants.

**Gel Electrophoresis**

Proteins and restriction endonuclease generated DNA fragments were resolved on polyacrylamide and agarose gels, respectively. The method used for polypeptide separation was that of Laemmli (1970). A standard procedure described by Maniatis et al. (1982) was followed for the resolution of restriction fragments.

**Polyacrylamide Gel Electrophoresis**

A gel-slab (13X13X0.2 cm) consisting of 1.5 cm long stacking and 11.5 cm long separating parts was prepared. For the separating portion, 10% acrylamide and 0.8% methylene-bisacrylamide (Sigma) in 350 mM Tris-HCl (pH 8.8) containing 0.1% SDS were polymerized chemically by addition of 0.025% of tetramethylene
diamine (TEMED) and ammonium persulfate. 3% acrylamide and 0.08% bisacrylamide in 0.125 M Tris-HCl (pH 6.8) with 0.1% SDS were polymerized similarly to form the stacking part of the gel. 30-40 μg of protein samples were mixed with equal
volumes of 2X loading buffer (0.0625 M Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol and 0.001% bromophenol blue) and heated in a boiling water bath for 5 min prior to application. The electrode buffer was composed of 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS. SDS was excluded from gels and buffers when non-denaturing conditions were required. All gels were run at 100 volts until the tracking dye reached approximately 1 cm from the lower end. After electrophoresis, the gels were fixed in 50% trichloroacetic acid (TCA) for 12 hours prior to staining with 0.1% Coomassie brilliant blue. They were then diffusion-destained with 7% acetic acid containing 5% methanol. When necessary, gels were silver-stained using procedures provided by the manufacturers of the reagents (Bio-Rad). The molecular weights of unknown polypeptides were estimated with the aid of marker proteins.

Activity-staining

p-Nitrophenol, a yellow colored compound, is a product of the reaction between alkaline phosphatase and its substrate p-nitrophenyl phosphate. This reaction was utilized to identify alkaline phosphatase among a host of other proteins on polyarylamide gels. Freshly run non-denaturing gels were rinsed thoroughly with 1.5 M Tris-HCl (pH8.0), overlayed with 1% agarose containing 3.0 mM p-nitrophenyl phosphate and incubated at 37°C for 10 min. Alkaline phosphatase bands were detected as clearly visible yellow spots.

Agarose Gel Electrophoresis

A "mini" horizontal electrophoretic apparatus (GNA-100, Pharmacia) was used to analyze the restriction-fragment patterns.
Agarose (0.8% final concentration) was dissolved in TBE buffer (90 mM Tris-HCl pH 8.2, 2.5 mM EDTA, 89 mM boric acid) in a boiling water bath. The solution was cooled to 50°C before the gel was cast in an acrylic holder (82x106 mm). DNA samples were mixed with 1/3 volume of the 6X loading buffer (0.25% bromophenol blue, 40% sucrose in deionized water) and 15 μl were added to each of the wells. Electrophoresis was performed at constant 40 Volts (4 V/cm) until the tracking dye reached within 0.5 cm from the anode-edge. The gels were stained with an aqueous solution of ethidium bromide (5 μg/ml) and DNA fragments were visualized using an violet transilluminator (La Jolla Scientific Co).

**Antibodies Against Macrofiber Cell Surface**

Macrofibers of various conformational states were washed twice with saline and the structures suspended in saline were emulsified in an equal volume of Freund’s incomplete antigen (85 ml mineral oil and 15 ml of Arlacel A; Sigma) using a double hub needle. The freshly prepared emulsions served as antigens. Healthy New Zealand white rabbits, weighing 2-3 kg, were injected subcutaneously with 350-400 mg antigen (wet weight). The animals were given two booster injections, each 10 days apart, prior to bleeding. The blood, which was obtained using standard methods, was allowed to stand at room temperature for 90 min and then centrifuged at 2000 rpm for 30 min. The supernatant was carefully collected and incubated at 56°C for 30 min to inactivate complements.
To obtain LH specific antibodies, 500 µl of undiluted serum was adsorbed onto freshly grown RH macrofiber for 120 min at room temperature after which the cells were removed by centrifugation. The supernatant was allowed to react with phosphate buffer-saline (PBS) washed fragments of LH macrofibers in a 90 µl reaction mixture. The antibody coated cells were washed four times with PBS and incubated with 90 µl of fluorescein-conjugated goat anti-rabbit antibodies (1:30 dilution; Cappel Laboratories) for 90 min at room temperature. The samples were spread on clean glass slides, rinsed gently with PBS and observed under a fluorescence-microscope equipped with incident illuminator (La Jolla Scientific Co. Inc., La Jolla)

Monoclonal antibodies were raised by Dr. David L. Lucas of the Department of Microbiology and Immunology using standard methods.
CHAPTER 3

RESULTS

The conformational states that the *B. subtilis* macrofibers exhibit in various growth environments have been proposed to represent the orientations of peptidoglycan (Mendelson, 1982b). Temperature-induced hand inversion, the prime subject of the present study, is an example in extremity of such twist-adjustment response. The strains used for investigation of the biochemical mechanism underlying inversion phenomenon are described in Table 1. FJ7, a lyt− mutant derived from wild type strain 168 by chemical mutagenesis, is capable of helix hand inversion at 48°C (hence switchable). PS6µB and 8-4A, derivatives of FJ7, were isolated as mutants which formed LH and RH structures, respectively, at all temperatures and thus were considered impaired in the inversion process (henceforth referred to as "fixed" mutants) (Favre, 1984). In addition to growth temperature, the nutritional status of the culture medium and exogenously added factors, such as D-alanine and ammonium sulfate, exert considerable influence on helical organization. Therefore, both nutritional and temperature regimes were used to introduce alterations in the twist states through out the course of this study.

The problem of twist determination and inversion has been addressed at various levels of specificity. For a general physiological characterization, both purified walls and the cells derived
from RH and LH macrofibers were tested for their lysozyme sensitivity. To estimate the contribution of the relevant surface polymers in twist regulation, effects of various twist modulating agents on the steady state conformation were studied. This matter was further explored by a detailed chemical analysis of purified walls. The overall composition, ester-linked D-alanine substitution on teichoic acids and the extent of cross-linking were determined. Results of an attempt to identify the putative LTP (left-twist protein) and a method for insertional mutagenesis in the macrofiber system have also been presented. Finally, the relevance of these findings are discussed in the context of twist determination, maintenance and inversion.

Sensitivity of Macrofibers and Purified Cell Walls to Lysozyme

Certain biochemical modifications of peptidoglycan are known to alter its susceptibility to muramidases. The removal of O- and N-substituted acetyl groups, for example, results in the loss of resistance to lysozyme digestion (Brumfitt, Wardlow and Park, 1958; Araki et al., 1972). In macrofibers, LH structures produced by FJ7 at 48°C were found to be fivefold more sensitive to lysozyme relative to their 20°C grown RH counterpart (Mendelson, Favre and Thwaites, 1984). Thus, the changes that make LH helices a better substrate, must occur during the hand inversion process. Since muramidases specifically hydrolyze peptidoglycan it was argued that if the differential sensitivity is due to a unique biochemical identity of murein in LH structure, the purified walls should exhibit similar differences.
Lysozyme Sensitivity of Cell Walls

Cell walls purified from FJ7 grown at 20°C and 48°C were suspended (500-800 µg/ml) in phosphate buffer (100 mM, pH 7.0). The suspensions were prewarmed at 37°C prior to addition of lysozyme to a final concentration of 5 µg/ml. The kinetics of digestion was followed by optical density measurement (450 nm) at various time intervals. Identical wall suspensions incubated in parallel without addition of the enzyme served as controls for autolysis. As shown in panel B of Figure 3, the dissolution rate for walls from RH structures of FJ7 is slightly higher compared to that for LH forms. For 168 (Figure 3, Panel A), the rates of lysozyme-induced lysis are almost identical.

In order to determine if the cell surface of "fixed" RH and LH mutants respond differently to hydrolytic enzymes, walls purified from 8-4A and PS6µB were subjected to lysozyme digestion under conditions outlined above. The results are shown in Figure 4 (panel A, PS6µB and Panel B, 8-4A). The rate of loss of turbidity for both strains are comparable irrespective of the temperature at which macrofibers were grown. If the observations for both cell walls and intact macrofibers are valid then it seems likely that the failure to detect differential lysozyme sensitivity in walls isolated from LH and RH is due to loss of the factor(s) which render the former prone to and the latter resistant to muramidases during purification procedures. The most commonly used methods for wall isolation include an extensive digestion of crude wall preparation with proteases to remove surface associated polypeptides. That some of these proteins may enhance lysozyme binding to intact cells is a viable possibility.
Figure 3. Lysozyme sensitivity of cell wall purified from 168 and FJ7 grown at 20°C and 48°C.

Purified walls were suspended (500-800 μg/ml) in phosphate buffer (100 mM, pH 7.0). Lysozyme (5 μg/ml) was added to the prewarmed suspensions and mixtures were incubated at 37°C. The lysis was monitored by measuring optical density at 450 nm at various time intervals. Panels A: O, 168 (20°C) and Δ, 168 (48°C); panel B: D, FJ7 (20°C) and X, FJ7 (48°C). Smaller symbols represent autolysis-controls for corresponding samples.
Figure 3. Lysozyme sensitivity of cell wall purified from 168 and RJ7 grown at 20°C and 48°C.
Figure 4. Lysozyme sensitivity of cell walls purified from PS6μB and 8-4A grown at 20°C and 48°C.

The procedure was same as in Figure 3. Panel A: O, PS6μB (20°C) and △, PS6μB (48°C); panel B: □, 8-4A (20°C) and Ξ, 8-4A (48°C). Smaller symbols represent autolysis-controls for the corresponding sample.
Figure 4. Lysozyme sensitivity of cell walls purified from PS6μB and 8-4A grown at 20°C and 48°C.
Lysozyme Sensitivity of Whole Cells

If the factor(s) responsible for the higher sensitivity of LH macrofibers are lost during wall isolation, the undamaged cells derived from RH and LH structures should demonstrate the differential susceptibility to lysozyme. Freshly grown macrofibers of FJ7, PS6μB and 8-4A at 20°C and 48°C were collected and washed twice with saline by centrifugation at 3,000 rpm for 15 min at room temperature. The pellet was resuspended in phosphate buffer (100 mM, pH 7.0) and gently shaken with glass beads (3mm diameter) until all the macrostructures were converted to small filaments, making the suspension homogeneous. The remaining procedure was similar to the one described for lysozyme digestion of purified walls. Panel B of Figure 5 shows that the lysis patterns of the cells derived from FJ7 macrofibers produced at 20°C and 48°C are not significantly different. 168 grown at 48°C (Figure 5, Panel A), on the other hand, lyses at a rate considerably faster than its 20°C counterpart, especially during the early stage of digestion. Since the autolysis controls show similar differences in the rate of lysis, the increased sensitivity of 48°C cells to lysozyme may not necessarily reflect a biochemical alteration of the wall polymers at higher temperature. The kinetics of lysis for "fixed" mutants, shown in panel A of Figure 6, clearly indicates that both PS6μB and 8-4A cells are equally sensitive to lysozyme.

The helical orientation in FJ7 can be manipulated by changing the composition of the culture medium. For example, the LH structures produced at 20°C in T supplemented with ammonium sulfate (TN) invert to
Figure 5. Lysozyme-induced lysis of whole cells of the strains 168 and FJ7 grown in TB at 20°C and 48°C.

Cells from exponential-phase cultures, growing either as single cells (168) or macrofibers (FJ7; cellular filaments were isolated by gentle disruption of macrostructures), were harvested and washed twice with phosphate buffer (100 mM, pH 7.0) by centrifugation. Remainder of the procedure and symbols are same as in Figure 3. Panel A: 168; panel B: FJ7.
Figure 5. Lysozyme-induced lysis of whole cells of the strains 168 and FJ7 grown in TB at 20°C and 48°C.
Figure 6. Lysozyme-induced lysis of whole cells derived from PS6μB, 8-4A and FJ7 macrofibers.

PS6μB and 8-4A were grown in TB at 20°C. The LH and RH structural forms of FJ7 were obtained by cultivation in T supplemented with ammonium sulfate (TN) and D-alanine (TND), respectively. For experimental procedure, see Figure 5. Panel A: O, PS6μB and Δ, 8-4A; panel B: □, FJ7 (TN) and ▲, FJ7 (TND). Smaller symbols represent autolysis-controls for the corresponding samples.
Figure 6. Lysozyme-induced lysis of whole cells derived from PS6μB, 8-4A and FJ7 macrofibers.
RH orientation if D-alanine is present during growth (TND). The cell suspensions prepared from nutritionally induced RH and LH forms were tested for their susceptibility to lysozyme. The rates of lysis were found to be very similar (Figure 6, Panel B).

These results seem to contradict the observations made by Mendelson et al. (1984) using structurally unperturbed FJ7 macrofibers. The time required for disintegration of macrofibers into individual cells upon lysozyme addition was used as an index of sensitivity by these authors. Hence, in their experiments, the differential susceptibility of the intact RH and LH forms reflects the unique chemical nature of the septal region in LH macrofibers. If so, then only a small part of the cell surface would differ in the two helical forms. Since the contribution of septum to overall lytic pattern would be insignificant, the difference in lysozyme sensitivity of walls or cells derived from LH and RH structures might be impossible to detect.

**Effects of Twist-Influencing Agents on Helical Conformation**

Although both the helix hand and static twist of a given macrofiber-producing strain are primarily determined by its genetic background, these parameters can be affected by manipulating the culture conditions. The molecular mechanism by which macrofibers attain a new static twist value in an altered growth environment remains largely unknown. Growth in the presence of agents that modify twist and act on specific cellular targets have provided clues about
the cell surface components involved in determination and maintenance of helical organization. Induction of relaxation motions upon addition of lysozyme (Mendelson, Briehl and Favre, 1982), for example, indicates involvement of peptidoglycan in twist-maintenance. Wolfe (1985) noted that LH structures produced by FJ7 in SL (a semi-synthetic medium) at 20°C could be made to invert their helix hand if the medium was supplemented with D-alanine. Similarly, macrofibers growing as LH structures in T (TB devoid of NaCl and beef extract) at 20°C undergo inversion in response to the addition of D-alanine (Mendelson, personal communication). Since D-alanine is exclusively a peptidoglycan constituent, these findings strengthen the conclusions drawn from lysozyme experiments. In a separate study, the presence of trypsin in small quantities during active growth of FJ7 in TB (a rich medium) at 48°C was found to prevent the development of leftward twist (Favre et al., 1985). This observation suggests that the cell wall associated polypeptides may play an important role in the determination of helical conformation. Whereas the addition of D-alanine and trypsin to growth medium counteract the attainment of left-twist, ammonium sulfate (N) enhances the LH orientation, perhaps by altering the electrostatic interactions within the cell wall matrix (Mendelson and Favre, 1987).

In order to determine how these compounds interact to shape the final conformational state, the effects of the simultaneous presence of two of these agents (an enhancer and an antagonist to a particular orientation) on twist development were studied. Fragments of macrofibers, cultured for at least two growth cycles in T, were seeded
in a fresh medium supplemented with various concentrations of either N/D-alanine or N/trypsin. After 15-18 hours of incubation at 20°C, the resultant helical structures were viewed under a phase contrast microscope for qualitative estimation of static twist. At least six such structures from each duplicate set were scored before a definite twist value was assigned. The scale used for twist assignment and the structural organizations corresponding to various twist values are shown in Figures 1a and 1b, respectively.

D-alanine–Ammonium Sulfate Interaction

FJ7 grows as left-handed helices with increasing static twist in response to the concentrations of N ranging from 0 to 60 mM (Figure 7, first row). The presence of D-alanine, on the other hand, induces formation of RH structures (Figure 7, first column). At low concentrations, ammonium sulfate seems to antagonize the influence of D-alanine but with increasing quantity of the former, the effect is synergistic (the lower half of the matrix). It is possible that higher amounts of N stimulate the uptake of D-alanine and thus the effect of the former is overridden. That D-alanine is a strong RH-effector is also suggested by the interaction matrix for PS6µB (Figure 8). Although this strain exhibits "fixed" LH phenotype in the temperature-induced inversion regime, it forms RH macrofibers in T supplemented, simultaneously, with higher amounts of ammonium sulfate and D-alanine (lower right region). Once again, the synergistic behaviour was observed at elevated concentrations of these agents. Figure 9 shows the results for 8-4A, a "fixed" RH derivative of FJ7. Although N tends
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Figure 7. Ammonium sulfate/D-alanine interaction matrix for RJ7.

Refer to the text for experimental design. Left- and right-handed orientation is designated with - and + signs, respectively. The numbers represent the degree of helical twist (see Figure 1 for the twist-scale).
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Figure 8. Ammonium sulfate/D-alanine interaction matrix for PS6pB.

Refer to the text for experimental details. The numbers represent the degree of helical twist (see Figure 1 for the twist-scale).
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Figure 9. Ammonium sulfate/D-alanine interaction matrix for 8-4A.

Refer to the text for experimental method. The numbers represent the degree of helical twist (see Figure 1 for the twist-scale).
tends to attenuate its right-handed organization in the absence of D-alanine, it is incapable of completely inverting the orientation (first row). Throughout the remainder of the matrix, macrofibers maintain a high positive twist value irrespective of the ammonium ion concentration. Even the lowest amount of D-alanine seems to overcome the effect of N. Since the former is present exclusively in the cell wall, these data suggest that the structural modification of peptidoglycan may be one of the most effective mechanisms by which macrofibers achieve various conformational states. Ammonium sulfate, on the other hand, is a rather nonspecific modulator which may exert its influence either by altering the charge status of wall polymers or by affecting the enzymes involved in peptidoglycan assembly.

Ammonium Sulfate-Trypsin Interaction

As mentioned earlier, steady state growth of FJ7 in the presence of proteases was found to prevent the development of leftward twist at 48°C (Favre, Karamata and Mendelson, 1985). Similar observations have been reported using the TB-S1 inversion regime (Wolfe, 1985) and the T system (Mendelson and Favre, 1987). In both instances, addition of trypsin abolishes attainment of LH conformation. Thus, proteases seem to represent yet another class of RH-effectors.

To determine if proteases are effectors as strong as D-alanine, FJ7 was grown in T containing various concentrations of ammonium sulfate and trypsin. After incubation for 15-18 hours at 20°C, twist values were assigned to the resultant structures as described earlier.
The interaction matrix for FJ7 (Figure 10) shows that in the absence of ammonium sulfate, macrofibers grow with moderately positive twist (+2.5) under the sole influence of trypsin (column 1). However, ammonium ions render the protease biologically inactive and consequently, the structures corresponding to each position in the remainder of the matrix, exhibit negative twist of various degrees. Although the response of PS6µB to the combination of these modulators is quite similar to that of FJ7 (Figure 11) the former, does not cross over to the RH quadrant of the twist spectrum even at 200 µg/ml of trypsin (column 1). The structures remain disorganized when proteases are used in concentrations beyond this range (data not shown). Once again, trypsin fails to influence the helical organization in the presence of ammonium sulfate. Using the Azocoll assay, it has been shown that ammonium ions do not significantly diminish the protease activity. Thus, the loss of trypsin's twist modulating activity in the presence of ammonium sulfate may either be due to protection of the relevant target or substitution of its function by these ions.

The behavior of 8-4A is intriguing in this context. The macrofibers tend to be somewhat less organized with increasing concentration of ammonium sulfate (Figure 12, first row). However, the presence of trypsin restores the static twist to values greater than their characteristic T-values. Thus, unlike in the case of FJ7 and PS6µB, ammonium ions are unable to counteract the effects of trypsin on 8-4A. A reasonable explanation for this observation is lacking at present.
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Figure 10. Ammonium sulfate/trypsin interaction matrix for RJ7.

Refer to the text for experimental design. The numbers represent the degree of helical twist (see Figure 1 for the twist-scale).
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Figure 11. Ammonium sulfate/trypsin interaction matrix for PS6μB.

Refer to the text for experimental method. The numbers represent the degree of helical twist (see Figure 1 for the twist-scale).
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Figure 12. Ammonium sulfate/trypsin interaction matrix for 8-4A.

Refer to the text for experimental procedure. The numbers indicate the degree of helical twist (see Figure 1 for the twist-scale).
The interaction matrices discussed above suggest that D-alanine is a strong "right-factor" whose action on macrofiber-organization is exclusive. However, being a "right-factor" does not always make a twist modulator more effective because influence of trypsin is easily overcome by low concentrations of ammonium ions. In a separate study, involving strain FJ7, the latter was found to completely override the effects of magnesium sulfate, a compound whose presence in the growth medium tends to make macrofibers right-handed (Mendelson and Favre, 1987). Since peptidoglycan is the major stress bearing polymer on the cell surface, modifications in its structure would be expected to have significant bearing on the helical organization. The fact that D-alanine is a muropeptide constituent is perhaps what makes it such a dominating effector.

**Intestinal Alkaline Phosphatase and Helical Organization**

During the course of investigation, it was discovered that alkaline phosphatase (AP) from sheep intestine (purchased from Sigma) prevents FJ7 macrofibers from developing leftward twist at 48°C. Although qualitatively similar to trypsin, the effect of alkaline phosphatase on twist was greater. To determine the relationship between the amount of enzyme and the extent of change in twist, fragments of structures that had been passed twice in TB, supplemented with 20 mM MgSO₄, were transferred to fresh, prewarmed medium containing various concentrations of AP. Incubation was continued for three hours at 48°C after which the macrofibers were scored for their
twist values as described earlier. The enzyme was excluded from the control plates which were otherwise treated the same. Figure 13 shows the effect of varying amounts of AP on helical orientation of FJ7 at 48°C. Each data point on the curve represents a value derived from at least two identical experiments. In the absence of AP, the macrofibers produced at 48°C are LH structures of moderate twist (-2) but the orientation becomes increasingly right-handed as the amount of AP in the growth medium increases.

To determine if the twist-modifying activity is common to all alkaline phosphatases, enzymes from various sources were tested. The assay was performed in 6-well plates, each well containing 3 ml of TB supplemented with equal amounts of enzyme units. The remainder of the procedure was identical to the one stated above. As shown in Table 2, FJ7 grows in the presence of bacterial- and kidney-APs without any apparent change in its helix-phenotype. Intestinal enzymes, on the other hand, effectively inhibit the development of leftward orientation irrespective of their mammalian origin.

It is possible that the observed biological activity of AP is due to the salts that are usually added to enzyme preparations for the purpose of maintaining appropriate ionic strength. To rule out this possibility, sheep intestinal AP was extensively dialyzed against deionized water and the dialysate was assayed for its influence on twist. Post-dialysis retention of both the biological and enzyme activities suggest that the accessory factors such as buffer of other ions are not responsible for the effect (data not shown).
Figure 13. Effect of alkaline phosphatase (AP) on twist development in FJ7.

FJ7 was grown in TB medium containing various concentrations of alkaline phosphatase (source, sheep intestine) at 48°C for 3 hours. The twist of the resultant macrofiber was estimated as described in the text. DO, disorganized; RH, right handed; LH, left handed. The numbers in parentheses represent the degree of helical twist.
Figure 13. Effect of alkaline phosphatase (AP) on twist development in RJ7.
<table>
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<tr>
<th>SOURCE OF ALKALINE PHOSPHATASE</th>
<th>AMOUNT USED(^a) (UNITS/3ML)</th>
<th>HELIX PHENOTYPE (^b)</th>
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\(^a\)One unit of enzyme liberates one micromole of p-nitrophenol at 25\(^\circ\)C, pH 8.0, under specified conditions (see text for details).

\(^b\)Helix phenotype determined after growth at 48\(^\circ\)C for 3 hours in TB containing indicated amounts of alkaline phosphatase. RH and LH, same as in Table 1; DO, disorganized. The numbers in parentheses indicate the degree of twist (see Figure 1 for the twist-scale).
Effect of Denaturation on the Biological Activity of AP

The charge status of cell wall is believed to affect its physical properties and, eventually, shape determination in Gram-positive bacteria. Hence, it is not surprising that exposure to acidic pH (pH 4.0 and below) induces instantaneous rotational movements in macrofibers. Furthermore, steady state growth at extremes of pH modify twist states significantly (Wolfe, personal communication). In light of these observations, it could be argued that the twist-modifying effect of AP may be a result of the interaction of its molecular charges with cell surface polymers rather than its phosphatase activity. To test this hypothesis, AP suspension was treated with 8 M urea and 10% β-mercaptoethanol as described in Materials and Methods section. A sample containing appropriate amounts of denaturing agents (without the enzyme) was dialyzed in parallel and used as a control (from hereon referred to as the blank).

As expected, the untreated sample exhibited its capacity to preclude left-twist development in FJ7 at 48°C and the denatured enzyme had lost all traces of both phosphatase and biological activities (Table 3). These results suggest that the biological effect of AP is dependent on its enzyme activity instead of its molecular charges. Moreover, although the degree of leftward twist is identical in FJ7 and PS6uB growing at 48°C and 20°C, respectively (column 1), the active AP converts the former to RH structures but the latter is affected only mildly. This observation indicates inherent differences between the two strains, one of which is "fixed" and the other switchable.
Table 3. Biological activity\(^a\) of sheep intestinal alkaline phosphatase inactivated by urea and \(\beta\)-mercaptoethanol.

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<td>DO(0)</td>
<td>LH(-1)</td>
<td>LH(-4)</td>
</tr>
<tr>
<td>8-4A</td>
<td>20</td>
<td>RH(+6)</td>
<td>RH(+6)</td>
<td>RH(+6)</td>
<td>RH(+6)</td>
</tr>
</tbody>
</table>

\(^a\)Refers to the influence of alkaline phosphatase on the development of left-twist.

\(^b\)See text.
Biological Activity of Various Intestinal APs

Alkaline phosphatases from sheep, dog and calf intestine were compared for the extent to which they affect the macrofiber organization. Small fragments of FJ7 structures grown in TB at 20°C were seeded in prewarmed medium containing varying concentrations of AP (Table 4, column 1 and 2) from the sources stated above. As shown in Table 4, whereas sheep and dog intestinal alkaline phosphatases cause the structures to grow with right-handed twist, calf intestinal AP fails to "push" the helices into the RH quadrant of the twist spectrum. Since the phosphatase activities of the test solutions from the various sources were identical, the differential ability to modify twist might be due to a difference in their affinity for the relevant target.

Heat Inactivation Kinetics of Sheep Intestinal AP

Commercial enzyme preparations are often contaminated with trace amounts of proteolytic enzymes. Since growth in the presence of trypsin significantly alters the helical orientation of the LH macrofibers, it was considered necessary to estimate the amount of proteases in alkaline phosphatase preparations. 100 μl of enzyme suspension (approximately 1 unit) was used in an Azocoll assay. The color was allowed to develop at 48°C for 60 min and the optical density was measured at 520 nm. All intestinal phosphatase preparations that acted as twist modulators were also found to contain detectable levels of protease (Table 5). The kidney and bacterial enzymes which exhibit no effect on macrofiber organization are relatively low in proteolytic
Table 4. Biological activity of intestinal alkaline phosphatase from various sources.

<table>
<thead>
<tr>
<th>CONCENTRATION OF ALKALINE PHOSPHATASE (µg/ml)</th>
<th>AMOUNT OF ALKALINE PHOSPHATASE USED (units/ml)</th>
<th>HELIX PHENOTYPE^1 AFTER GROWTH IN AP FROM SHEEP INTESTINE</th>
<th>HELIX PHENOTYPE^1 AFTER GROWTH IN AP FROM DOG INTESTINE</th>
<th>HELIX PHENOTYPE^1 AFTER GROWTH IN AP FROM CALF INTESTINE</th>
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</thead>
<tbody>
<tr>
<td>20</td>
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<td>DO(0)</td>
<td>LH(-3)</td>
</tr>
<tr>
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<td>DO(0)</td>
<td>DO(0)</td>
<td>DO(0)</td>
</tr>
<tr>
<td>60</td>
<td>0.18</td>
<td>DO(0)</td>
<td>DO(0)</td>
<td>DO(0)</td>
</tr>
<tr>
<td>80</td>
<td>0.24</td>
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<td>DO(0)</td>
<td>DO(0)</td>
</tr>
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<td>RH(+1)</td>
<td>DO(0)</td>
</tr>
<tr>
<td>120</td>
<td>0.36</td>
<td>RH(+3)</td>
<td>RH(+2)</td>
<td>DO(0)</td>
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</tbody>
</table>

^1 FJ7 was used as the test organism. Conditions for the assay of biological activity were same as in Table 2.
Table 5. Protease activity in alkaline phosphatase (AP) preparation from various sources.

<table>
<thead>
<tr>
<th>SOURCE OF ALKALINE PHOSPHATASE</th>
<th>SHEEP INTESTINE</th>
<th>DOG INTESTINE</th>
<th>CALF INTESTINE</th>
<th>BOVINE KIDNEY</th>
<th>E. coli</th>
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</thead>
<tbody>
<tr>
<td>PROTEASE ACTIVITY</td>
<td>1.12</td>
<td>1.08</td>
<td>1.19</td>
<td>0.17</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Protease activity expressed in trypsin equivalent (ng/μg AP). For definition see Materials and Methods.
activity. Therefore, the observed "right-factor" property of intestinal APs may be solely due to their protease content. However, this correlation seems weak since calf intestinal AP, although highest in its protease content, is the weakest of the intestinal enzymes in its influence on macrofibers.

That the twist modulation is independent of the protease contaminants is further supported by the heat inactivation kinetics. 10 mg/ml suspension of sheep AP was incubated at 60°C. Samples withdrawn at various time intervals were examined for their AP, protease and biological activity, as described previously. As shown in panel A of Figure 14, within 10 min of exposure to 60°C, the suspension loses 57% of its initial AP activity, while the contaminating proteases remain virtually unaffected (Panel B). The loss of potential to modify twist (Panel C) within this period parallels the decline of the former. Whereas the majority of the AP and biological activity disappears by the end of the incubation period, the test solution retains approximately 50% of its proteolytic strength. This experiment clearly suggests that the effect of intestinal alkaline phosphatases on the development of leftward orientation cannot be attributed to the presence of proteases.

Purification of Alkaline Phosphatase

To verify the conclusions from the heat inactivation experiment, attempts were made to purify AP activity away from the proteolytic enzymes. 15 mg of dog intestinal AP was applied to a hydroxylapatite column (see Material and Methods for details). Elution
Figure 14. Heat inactivation kinetics of various activities exhibited by alkaline phosphatase preparation.

Dog intestinal alkaline phosphatase (10mg/ml) was incubated at 60°C. At various time intervals samples were withdrawn and assayed for phosphatase (panel A), protease (panel B) and biological activities (panel C). See Materials and Methods for experimental details and definitions. Helix phenotype, same as in Figure 13.
Figure 14. Heat inactivation kinetics of various activities exhibited by alkaline phosphatase preparation.
with the initial buffer resulted in appearance of a large peak, designated UNF-l, which contained most of both the phosphatase and protease activities (Figure 15, Panel A). Upon continued elution with a linear salt gradient (10 to 300 mM NaCl) another protein-peak, CF-l, was obtained (Panel B). The material from this peak exhibited neither AP nor protease nor "right-factor" activity (data not shown). UNF-l was further fractionated on a DEAE-cellulose column. The elution profile is shown in Figure 16. The contents of the peaks, labeled F-I, F-II and F-III, were extensively dialyzed and tested for various activities.

Table 6 shows the results of these analyses. A single passage through the hydroxylapatite column leads to at least three fold increase in the specific activity of phosphatase (from 2.3 to 7.4 units/mg protein). Fractionation on DEAE-cellulose elevates this value to 15.8 units/mg protein indicating overall seven-fold purification. Whereas F-I, exhibiting strongest AP activity, is virtually free of proteases, F-III contains most of the latter with no detectable amount of phosphatase. Since fraction F-I displays undiminished twist-modulating potential it can be concluded that the ability of intestinal phosphatases to inhibit the development of leftward twist is independent of their protease content. While the biological activity of F-III is in compliance with the previously observed trypsin effect (Favre et al., 1985), the influence on the macrofiber organization of the remaining samples, unfractionated AP, UNF-I and F-II is probably due to action of both phosphatase and proteolytic enzymes.
Figure 15. Hydroxylapatite chromatography of alkaline phosphatase.

Dog intestinal alkaline phosphatase was fractionated on a hydroxylapatite column under the stated conditions. Protein content in each fraction was estimated by measurement of optical density (OD) at 280 nm. The peaks designated as UNF-1 and CF-1, were assayed for both phosphatase and protease activities (see Table 6 and the text).
Figure 16. Fractionation of UNF-I on DEAE-cellulose (DE-52).

UNF-I was chromatographed on a DE-52 column under the state condition. The peaks, F-I, F-II and F-III, were assayed for phosphatase, protease and biological activities (see Table 6).
Table 6. Phosphatase, protease and biological activities in various fractions obtained from the dog intestinal alkaline phosphatase.

<table>
<thead>
<tr>
<th>ALKALINE PHOSPHATASE ACTIVITY</th>
<th>FRACTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog intestine alkaline phosphatase</td>
<td>UNF-I</td>
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<tr>
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<td>7.4</td>
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</table>

<table>
<thead>
<tr>
<th>PROTEASE ACTIVITY</th>
<th>FRACTIONS</th>
</tr>
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<tbody>
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<td>7.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BIOLOGICAL ACTIVITY</th>
<th>FRACTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO(C)</td>
<td>DO(C)</td>
</tr>
</tbody>
</table>

- \( ^{a} \) Dog intestinal alkaline phosphatase prior to fractionation
- \( ^{b} \) Same as in Table 2.
- \( ^{c} \) Same as in Table 5.
- \( ^{d} \) Same as in Table 3. The designations for twist are as described in Table 1 and 2. Condition for the assay of biological activity were same as in Table 2.
The extent of purification was further examined by non-denaturing polyacrylamide gel electrophoresis. Lane 5 in panel A of Figure 17 shows the pre-fractionation protein profile of dog intestinal AP. The protein band indicated by an arrow was present predominantly in fraction CF-I. Lanes 1, 2, 3 and 4 represent the polypeptide pattern of F-III, F-II, F-I and UNF-1, respectively. To identify the band corresponding to phosphatase, an identical gel was stained with its substrate (p-nitrophenyl phosphate), schematic diagram of which is shown in panel B (Figure 17). Both unfractionated AP and UNF-I (lanes 6 and 7) contain a protein in the region close to the origin that reacts with the substrate. Hence, the topmost band in lane 3 (Panel A) is alkaline phosphatase with no parallel in lane 1. Since fraction F-III is rich in proteolytic activity, the most prominent protein band in lane 1 must be the peptidase. Complete absence of this protein from lane 3 indicates that F-I is free of such contaminants. These results are in perfect agreement with the enzyme-activity measurements compiled in Table 6.

If the enzyme activity of intestinal alkaline phosphatase is indeed responsible for its interference with the process of helix hand inversion, what is the nature of its target? Since phosphatases are nonspecific phosphomonoesterases, the cellular substrate must contain ester-linked phosphate groups. Although a small fraction of the muramic acid residues in murin are phosphorylated, teichoic acids are the major phosphate containing polymers and hence, could be a possible target of AP. Cell walls purified from 168 and FJ7 were digested with
Alkaline phosphatase was chromatographed on hydroxylapatite and DEAE-cellulose columns. The fractions obtained were analyzed by non-denaturing polyacrylamide gel electrophoresis. Panel A: lane 1, F-III; lane 2, F-II, lane 3, F-I; lane 4, UNF-1; lane 5, unfractionated AP. The prominent protein bands in lane 1 and 3 correspond to protease and phosphatase respectively (see also Table 6). The arrow indicates the polypeptide exclusively present in CF-1. Panel B: schematic representation of a gel stained for phosphatase activity (see Material and Methods for experimental details). The cross hatched areas represent the yellow spots visualized in an actual gel due to reaction of AP with its substrate. Lane 6, unfractionated AP; lane 7, UNF-1; lane 8, CF-1.

Figure 17. Protein profile and activity staining of various fractions from dog intestinal alkaline phosphatase (AP).
sheep intestinal AP (3 units/mg cell wall in 50 mM Tris-HCl buffer, pH 8.0) at 48°C. After three hours, the insoluble material was removed by centrifugation and the supernatant was assayed for its phosphate content. Less than 1% of the total phosphate in the wall was released during AP treatment, an amount that would seem unlikely to have profound effects on macrofiber orientation. Further investigation is required for the identification of the AP target.

**Cell Wall Composition as a Function of Twist**

There is ample evidence for an intimate involvement of cell surface in a variety of cellular functions and responses. It is expected, therefore, that drastic changes in its biochemical composition would cause sizable modification of a cell's behavior. Shape determination and lysozyme sensitivity have already been discussed in this context. In *B. subtilis* macrofibers, induction of relaxation motion by muramidase has implicated peptidoglycan in twist maintenance (Mendelson et al., 1982). Hence, it is appropriate to ask if a shift in chemical identity of the surface polymers underlie the process of helix hand inversion. This possibility was investigated at the level of overall composition, the extent of ester-linked D-alanine substitution and the degree of cross-linking. No attempts were made to measure the glycan chain length for reasons discussed earlier.

**Cell Wall Composition (Temperature Regime)**

If the helical organization represents the inherent orientation of cell surface polymers, the hand inversion in FJ7 at 48°C may be a
consequence of modifications in their chemical structure induced by
growth at elevated temperature. Purified cell walls from FJ7 grown at
20°C (RH) and 48°C (LH) were subjected to biochemical analysis. The
constituent components of the three major polymers, peptidoglycan,
teichoic acid and teichuronic acid were estimated. The composition of
the murein layer from 168 cultivated under similar culture conditions
served as a wild type control. It is evident from Table 7 that the
48°C grown 168 cells contain higher amounts of amino sugars (>1.5
times) and amino acids (at least 1.2 times) relative to their 20°C
counter part. Majority of the teichoic and teichuronic acid
constituents, phosphate, glycerol, glucose and glucuronic acid, are
present at not too significantly different levels. The most striking of
all changes, however, is the disappearance of galactosamine at 48°C.

The changes noted in the overall composition of walls isolated
from temperature-induced RH and LH forms of FJ7 were qualitatively
similar with few exceptions. Among peptidoglycan components, the
concentration of muramic acid and alanine was greater at 48°C; although
the extent was lower compared to 168. The other elements were found to
be at values that are within 80% of the ones reported for 20°C grown
structures. Unlike the wild type strain, growth at high temperature
does not lead to complete loss of galactosamine in FJ7. Instead, the
level depresses to 25% of the low-temperature value. Both the
increased content of murein and the reduction in galactosamine have
been previously observed in conditional rod- mutants of B. subtilis
(Rogers and Taylor, 1978). Whereas the former was interpreted as a
Table 7. Composition of cell walls from autolytic enzyme deficient strains and the wild type 168 (temperature regime; set-I).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>NMOLES/MG CW&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
</tr>
<tr>
<td>MURAMIC ACID</td>
<td>358</td>
</tr>
<tr>
<td>GLUCOSAMINE</td>
<td>349</td>
</tr>
<tr>
<td>ALANINE</td>
<td>914</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>587</td>
</tr>
<tr>
<td>DIAMINOPIMELIC ACID</td>
<td>635</td>
</tr>
<tr>
<td>PHOSPHATE</td>
<td>1720</td>
</tr>
<tr>
<td>GLYCEROL</td>
<td>832</td>
</tr>
<tr>
<td>GLUCURONIC ACID</td>
<td>200</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>242</td>
</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>134</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell wall.
consequence of derepression of peptidoglycan synthesis, the latter was considered a change not too significant in the context of drastic morphological aberrations. However, due to their high negative charge, teichoic and teichuronic acid may be expected to affect the orientation of the glycan chains during surface assembly. Thus it was reasoned that galactosamine, being a component of teichuronic acid, could influence peptidoglycan orientation by its absence and eventually lead to an inversion of helix hand in macrofibers. If there is any validity to this argument, "fixed" RH mutants should contain normal levels of galactosamine at all temperatures. In "fixed" LH mutants, on the other hand, it should be present in consistently lower amounts. To test this hypothesis, cell walls isolated from 8-4A and PS6μB were analyzed. The results (Table 7) show that the cultivation of "fixed" mutants at 48°C leads to 75% reduction in their galactosamine content irrespective of the helical orientation. Hence, loss of this amino sugar seems merely a consequence of growth at high temperature and is probably irrelevant to the determination of twist state.

Among other alterations, a significant depression in the amount of glucose (70%) and glucuronic acid (50%), compared to 168 and FJ7, is apparent. It is not clear at present if these changes are related to the non-switchable phenotype of these mutants. The values of the remaining components in PS6μB and 8-4A, though not identical, are comparable with the exception of alanine. The molar proportions of various wall constituents for each strain are shown in Table 8. Considerable departure from the theoretical ratio is evident. For
Table 8. Molar ratios of cell wall components (temperature regime; set-I).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>MOLAR RATIO COMPARED WITH GLUTAMIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
</tr>
<tr>
<td>MURAMIC ACID</td>
<td>0.61</td>
</tr>
<tr>
<td>GLUCOSAMINE</td>
<td>0.60</td>
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<tr>
<td>ALANINE</td>
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<tr>
<td>GLUTAMIC ACID</td>
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</tr>
<tr>
<td>DIAMINOPIMELIC ACID</td>
<td>1.08</td>
</tr>
<tr>
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</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>0.23</td>
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</tbody>
</table>
example, according to the proposed chemical structure, peptidoglycan should contain an amount of alanine that is twice that of glutamic acid. Though the ratio ranges from 1.4 to 1.9 in this analysis, the variation of this extent is not uncommon in the literature. No attempt has ever been made to explain these persistent anomalies. In the current investigation, the departure from the theoretical value is more pronounced in macrofiber-forming strains.

Whatever the absolute values and molar ratios, the differences among them do not seem to be correlated to the conformational states. The observed variation could either be due to the pleiotropic effects of mutations in the genes that govern twist or a consequence of the mutations that bear no relationship to hand determination. To confirm lack of correlation, similar analyses were performed on a second set of walls purified from the various strains. The estimated amounts of cell wall elements and their molar ratios are listed in Tables 9 and 10, respectively. Although the numerical values are somewhat different from those obtained from the previous analysis, the deductions remain unaltered.

Cell Wall Composition (Nutrition Regime)

The early studies concerning the kinetics of helix hand inversion in rich medium, TB, were conducted using temperature as an inducing agent (Favre, 1984). However, the effects of a shift in growth temperature on cell physiology are of a general nature which, although unrelated to the cellular phenomenon under study, may complicate interpretation of the experimental data. The metabolic
Table 9. Composition of cell walls from autolytic deficient enzyme strains and the wild type 168 (temperature regime; set-II).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>NMOLES/MG CW</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168</td>
<td>FJ7</td>
<td>PS5μB</td>
<td>8-4A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>48°C</td>
<td>20°C</td>
<td>48°C</td>
<td>20°C</td>
<td>48°C</td>
<td>20°C</td>
<td>48°C</td>
</tr>
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<td>452</td>
<td>655</td>
<td>421</td>
<td>446</td>
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<tr>
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<td>134</td>
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<td>327</td>
<td>85</td>
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Table 10. Molar ratios of cell wall components (temperature regime; set-II).

<table>
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<th>168</th>
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</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>1.3</td>
<td>-</td>
<td>0.68</td>
<td>0.09</td>
</tr>
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rates, phospholipid composition of the cell membrane and thus fluidity, ribosomal content and eventually the growth rates are but some of the parameters that are modified in response to the altered environment (Marr, Nelson and Clark, 1963; Herendeen et al., 1979).

Effects of temperature on enzyme activity is well documented and wall-biosynthetic enzymes are no exception. Thus, in context of the macrofiber system, twist-unrelated changes in the chemical composition of cell surface due to growth at 48°C are expected. In order to alleviate the problems associated with the use of temperature, change in the nutrient status was used as a means to induce inversion (Wolfe, 1985; Mendelson and Favre, 1987). FJ7 macrofibers were cultivated in T medium which was supplemented with 30 mM ammonium sulfate (TN) to enhance the LH organization. To grow RH structures, the strain was propagated in the presence of both ammonium sulfate and 4.5 mM D-alanine (TND). As it was mentioned earlier, the latter is a dominating RH-factor which overrides the LH influence of the former. The cell walls were purified and analyzed in the standard manner (see Materials and Methods for detail). Once again, the murein layer isolated from 168 grown under identical growth conditions was used as a wild type control.

The result of this analysis is shown in Tables 11 and 12. The content of various peptidoglycan constituents in TN-grown 168 cells are within 90% of the values estimated for organisms cultured in the presence of D-alanine. The difference in the levels of teichoic and teichuronic acid components of these cells is of even lesser magnitude,
Table 11. Composition of cell walls from autolytic deficient strain FJ7 and wild type 168 (nutrition regime).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>NMOLES/MG CW</th>
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<th></th>
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</thead>
<tbody>
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<td></td>
<td>168</td>
<td>FJ7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TN</td>
</tr>
<tr>
<td>MURAMIC ACID</td>
<td>354</td>
<td>376</td>
<td>346</td>
</tr>
<tr>
<td>GLUCOSAMINE</td>
<td>490</td>
<td>512</td>
<td>415</td>
</tr>
<tr>
<td>ALANINE</td>
<td>656</td>
<td>701</td>
<td>789</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>531</td>
<td>622</td>
<td>386</td>
</tr>
<tr>
<td>DIAMINOPIMELIC ACID</td>
<td>420</td>
<td>435</td>
<td>540</td>
</tr>
<tr>
<td>PHOSPHATE</td>
<td>1750</td>
<td>1650</td>
<td>2150</td>
</tr>
<tr>
<td>GLYCEROL</td>
<td>1040</td>
<td>1160</td>
<td>1700</td>
</tr>
<tr>
<td>GLUCURONIC ACID</td>
<td>120</td>
<td>134</td>
<td>120</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>177</td>
<td>177</td>
<td>513</td>
</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>545</td>
<td>450</td>
<td>204</td>
</tr>
</tbody>
</table>

<sup>a</sup>TN refers to T medium supplemented with ammonium sulfate (30 mM). TND was prepared by addition of D-alanine (final concentration 4.5 mM) to TN. FJ7 grows as LH and RH macrofibers, respectively, in these media.
Table 12. Molar ratios of cell wall components (nutrition regime).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>MOLAR RATIO COMPARED WITH GLUTAMIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>TN</td>
</tr>
<tr>
<td>MURAMIC ACID</td>
<td>0.59</td>
</tr>
<tr>
<td>GLUCOSAMINE</td>
<td>0.9</td>
</tr>
<tr>
<td>ALANINE</td>
<td>1.26</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
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</tr>
<tr>
<td>DIAMINOPIMELIC ACID</td>
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</tr>
<tr>
<td>PHOSPHATE</td>
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<tr>
<td>GLYCEROL</td>
<td>1.95</td>
</tr>
<tr>
<td>GLUCURONIC ACID</td>
<td>0.23</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>0.33</td>
</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>FJ7</td>
</tr>
<tr>
<td></td>
<td>TN</td>
</tr>
<tr>
<td>MURAMIC ACID</td>
<td>0.9</td>
</tr>
<tr>
<td>GLUCOSAMINE</td>
<td>1.55</td>
</tr>
<tr>
<td>ALANINE</td>
<td>2.0</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>1</td>
</tr>
<tr>
<td>DIAMINOPIMELIC ACID</td>
<td>1.4</td>
</tr>
<tr>
<td>PHOSPHATE</td>
<td>5.57</td>
</tr>
<tr>
<td>GLYCEROL</td>
<td>4.4</td>
</tr>
<tr>
<td>GLUCURONIC ACID</td>
<td>0.31</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>1.33</td>
</tr>
<tr>
<td>GALACTOSAMINE</td>
<td>0.53</td>
</tr>
</tbody>
</table>
with the exception of galactosamine. In FJ7, the amounts of muramic acid and diaminopimelic acid in RH structures are 25% and 8% (respectively) higher than the LH macrofibers but the increase in glucosamine and glutamic acid content exceeds 50%. This lower value of glutamic acid in LH forms of FJ7 is also responsible for its relatively higher molar ratios (Table 12). The status of anionic polymer ingredients, with the exception of galactosamine, remains comparable in the two media. Once again, owing to their nonspecific nature, these compositional differences fail to provide any rational clue to the mechanism of D-alanine induced inversion. Perhaps its influence is more specific than a general modulation of peptidoglycan composition.

The two most striking features of the biochemical composition of cells grown at various temperatures were the derepression of murein biosynthesis and reduction in the level of galactosamine at 48°C. The absence of these changes in nutritionally manipulated conformational states supports the conclusion that these alterations are not relevant to the inversion process.

Ester-linked Alanine Substitution

The fact that D-alanine is found exclusively in the cell wall makes it a twist modulator of narrow target specificity. It is expected, therefore, that the prime targets of its action would be the surface polymers that contain this amino acid as one of the constituents, i.e. peptidoglycan and teichoic acids. Since no twist-correlated changes were detected in D-alanine induced RH structures, it was logical to question if modification in the state of D-alanine
substitution on teichoic acid was the mechanism underlying the inversion process. Cell walls isolated from 168, FJ7, F86μB and 8-4A grown at 20°C and 48°C were subjected to alkali hydrolysis at room temperature for 6 hours. This treatment causes release of ester-bonded D-alanine. At the end of the incubation period, walls were centrifuged and alanine was estimated in the pellet and supernatant as outlined before. The extent of substitution was calculated by dividing the amount released in the supernatant by the total alanine content (supernatant + pellet). Cell walls purified from TN- and TND-grown wild type and FJ7 cells were analyzed similarly. Amounts <5 nmoles/mg cell wall were considered too low and were not included in the final calculation.

It is evident from Table 13 that the D-alanine substitution on teichoic acid of 168 cells cultured at 48°C is at least 2.5 times higher than their 20°C counterpart. The extent of substitution is 5-8 times lower for all other strains irrespective of the growth temperature and has no apparent correlation with the conformational states. Although the anionic polymers of the wild type cells are largely devoid of the ester-linked amino acid, FJ7 walls contain low amounts of this substituent in the nutrition regime (Table 14). Growth in TND tends to increase the level of alkali labile alanine by a factor of 2 in FJ7 but the absolute values are extremely low. The significance of these small differences in twist determination remains speculative.

In any case, the results indicate that modulation of the extent of ester-linked alanine substitution is probably not the mechanism underlying hand inversion.
Table 13. Ester-linked alanine content of cell walls purified from various strains (temperature regime).

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GROWTH TEMP. °C</th>
<th>NMOLES OF ALANINE/MG CW</th>
<th>% ESTER LINKED ALANINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SUPERNATANT a</td>
<td>PELLET a</td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>20</td>
<td>46.</td>
<td>724</td>
</tr>
<tr>
<td>168</td>
<td>48</td>
<td>195</td>
<td>978</td>
</tr>
<tr>
<td>FJ7</td>
<td>20</td>
<td>&lt;5</td>
<td>547</td>
</tr>
<tr>
<td>FJ7</td>
<td>48</td>
<td>7</td>
<td>843</td>
</tr>
<tr>
<td>P56µB</td>
<td>20</td>
<td>15</td>
<td>665</td>
</tr>
<tr>
<td>P56µB</td>
<td>48</td>
<td>16</td>
<td>847</td>
</tr>
<tr>
<td>8-4A</td>
<td>20</td>
<td>10</td>
<td>797</td>
</tr>
<tr>
<td>8-4A</td>
<td>48</td>
<td>12</td>
<td>705</td>
</tr>
</tbody>
</table>

^aSupernatant refers to the fraction that contained alanine released by alkali treatment of purified cell walls. The residual material, recovered by centrifugation, was designated as the pellet.
Table 14. Ester-linked alanine content of cell walls purified from 168 and FJ7 (nutrition regime).

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GROWTH MEDIUM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NMOLES OF ALANINE/MG CW</th>
<th>% ESTER LINKED ALANINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>168 TN</td>
<td>&lt;5</td>
<td>758</td>
<td>-</td>
</tr>
<tr>
<td>168 TND</td>
<td>&lt;5</td>
<td>825</td>
<td>-</td>
</tr>
<tr>
<td>FJ7 TN</td>
<td>8</td>
<td>710</td>
<td>1</td>
</tr>
<tr>
<td>FJ7 TND</td>
<td>15</td>
<td>758</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Same as in Table 11.

<sup>b</sup>Same as in Table 13.
Cell Wall Associated Proteins

In addition to the muropeptide matrix and anionic polymers, the Gram-positive cell wall contains noncovalently associated polypeptides. Some proteins form a crystalline array on the surface, such as the T-layer of Bacillus sphaericus (Sleyter and Messner, 1983), while others are distributed throughout the structural network of the cell envelope. Some of the biosynthetic and remodelling enzymes are to be expected among the latter if the cell surface is to keep in step with the dynamics of growth and division (Rogers, Perkins and Ward, 1980). The question as to whether these proteins influence the physical orientation of glycan chains has never been addressed.

The possibility that protein factor(s) are involved in the development of leftward twist in FJ7 was first suggested by Favre et al. (1985). In one of the experiments, structures growing in TB at 20°C were transiently exposed to 48°C. When returned to the original temperature, the macrofibers underwent a short-lived inversion (RH to LH) before assuming their characteristic 20°C-orientation i.e. RH. No such inversion was observed if the high temperature pulse was given in the presence of chloramphenicol. This phenomenon was found to be asymmetric since inhibition of protein synthesis did not block the left-to-right inversion. Furthermore, steady state growth in TB supplemented with low concentrations of trypsin (200 μg/ml) caused structures to shift towards the RH-quadrant of the twist spectrum. From these experiments it was concluded that the protein(s) required for attainment of left twist is located on the cell surface. This
unidentified factor(s) has been designated as LTP or left-twist protein. Similar conclusions were reached by Wolfe (1985) who used a nutrition-regime to induce inversion.

In the current investigation, an attempt was made to identify the LTP. Due to its proposed surface location, a characterization of only wall associated proteins was undertaken. The intact macrofibers, grown in appropriate culture conditions, were extracted with 5 M lithium chloride, as described previously. The polypeptides were analyzed by SDS gel electrophoresis. A 200 Kdal protein (Figure 18, lane 2) was observed in the extract prepared from 48°C-cultivated wild type cells. Although present in small amounts at high temperature, it was completely absent from 20°C preparations (lane 1). PS6µB, a "fixed" LH mutant with fairly high static twist value, contains significantly large quantities of this polypeptide (lanes 5 and 6) at both 20°C and 48°C. Despite a dramatic reduction in the overall protein yield in this strain, the high intensity of the 200 Kdal band suggested that it may be of importance in the maintenance of left-twist. In FJ7, a protein band of similar molecular weight is seen in the extracts from macrofibers cultivated at 48°C, a condition that favours LH orientation (lane 4). Since requirement of LTP has been established in nutrition-induced inversion, it was logical to determine the status of this protein in cells grown in T containing various salts that modify helical twist. The 200 Kdal polypeptide is, once again, seen in FJ7 cultured at 20°C in 20 mM of each ammonium sulfate, sodium chloride and sodium sulfate (Figure 19, lanes 1, 2 and 4). In the presence of
Figure 18. Protein profile of the lithium chloride extracts prepared from various strains.

Wall associated polypeptides were extracted with 5 M LiCl from cells grown in various conditions and were analyzed by SDS polyacrylamide gel electrophoresis (10%). Suspensions containing 30-40 μg of proteins were applied to each lane. Lane 1, 168 (TB, 20°C); lane 2, 168 (TB, 48°C); lane 3, FJ7 (TB, 20°C); lane 4, FJ7 (TB, 48°C); lane 5, PS6uB (TB, 20°C); lane 6, PS6uB (48°C); lane 7, A734 (TN, 20°C); lane 8, A734 (TND, 20°C); lane 9, B-4A (TB, 20°C); lane S, molecular weight standards (K=1000 dalton). The arrows indicate the twist-correlated 200 Kdal protein (see text).
Figure 19. Protein profile of lithium chloride extracts prepared from FJ7 grown in presence of various salts.

FJ7 was cultivated in T supplemented with a variety of electrolytes (20 mM). The LiCl extracts were prepared and analyzed as described in Figure 18. Lane 1, ammonium sulfate (LH); lane 2, sodium chloride (LH); lane 3, magnesium chloride (RH); lane 4, sodium sulfate (RH); lane S1, bovine serum albumin; lane S2, ovalbumin. The arrow indicates the twist-correlated 200 Kdal protein. RH and LH in parentheses refer to the steady state helix phenotype of FJ7 when grown in presence of the corresponding salt.
these compounds this strain forms LH helices of moderate twist. The band is conspicuously absent from structures produced in 20 mM magnesium chloride, a salt that favours RH organization (lane 3).

Effect of Protease on Surface-Protein Profile

If the 200 Kdal protein is an integral part of the twist maintenance (or determination) scheme its destruction should exert considerable influence on macrofiber conformational state. The "right-factor" activity of trypsin is compatible with this idea. To test the validity of this notion, FJ7 cells were cultivated in TN medium containing trypsin (2 µg/ml). The enzyme was excluded from the control experiment. Since proteases prevent the development of leftward twist, the structures assume RH orientation in their presence. The lithium chloride extracts were prepared and analyzed as before. Whereas the control sample contains a detectable amount, the trypsin-grown cells have lost all traces of the 200 Kdal protein (Figure 20, lanes 1 and 2). Similar results were obtained using the strain PS6µB which forms LH helices of high twist in T medium but loses its organization when cultured in the presence of proteases (Figure 20, lanes 3 and 4). Since bands of far less intensity persist beyond trypsin treatment, the disappearance of the polypeptide in question does not seem to be a result of nonspecific degradation.

Trypsin-Digestion Kinetics of LiCl Extracts

The high trypsin sensitivity of the 200 Kdal protein could either be due to its location on the cell surface resulting in easy
Figure 20. The effect of growth in presence of trypsin on the wall associated proteins.

FJ7, PS6uB and 8-4A were grown in T containing trypsin (2 ug/ml) at 20°C. To enhance the production of 200 Kdal protein in FJ7, the medium was supplemented with ammonium sulfate (30 mM). LiCl extracts were prepared and analyzed as stated in Figure 18. The extracts made from cells grown without trypsin were used as controls. Lane 1, FJ7 control; lane 2, FJ7 with trypsin; lane 3, PS6uB control; lane 4, PS6uB with trypsin; lane 5, 8-4A control; lane 6, 8-4A with trypsin; lane S, molecular weight standards. The arrow indicates the position of twist-correlated 200 Kdal protein.
accessibility to the exogenously added enzymes or its amino acid composition, rendering it inherently susceptible to proteases or both. To address this issue, 50 µl (350 µg protein) of LiCl extracts prepared from TN-grown FJ7 were subjected to trypsin digestion (0.1 µg/ml final concentration) at 37°C. Samples were withdrawn at various time intervals and analyzed on SDS-polyacrylamide gels, as described previously. As shown in Figure 21, the intensity of the 200 Kdal band diminishes with increasing incubation time, eventually leading to a complete loss. The preferential digestion of this protein suggests that its sensitivity to proteases in intact cells is perhaps due to its unique composition. However, no prediction can be made from these experiments about its cellular location. In a parallel study, a protein preparation from PS6µB was treated with trypsin under the conditions outlined above. The protein profiles of the digest at various time points are shown in Figure 22. Once again, the 200 Kdal band in this strain disappears but only after 50 min of incubation (lane 6) while the other polypeptides persist. Hence, in PS6µB this protein seems relatively protease resistant in comparison to its FJ7 analogue. The ramification of this differential sensitivity is not clear at present.

From these results, there appears to be a strong correlation between the presence of this protein and attainment of LH orientation. However, some discrepancies, however, need to be resolved before a twist-determining function may be assigned. First, 8-4A, a "fixed" RH mutant in the temperature regime, contains a protein of similar
Figure 21. Trypsin sensitivity of the 200 Kdal protein in LiCl extracts prepared from FJ7.

LiCl extracts were made from FJ7 grown in T supplemented with ammonium sulfate at 20°C. 50 ul (350 ug protein) of this suspension were digested with trypsin (0.1 ug/ml) at 37°C in Tris HCl (50 mM, pH 8.0). Samples (7 ul) were withdrawn at various time intervals were analyzed as described (see Materials and methods). Lane 1, 0 min; lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 20 min; lane 6, 25 min; lane 7, 30 min; lane 8, 40 min. The arrow indicates position of the 200 Kdal protein.
Figure 22. Trypsin sensitivity of the 200 Kdal protein in LiCl extracts prepared from PS6uB.

Wall associated proteins were extracted from T-grown PS6uB at 20°C. The extract was digested with trypsin as described in Figure 21. Samples were withdrawn at various time intervals and analyzed by SDS polyacrylamide gel electrophoresis. Lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 40 min; lane S, molecular weight standards. The arrow indicates the location of the 200 Kdal protein.
molecular weight at 20°C (Figure 18, lane 9). In addition, the same band is present in the extracts made from 734 (a strain closely related to FJ7) grown in T medium supplemented with D-alanine (Figure 18, lane 8) and magnesium sulfate (data not shown). Both compounds are conducive to formation of right-handed structures. Although some of these anomalies may be explained (discussed below), it is rather premature to draw definite conclusions about the function of this protein. Nevertheless, the correlation of its presence with development of LH conformation is apparent.

Insertional Mutagenesis

Indigenous transposable elements have been extensively used for genetic analysis in E. coli and other organisms (Kleckner, Roth and Botstein, 1977). Since transposons provide greater advantage over the conventional genetic methods, efforts have been made to introduce some of the well characterized enteric bacterial elements into cells of unrelated species (Kuner and Kaiser, 1981; Ely and Croft, 1982). Recently, Youngman et al. (1983) have described a method for insertional mutagenesis in B. subtilis using a Streptococcus transposon, Tn917. This element confers resistance to macrolides, lincosamide and streptogramin B antibiotics (MLS). The selection can also be made for resistance to erythromycin (1 µg/ml) and lincomycin (25 µg/ml). These authors have cloned Tn917 on a plasmid with temperature sensitive replication and a gene for chloramphenicol resistance. The resultant 12.4 kb plasmid has been designated pTV1. In the current investigation, pTV32ts, a 15.6 kb derivative of pTV1
that carries the β-galactosidase gene (Figure 23), was used for insertional mutagenesis.

Although mutants impaired in temperature-induced helix hand inversion are available, the reasons to utilize Tn917 for isolation of more of such mutants are three fold: 1) to develop a method for insertional mutagenesis in macrofiber producing strains 2) to isolate "fixed" mutants without too many irrelevant mutations for identification of LTP and 3) once insertions in the genes responsible for inversion are isolated, mapping of these genes will be far less laborious considering the fact that at present the only way to distinguish a RH macrofiber from LH is to view individual structures under a phase contrast microscope.

Transformation of FJ7 with pTV32ts

The plasmid, pTV32ts, was isolated from a strain with the same designation (provided by Dr. U. N. streips of the University of Louisville, Kentucky) using an alkaline extraction procedure. Being the most extensively studied strain in regards to macrofiber formation, FJ7 spheroplasts were transformed with this plasmid. Upon regeneration erythromycin (Em) and chloramphenicol (Cm) resistant clones were selected. To confirm the presence of pTV32ts in the transformants, a plasmid preparation from the first of these isolates, referred to as FJ7US1, was digested with EcoRI and BamHI. Preparations from the parent strain FJ7 and the similarly treated Louisville-strain served as controls. As predicted from the restriction map, EcoRI treatment of both pTV32ts and the plasmid isolated from FJ7US1 yields a unique
Figure 23  Physical map of the plasmid pTV32ts.
fragment (Figure 24, lanes 3 and 6), whereas digestion with BamHI results in generation of two bands of nonidentical size (lanes 2 and 7). Discrete bands were detected in the lane corresponding to EcoRI digest of FJ7 (lane 5). Clearly, the FJ7 transformant (i.e. FJ7US1) harbours pTV32ts. The size of the plasmid, however, was found to be 18 Kb as determined from a standard curve made by using HindIII digested λCI857Sam7 DNA (lane 4), instead of the reported value, 15.6 Kb (Streips, personal communication). Considering the nature of ensuing experiments, it was not considered necessary to resolve this anomaly.

Helical Properties of FJ7US1

To determine if the presence of pTV32ts influence the helix phenotype, FJ7US1 was grown in various culture conditions and the state of helical organization was compared to its parent strain, FJ7. The growth medium for the former was always supplemented with appropriate antibiotics (Em and Cm) to maintain the selection pressure. The results of these studies show that, other than its behaviour in T and TN, the helix phenotype of FJ7US1 is almost identical to its parent (Table 15). Two other isolates of FJ7 series containing the plasmid were tested. Similar to FJ7US1, both strains produced structures composed of edison filaments and swollen cells in T and TN (data not shown). To confirm if this response is solely due to the presence of pTV32ts, FJ7US2 was cured by cultivation in TB at 48°C, the restrictive temperature for the plasmid replication. Clones, sensitive to both Em and Cm, were selected and examined for their helical properties in various growth conditions. The helix phenotype of the cured strain
Figure 24. Agarose gel electrophoretic separation of EcoRI- and BamHI-generated fragments of the plasmid pTV32ts.

The miniscreen preparations from various strains were subjected to digestion by restriction endonucleases and analyzed on a 0.8% agarose gel. Lane 1, undigested plasmid sample pTV32ts; lane 2, BamHI digest of pTV32ts; lane 3, EcoRI digest of pTV32ts; lane 4, cI857Sam7 digested with HindIII to provide molecular size markers; lane 5, EcoRI digest of FJ7; lane 6, EcoRI digest of FJ7US1; lane 7, BamHI digest of FJ7US1. The numbers indicate size of restriction fragments in Kb (kilobases).
Table 15. The helix phenotype of RJ7US1 in various growth conditions.

<table>
<thead>
<tr>
<th>GROWTH MEDIUM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TEMPERATURE</th>
<th>HELIX PHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>20°C</td>
<td>RH(+2)</td>
</tr>
<tr>
<td>TB+MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>20°C</td>
<td>RH(+4)</td>
</tr>
<tr>
<td>T</td>
<td>20°C</td>
<td>stressed cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCND (b)</td>
</tr>
<tr>
<td>T+(NH₄)&lt;sub&gt;2&lt;/sub&gt;SO₄</td>
<td>20°C</td>
<td>stressed cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCND</td>
</tr>
<tr>
<td>T+MgSO₄</td>
<td>20°C</td>
<td>RH(+3)</td>
</tr>
<tr>
<td>TB</td>
<td>48°C</td>
<td>LH(-3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>MgSO<sub>4</sub> and (NH₄)<sub>2</sub>SO₄ were added in the final concentrations of 20 and 30 mM respectively.

<sup>b</sup>Helix-hand could not be determined due to distorted morphology.
closely resembled that of the parent, FJ7. No explanation for these intriguing findings can be offered at present.

Mutagenesis

The first of the isolates, FJ7USl, was grown in antibiotic media #3 containing Em (1 μg/ml) and Cm (10 μg/ml) for 18 hours at 33°C. The culture was then diluted 100 fold in fresh medium, prewarmed at 48°C, and growth was continued for 24 hours. At the end of the incubation period, 5 ml of culture was treated with lysozyme and spheroplasts were regenerated on DMB plates supplemented with appropriate antibiotics, as outlined earlier. A collection of Cm-sensitive clones which exhibited resistance to erythromycin constituted the insertional-mutant bank.

Cells from each clone were transferred to 24-well microtiter plates containing 500 μl of TB per well supplemented with erythromycin. Fragments of rudimentary macrofibers that had formed in every well after 18 hours of incubation at 20°C were seeded into two fresh plates; one of which was incubated at 20°C for 18 hours and the other at 48°C for 3 hours. The structures produced by individual clones were then scored for their helical phenotype. The primary goal of this search was to isolate mutants that were impaired in the temperature-induced inversion of helix hand. Although isolates exhibiting altered static twist within a given quadrant were frequent, none of the twelve hundred clones screened, so far, possessed the desired phenotype. Due to time constraint, however, the search could not be completed.
Antibodies Against Macrofiber

An effective approach to identify the twist specific cellular component is to raise antibodies that would bind exclusively to either of the conformational states (RH or LH). Once obtained, these antibodies can be utilized to isolate and characterize the unique antigen by a combination of biochemical and immunological techniques.

Healthy New Zealand white rabbits were immunized individually against 168 (grown in TB at 20°C) and temperature-induced RH and LH structural forms of FJ7 (for detail see Materials and Methods). The resulting antisera were incubated at 56°C for 30 min to inactivate the complement factors. Due to the suspected cell surface location of the twist specific component, activity of the antisera was determined by their capacity to bind whole cells in macrofibers. To avoid non-specific entrapment of the unbound fluorescent antibodies within the inter-strand spaces of intact structures, filaments from gently disrupted multistrand helices were used as antigens. The extent of binding was monitored using the double-layer (sandwich) immunofluorescence technique. The control samples, i.e. pre-bleeding serum and fluorescein-conjugated antibody, showed no affinity for any of the strains but the antisera were found to react strongly with their respective antigens and exhibited easily detectable cross reactions. These results suggested that the surface-antigen composition of RH and LH structures are not drastically different.

To determine if any of the antisera contained twist specific antibodies, they were adsorbed onto 168 and FJ7 grown at 20°C and 48°C.
Table 16. Indirect immunofluorescence of 168 and FJ7.

<table>
<thead>
<tr>
<th>CELLS TESTED</th>
<th>IMMUNOFLUORESCENCE (QUALITATIVE)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>AS(168)(^a) ON</td>
<td>AS(FJ7(RH))(^b) ON</td>
</tr>
<tr>
<td>FJ7(RH)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FJ7(LH)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)Antiserum prepared in rabbits by immunization with 168 grown in TB at 20\(^\circ\)C.

\(^b\)Antisera prepared in rabbits by immunization with temperature-induced RH and LH forms of FJ7.
Figure 25. Indirect immunofluorescence staining of LH structural forms of FJ7.

FJ7 was grown in TB at 48°C. The cellular filaments derived by gentle disruption of LH macrofibers were challenged with AS [FJ7 (LH)] pre-adsorbed on FJ7 (RH) (see Table 17 for explanation), washed with PBS and stained with fluorescein conjugated goat anti-rabbit antibodies. (A) Phase contrast micrograph (B) same filaments upon UV illumination.
The unadsorbed fraction was then examined for its ability to bind various strains. As shown in Table 16, antibodies raised against 168 and FJ7 (RH) lose their binding activity, if pre-adsorbed onto any of the test strains. However, FJ7 (LH) antiserum, pre-reacted with 168 and FJ7 (RH), exhibits weak but significant binding to LH antigen (Table 16 and Figure 25). Adsorption of this antiserum onto LH structures, prior to the binding assay, completely abolishes its affinity for the LH form. These findings indicate that the 48°C grown left-handed forms of FJ7 possess a unique antigenicity. However, the low antibody titer limited further investigation.

Encouraged by these results, attempts were made to raise left-hand specific monoclonal antibodies by Dr. David L. Lucas of the Department of Microbiology and Immunology. The antibodies were tested for their specificity in an enzyme-linked immunoadsorption assay (ELISA) using intact cells as the antigen. None of the 400 clones analyzed from two independent fusions exhibited specificity to any of the conformational states.
CHAPTER 4

DISCUSSION

The cell surface in procaryotes has been implicated in the control of major functions including growth, division and shape maintenance. However, its role is most strikingly realized in the context of cellular morphogenesis. A number of systems displaying morphological aberrations under special circumstances, genetic or physiological, have been studied previously for elucidation of the biochemical basis of shape determination (Rogers et al., 1980, Spratt, 1977; Boylan et al., 1972; Rogers et al., 1970, Krulwich and Ensign, 1968). Unfortunately, the principles that govern transformation of cell shape remain largely elusive. One of the reasons cited for this failure is the rudimentary understanding of the organizational and dynamic aspects of cell walls.

The macrofiber system of *B. subtilis* provides a unique experimental approach in which the dynamics of cell surface structure can be explored in terms of the organization it assumes in response to external stimuli. These changes, although too minute for detection in single cells, are amplified in macrofibers owing to their macroscopic dimensions. One example of the insights this system has already provided is the process of helix hand inversion. Whereas exponentially growing wild type cells exhibit no apparent change in morphology when transferred from 20°C to 48°C, a shift in the growth temperature of a
lyt^- derivative growing as macrofibers is accompanied by a drastic and clearly perceptible alteration in its helical orientation. These minute adjustments of cell surface topology in response to growth environment would be impossible to visualize in single cell cultures.

Helix hand inversion, a corollary in the broader context of twist-determination, serves as an appropriate model system to study structural dynamics of bacterial walls. Hence, a biochemical characterization of macrofiber cell surface, an essential step towards elucidation of the mechanism of inversion, was to constitute the main objective of this investigation. In the first phase, relatively indirect approaches were used to study parameters that are relevant to both inversion and determination of twist. Included were a comparison of lysozyme sensitivity of various strains, interactions of the twist-modulating agents and effect of alkaline phosphatases on twist-development. To detect any gross changes that accompany inversion of helix hand, a complete compositional analysis of cell walls was conducted. The sacculi for these analyses were isolated and purified from strains whose helical orientation was modified using either temperature- or nutrition-regime. More specific parameters such as ester-linked alanine substitution and the extent of cross-linking were also examined. Since requirement of a surface protein in the attainment of leftward twist had already been suggested (Favre et al., 1985; Wolfe, 1985), profiles of wall-associated polypeptides from various structural forms were analyzed for identification of the left-twist protein. In order
to facilitate genetic analysis of the inversion process, a *Streptococcus* transposon, Tn917, was used for insertional mutagenesis in the macrofiber system.

The initial contention that LH and RH forms must possess considerably different chemical identity was based on the early experiments in which intact LH structures were found relatively sensitive to degradation by lysozyme (Mendelson, Favre and Thwaites, 1984). If the differential sensitivity is indeed due to chemical modifications of peptidoglycan, the cell envelopes isolated from the two structural forms should reflect similar differences. In the current investigation, however, the cell walls purified from the temperature-induced left- and right-twist states of FJ7 lysed at almost identical rates (Figure 3, Panel B). Likewise, sacculi prepared from the "fixed" mutants with LH and RH orientation (PS6μB and 8-4A) exhibited similar dissolution patterns (Figure 4). Thus, there seems a disparity in the degree of sensitivity of the intact macrofibers and their walls to muramidase. However, some arguments can be advanced to explain this apparent anomaly. First, it is possible that the surface component which renders LH structures relatively prone to lysozyme may be lost during the harsh treatments employed for wall purification. Secondly, Mendelson et al. measured lysozyme-susceptibility in terms of disintegration of macrofibers i.e. lateral cleavage of septa. Hence differential sensitivity of RH and LH forms in their experiments may represent uniqueness of the septal region alone. Since in *B. subtilis* cross-wall constitutes merely 15% of the entire surface area (Rogers, Ward and Burdett, 1978), it is unlikely to exert detectable influence
on the overall dissolution rate of purified walls. Therefore, the results of this study are not in contradiction with Mendelson et al.'s finding; instead, together they emphasize the importance of septum in the determination of susceptibility to muramidase of intact macrofibers (measured in terms of structural disintegration) and possibly in governing twist.

To test the first possibility, i.e. the loss of a surface component during purification, macrofibers, produced by growth of various strains at 20°C and 48°C, were gently disintegrated. The resulting cellular filaments (consisting of visually undamaged cells) were examined for their sensitivity to lysozyme. None of the four strains (168, FJ7, FS6uB and 8-4A) show any indication of conformation dependent differential susceptibility (Figures 5 and 6). Furthermore, the results appear to be independent of the effector used to modify twist (D-alanine or temperature). Therefore, either the method for macrofiber disruption is not sufficiently gentle or the mechanical integrity of a structure is extremely important in determining its response to hydrolytic enzymes. If the helical twist in macrofibers results from an accumulation of stress during length elongation, it would be expected that the glycan chains of cells within LH and RH structures are strained differently. These nonsimilarly stressed configurations may not be equally sensitive to muramidases (hence the differential susceptibility). Upon disintegration of macrofibers, the wall polymers in both LH and RH structures would return to a common relaxed state and will be equally sensitive to lysozyme. Since
testing of this hypothesis was beyond the scope of the present investigation, no experimental data exist in its favor.

The modifications that make left- and right-handed macrofibers different in their response to hydrolytic enzymes such as lysozyme may only be, if at all, a subset of the entire twist-determining scheme. Hence, it is more pertinent to ask which other surface polymers, in addition to peptidoglycan, participate in controlling twist. The compounds whose presence in the growth medium modify conformational state provided the preliminary clues. Whereas D-alanine (Wolfe, 1985), trypsin (Favre et al., 1985) and magnesium sulfate (Mendelson and Favre, 1987) exhibit "right-factor" activity, ammonium sulfate enhances the development of left-twist. These agents have different cellular targets suggesting that various surface polymers are involved in twist regulation. Thus, peptidoglycan and teichoic acid (D-alanine effect), wall associated proteins (trypsin effect) and electrostatic status (ion effect) must interact in a way not yet understood to govern the eventual helical orientation of a given strain. The influence of growth at various temperatures on helix hand has already been mentioned in this context.

Peptidoglycan and teichoic acids were the first focus of attention. Cell walls purified from 168, FJ7, PS6uB and 8-4A grown at 20°C and 48°C were subjected to biochemical analysis. The two striking features that emerge from the results (Tables 7 and 9) are: 1) derepression of peptidoglycan synthesis at 48°C and 2) a drastic reduction in the galactosamine content at high temperature. Both
changes turned out to be a temperature effect. Other compositional
differences such as lower glucose content in the "fixed" mutants were
also noted. However, none of the differences seemed to be confor-
mational state specific. The molar ratio of wall components (Tables 8
and 10) also failed to reveal any pertinent clue. Since changes in
growth temperature influence the overall cell physiology leading to
alterations unrelated to twist determination, it was considered
appropriate to induce hand-inversion using an effector with a compara-
tively specific target. Both 168 and FJ7 were cultivated in TN and TND
(N and D refer to ammonium sulfate and D-alanine, respectively) at
20°C. The walls purified from these strains were analyzed for gross
composition. Once again, the differences in the content of various
components (Tables 11 and 12) bore no correlation with helical
orientation. The structural features of Gram-positive envelope most
likely to be influenced by growth in presence of D-alanine are the
extent of cross-linking and alanine substitution on teichoic acid.
Although the results of cross-linking analysis have not been included
because of high variability, the walls from cells grown in various
culture conditions were analyzed for alanine substitution on anionic
polymers. None of the strains, with exception of the wild type, appear
to contain significant amounts of ester-linked alanine (Tables 13 and
14). In 168, increased alanine content in teichoic acid at 48°C may
merely be a temperature effect. These observations suggest that a
gross alteration in wall composition is perhaps not the mechanism
employed by cells to regulate twist.
Prior to discussing the possible mechanisms underlying helix-inversion, a comment regarding the variability of results obtained from analysis of two sets of wall preparations must be made (Tables 7 and 9). The variance in the content of wall components is especially pronounced in the macrofiber-forming strains. Even though each set of hydrolysates was analyzed with an amino acid analyzer using two different brands of ion exchange resin, the observed fluctuation could not have been introduced by the instrument alone for the following reasons: 1) the peak areas corresponding to standards were always comparable, if not identical, for both column materials, 2) no drastic variations in the values of internal controls were detected and 3) the standard curves used in the chemical and enzymatic procedures remained almost invariable throughout. Since structural heterogeneity of wall polymers is growth phase dependent, (Pedro and Schwarz, 1981; Glauner and Schwarz, 1983), it is conceivable that the observed fluctuations might, partly, be due to an inconsistency in the age (i.e. stage of growth) of duplicate cultures at the time of harvesting. Since macrofibers are not suited for optical density measurements, the decision regarding an appropriate harvesting-time was based on visual estimation of growth. Thus, two independently grown cultures of the same strain may have been in different growth phases at the time of cell-collection; an outcome of which could be slightly different wall compositions. Despite these variations, however, the results of both analyses led to similar conclusions.

We now return to the question of possible mechanisms underlying the process of inversion. Since D-alanine is the strongest right-
factor with cell wall as its exclusive target, it is rather unusual that growth in its presence perturbs wall composition so insignificantly. That D-alanine may promote hand-inversion via chemical modifications within a small but specific region of the wall is a distinct possibility. Though crucial to the force interactions that ultimately determine helical orientation, the altered constitution of this sector may go undetected in an average chemical analysis designed to reveal only the gross compositional changes. There is precedent for the septal region to be a likely candidate for this role. It has been proposed that despite its relatively minor contribution to the surface area of Gram-positive rods, the effect of septum on the hierarchy of helical folds may be of considerable magnitude (Mendelson, 1982b). The author observed every subsequent fold in an actively growing macrofiber to be of the same helix hand, unlike an overwound string in which every folding cycle results in a string orientation opposite to the preceding one. Thus, it was suggested that "...only the equivalent of negative twist..." can drive the folding process typical of macrofibers and that the negative twist "...arises when cell septa are produced within macrofibers". If the septal region is indeed an acute functional unit of the folding scheme, the impact of its altered chemical structure on the final helical orientation may not be unprecedented. Testing of this hypothesis would, however, impose some experimental difficulties; the most obvious one being the isolation of cross-walls with insignificant contamination from the cylindrical region. Although Lysozyme digestion of the latter has been used (Fan et al., 1974) to obtain
hemispherical caps for turnover studies, the preparation would be unsuited for the biochemical analysis of septa. In mini-cells, the majority of the surface material is constructed from the septal wall. Thus, analysis of mini-cells produced by the LH and RH macrofibers, carrying appropriate mutations, may be an alternative approach to study the changes in cross wall structure that accompany hand-inversion. Macrofiber-forming strains of \textit{B. subtilis} carrying divIV-B1 mutation, CG6D and BL8, are already available.

In addition to modifications of the carbohydrate matrix, experiments by Favre et al. (1985) and Wolfe (1985) have demonstrated involvement of a surface protein(s) in both temperature- and nutrition-induced inversions. To identify such a protein, LiCl-extracts prepared from strains cultivated in various conditions were examined. A 200 Kdal protein was consistently present in extracts from 168 and FJ7 grown at 48°C. A band of similar molecular weight was also found in PS6pB, a "fixed" LH mutant, irrespective of the growth temperature. FJ7 cultivated in T containing ammonium sulfate (30 mM), sodium chloride (20 mM) and sodium sulfate (20 mM), all of which supported formation of LH macrofibers, also contains an identical polypeptide (Figure 18). The cells growing in T supplemented with magnesium chloride, a salt whose presence is conducive to production of RH helices (Figure 18, lane 3), conspicuously lacked this protein. Thus, the presence of the 200 Kdal polypeptide seems to be correlated with the development of leftward twist.
Except the protein profile of FJ7 (grown at 20°C and 48°C), the data is strongly supportive of the correlation mentioned above. Due to extremely low yield of the surface proteins in this strain, lanes (lanes 3 and 4 in Figure 18) had to be over loaded in order to determine the presence of the polypeptide in question. In spite of excessive loading, however, the intensity of the 200 Kdal protein is very low. The problem of low yield of wall associated proteins was found common among fiber-forming strains. It is not clear at present whether lyt- mutants synthesize these polypeptides at a lower level or a substantial fraction of the normal complement is released to the surrounding medium due to their lower affinity towards the autolytic enzyme deficient cell wall.

Even though the existence of this protein is conformational state correlated, it does not qualify for the role of left-twist protein owing to its presence in: 1) 8-4A, a "fixed" RH mutant and 2) FJ7 cultivated in T supplemented with magnesium sulfate, a strong RH factor (data not included). Furthermore, growth of PS6uB in T containing ammonium sulfate (30 mM) causes reduction in the amount of the 200 Kdal polypeptide while the resulting LH structure attains a high static twist (data not shown). The first of these discrepancies may be explained in light of the possibility that 8-4A is a double mutant (Wolfe, personal communication). One of the mutations may be within the structural gene for the 200 Kdal protein resulting in loss of its left-twist activity, thus leaving RH orientation of 8-4A unperturbed. The anomaly relating to PS6uB can be justified if the
binding of the protein to cell surface is twist state dependent. Ammonium sulfate, being a strong left-effector, would "drive" the structures to the LH-extreme of the twist spectrum and thereby could limit their affinity to the 200 Kdal polypeptide. A complete resolution of these anomalies must await further experimentation.

How does a surface associated polypeptide such as the 200 Kdal protein or LTP modify helical orientation? A structural interaction between a protein and glycan strands could, conceivably, induce a change in conformation of the latter eventually leading to alteration in twist. Intuitively such a polypeptide would be distributed over the entire cell surface to hold the whole matrix in a specific orientation. A similar effect could also be exerted by its binding to some specific region within the wall which in turn governs the final twist. Alternatively, the modulator protein may be one of the surface remodeling enzymes that modify peptidoglycan orientation by the hydrolytic and re-synthetic activities similar to the ones described by Chatterjee et al. (1977) in a related context. The genus Bacillus is known to secrete a number of extracellular enzymes, including proteases, some of which are coordinately regulated with sporulation (Priest, 1977; Hoch, 1976). It is likely that the twist-correlated protein may be a protease whose activity controls the onset of a particular conformational state. However, based on the experiments using protease deficient mutants, Mendelson and Favre have argued against this possibility (1987).

That electrostatic interactions are also important in determination of twist is clearly indicated by the effects of electro-
lytes on helical organization. Some neutral salts induce instantaneous turning motions perhaps by acting at the level of twist maintenance (Mendelson et al., 1985), whereas others require continued growth to bring about drastic changes in helical orientation. Magnesium and ammonium sulfate are two prime examples of the latter category. It is debatable as to whether the effect of electrolytes on twist determination is a manifestation of alterations in the cell wall composition caused by cultivation in a varying ionic environment (Ellewood and Tempest, 1972) or not. It would seem less likely since the chemical differences detected in temperature- or nutrition-induced LH and RH forms were found not to be twist related. However, these effectors could disrupt the normal charge status of surface polymers by binding to the negatively charged teichoic acids. At least in _B. subtilis_ 168 where anionic polymers are co-assembled with glycan chains (Mauck and Glaser, 1972), the vicinal charge environment would be expected to influence spacial configuration of nascent subunits at the time of assembly. The teichoic acids themselves have been shown to lose their secondary structure in presence of salts (Doyle et al., 1974). Similar effects could be introduced in peptidoglycan matrix which carry both negative and positive charges at neutral pH. Such structural deformities caused by twist modulators should propagate throughout the cross-linked network when combined with continued synthesis and assembly of wall polymers ultimately causing a change in conformational state. That these interactions must be nonrandom in nature is indicated by the resulting structures which are highly
uniform. Both instantaneous and growth dependent effects of acidic pH are compatible with the role of electrostatic interaction in twist maintenance and determination.

During the course of investigation, some commercial intestinal phosphatases were shown to prevent the development of leftward twist in FJ7 at 48°C. Since these preparations were contaminated with proteases, it was imperative to determine which of the two activities was actually responsible for the effect on twist. Using differential heat inactivation of the phosphatase and protease activities (Figure 14) it was demonstrated that inhibition of twist development was due to the former. These conclusions were confirmed by fractionalation of dog intestinal phosphatase on hydroxylapatite and DEAE-cellulose columns (Figures 15 and 16). The fraction (F-I) containing most of the phosphatase, though virtually free of proteases, retained its biological activity (Table 6). As expected, F-III, with its high protease content, exhibited right-factor activity similar to trypsin.

Of all the twist modulators alkaline phosphatase is the most unusual. Teichoic acids, the predominant phosphate containing surface polymer, would seem an obvious target of this enzyme. However, the phosphatase treatment of purified walls released less than 1% of the total phosphate content. The pre-existing chains, therefore, would be an unlikely candidate for phosphatase action. There is evidence for derepression of cell-bound alkaline phosphatase in phosphoglucomutase-deficient mutants of B. licheniformis grown in $\text{PO}_4^{3-}$ limited conditions (Forsberg et al., 1973). Under these circumstances the cells lose the
majority of wall teichoic acids and undergo a morphological transformation (i.e. rod to sphere). The change in shape, however, was not observed until the loss of teichoic acid was nearly complete. These findings imply two important conclusions: 1) endogeneous alkaline phosphatase is somehow related to the teichoic acid metabolism and 2) a substantial reduction in the amount of charged polymers is required for a drastic alteration in shape. In the context of macrofibers, exogenously added phosphatase may exert its twist-modifying effect by interference with teichoic acids, possibly at the level of biosynthesis. The impact would not have to be large because helix-hand inversion is a less drastic cellular phenomenon than the rod-to-coccus transformation.

Do the various surface polymers implicated in the regulation of helical twist contribute equally to the final conformational state? Since each of these components are perhaps acted upon uniquely by RH or LH factors, it is also pertinent to ask if the effectors, discussed so far, possess comparable twist modifying potential. A cursory examination of interaction matrices indicates that although trypsin blocks the development of leftward twist in FJ7 growing in T, it is rendered completely ineffective in the presence of ammonium sulfate (Figure 10). The suppression of the right-factor activity of trypsin is not due to inhibition of its protease activity by ammonium sulfate. These findings imply that at least in the nutrition-regime, contribution of electrostatic interactions in attainment of left-twist is far greater than the surface proteins such as LTP and the 200 Kdal polypeptide.
D-alanine (4.5 mM), on the other hand, induces FJ7 to form RH helices irrespective of the ammonium sulfate concentration (Figure 7).

Similarly, PS6μB, a "fixed" LH mutant in the temperature-regime, achieves right-handed orientation when this amino acid is present in higher amounts (Figure 8). Hence, D-alanine appears to be the strongest right-factor. This observation is not surprising since D-alanine is a constituent of the covalently cross-linked surface matrix that bears most of the mechanical stress. Curiously, ammonium sulfate and D-alanine are antagonists when present in lower amounts but behave synergistically within the higher range of concentrations (Figure 8).

It is plausible that increasing ionic strength may enhance transport of the latter across the cytoplasmic membrane thereby stimulating its twist-modulating activity. Alternatively, ammonium sulfate at elevated concentrations could affect the D-alanine target, possibly the enzyme system that feeds nascent glycan chains into pre-existing wall, making it more prone to the effector.

To approach the problem of temperature-induced inversion from a genetic standpoint, Tn917, a Streptococcus transposon, was used to obtain insertional mutants for the reasons discussed earlier. FJ7US1, the strain harboring pTV32ts retained helix phenotype identical to the parent strain except when grown in T (Table 16). Unlike FJ7 which forms LH structures of moderately higher twist in this medium (supplemented with ammonium sulfate), FJ7US1 consistently produced stressed helices with morphologically distorted cells. Since growth in T is conducive to formation of left-handed helices, it was questioned
if the presence of pTV32ts interfered with attainment of LH orientation. This possibility was ruled out by cultivating FJ7US1 in TB containing ammonium sulfate and selective antibiotics. Since the structures formed under these culture conditions exhibited normal LH conformations, the production of stressed macrofibers in T must be due to its lower nutrition status accentuated by addition of erythromycin and chloramphenicol.

A small bank of insertional mutants was searched for mutants defective in temperature-induced hand inversion. Although, of 1200 clones screened, none were found to exhibit the desired phenotype, clones with altered static twist were not rare. It is hoped that with continued searching, inversion-impaired mutants will eventually be isolated.

Attempts to raise twist specific antisera were met with little success. Initial antibody-adsorption experiments exhibited marginal specificity to LH macrofiber surface. However, low antibody-titer barred further investigation. As mentioned earlier, efforts to isolate orientation-specific monoclonal antibodies were fruitless.

In conclusion, the results of the current investigation clearly emphasize a participation by various surface polymers in both twist determination and maintenance. Physiological and biochemical characterization of the macrofiber cell surface indicates that gross compositional changes, including the extent of ester-linked alanine substitution, are perhaps not the operating mechanisms underlying temperature- or nutrition-induced inversion. The differential lysozyme sensitivity observed in structurally unperturbed macrofibers was shown
to be absent from both walls and cells derived from LH and RH forms of macrofibers. These findings suggest that the changes accompanying the inversion process may be below the resolution of common chemical analysis. In support of an earlier suggestion that a surface protein may be required for a successful inversion, a lithium chloride extractable protein of 200 Kdal molecular weight was identified. Its presence in LH forms of macrofibers and high trypsin sensitivity are two crucial properties that this protein shares with the proposed LTP. Although the 200 Kdal protein participates in the development of left-twist, its effect is easily overcome by addition of D-alanine. This was clearly demonstrated by the protein profiles of LiCl extracts made from 734 grown in TN and TND (Figure 17, lanes 7 and 8). Despite the fact that TND-extracts contain the 200 Kdal protein, the structures produced in this growth medium are right-handed. Finally, methods for insertional mutagenesis in the macrofiber system have been standardized which should aid in genetic dissection of the inversion phenomenon.

From the foregoing discussion, a general scheme for twist-determination in _B. subtilis_ seems to be emerging. The data collectively indicate that the twist modulators utilized in this investigation act independently, although with varying degree of potential, on their respective targets instead of blocking a common twist determining pathway at different stages. It follows, then, that the final static twist of a given macrofiber is determined by the force-interaction to which each relevant surface component individually contributes. Any perturbation in this delicate balance could swing the
orientation, depending on the nature of the effector molecule, from its steady state position in either direction along the twist spectrum. An extremely large shift which spans the region of twist spectrum corresponding to zero twist will be what is known as hand-inversion.

As mentioned earlier, no twist correlated differences in walls isolated from LH and RH structural forms were found. If chemical modification of cell envelope is not the mechanism, how do macrofibers achieve distinct orientations? In context of shape determination in procaryotes, Koch (1986) has argued that it is possible to build different structural shapes from a common subunit simply by varying the spacial arrangement. An appropriate analogy would be construction of buildings with different forms using identical bricks. In macrofibers, then, both LH and RH forms could be attained utilizing chemically similar wall-subunits assembled in an orientation unique to each structure. To speculate further, the twist-modulators would induce changes in static twist or helix hand by modifying the conformation of wall polymers at the time of assembly. Obviously, experiments apart from the usual standard techniques, will have to be conducted to test these possibilities. In the meantime, a biochemical basis of twist determination, and hence inversion, cannot be ruled out. Methods such as high performance liquid chromatography, capable of detecting finer chemical changes in the bacterial cell wall (Glauner, 1984), should be employed to resolve the issue.

Much remains to be done before a full understanding of twist adaptation is reached. A thorough genetic analysis and generation of
conformational state-specific antibodies will be the most desirable tools for complete elucidation. However, the problem of helix hand inversion is only a subset of a much larger question. That is, what could be the physiological motive behind the perpetual twist adjustment in response to environmental stimuli? Is change in twist a signal setting off certain cellular responses or merely their end product? Though theoretical attempts to explore the cellular meaning of helical orientation have been made (Mendelson, 1982a), the state of knowledge is still premature for experimental endeavours. Nevertheless, the macrofiber system of *B. subtilis* provides a unique and effective approach to study the dynamic nature of the Gram-positive cell surface, comprehension of which is a pre-requisite for the functional integration of crucial cellular functions such as growth, division and shape determination.
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