THE INHIBITIONS OF CELL DIVISION AND AUTOLYSIS OF BACILLUS SP. #4 INDUCED BY GROWTH IN THE PRESENCE OF PARA-FLUOROPHENYLALANINE

By

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CHAPTER I

INTRODUCTION

The division of cells leading to an increase in cell number is a phenomenon common to all forms of life. Work in this laboratory over the past twenty-five years has been directed towards elucidation of the molecular mechanism of cell division, using bacteria as model systems. In terms of basic research, an understanding of the mechanism of bacterial cell division will go hand-in-hand with an understanding of the interaction of many essential cellular activities.

Interest in cell division was initiated in this laboratory with the finding that the addition of D-glucose to cultures of a strain of Erwinia carotovora, growing in nutrient broth, resulted in unusually long cells (Grula, 1960a). It was subsequently found that division of E. carotovora could be inhibited by various physical and chemical agents, including: D-serine, D-methionine, D-phenylalanine, D-tryptophan, D-threonine, D-histidine, penicillin, vancomycin, D-cycloserine, mitomycin c, S-dichloro-vinyl-L-cysteine, aminopterin, 5-fluorouracil, ultraviolet light, and fluoride ion (Grula, 1960b; Grula and Grula, 1962a, 1962b, 1964; Grula and Grula, 1976).

Another organism studied in this laboratory, because of the ease of working with its cell wall, is Micrococcus lysodeikticus. Inhibition of cell division in this organism by the previously mentioned treatments could not be demonstrated until a nutritional mutant of the organism was isolated. The mutant, M. lysodeikticus dis-II, was selected for its ability to utilize aspartic acid as its major source of carbon, nitrogen, and energy (Grula and King, 1970). Inhibition of cell division of M. lysodeikticus dis-II can be induced by treatment with D-serine, mitomycin c, penicillin, hydroxylamine, D-cycloserine, and suboptimal concentrations of magnesium ion (Grula and King, 1970; King and Grula, 1972).

Aside from inhibiting cell division, one factor that many of the inhibitors have in common (D-amino acids, penicillin, D-cycloserine, etc.) is their ability to modify cell wall mucopeptide and/or decrease mucopeptide synthesis in both of the organisms discussed above (Grula and Grula, 1964; Grula et al., 1965; King and Grula, 1972). Another parameter influenced by the division inhibitors is solute uptake. Transport has been shown to be altered in non-dividing cells of both E. carotovora and M. lysodeikticus (Grula et al., 1965; Grula and King, 1971). It has also been observed that non-dividing cells of E. carotovora leak periplasmic proteins, including a protein with ATPase activity (Grula and Hopfer, 1972). Grula and King (1971) have shown that non-dividing cells of M. lysodeikticus dis-

IIp$^+$ have an altered membrane protein composition as well as cell membrane proteins with altered conformation. Johnson and Grula (1980) have demonstrated that non-dividing cells of M. lysodeikticus have an altered membrane phospholipid composition. What all of these phenomena have in common is that they are indicative of damage to the cell membrane or result in conditions which could indirectly lead to cell membrane damage, as in the case of inhibited mucopeptide synthesis.

Another factor of significance is the ability to prevent or reverse the inhibition of cell division, under certain conditions. Inhibition of cell division induced by D-serine in E. carotovora can be prevented by addition of calcium ion, zinc ion, manganese ion, ammonium salts, p-aminobenzoic acid, and D- or L-alanine to the growth medium or by altering the initial pH of the medium to below pH 6.4 or above pH 7.4 (Grula and Grula, 1962a, 1962b). Hypertonic conditions can also prevent the inhibition of cell division (Grula and Grula, 1964). Chemical agents which, in high concentration, can prevent or reverse filament formation in E. carotovora include: propylene glycol, α-methyl D-glucoside, α-methyl D-mannoside, ethylene glycol, and NaCl. Pantoyl lactone can also prevent and reverse filament formation in E. carotovora. An interesting exception to prevention of filament formation by high osmotic pressure is the case of sodium (or potassium) chloride added together with penicillin. In this situation, the salt markedly

increases filament length. Studies with M. lysodeikticus have shown that pantoyl lactone, D- and L-alanine, glycine, and Carbowax-400 can all prevent, but not reverse cell division inhibition induced by D-serine in that organism (Grula and King, 1970).

Addition of pantoyl lactone to non-dividing cells of E. carotovora results in filament reversion, without restoration of the mucopeptide content to a normal level (Grula and Grula, 1964). The presence of pantoyl lactone was subsequently shown to prevent the loss of periplasmic proteins from E. carotovora and most of the changes of membrane proteins of M. lysodeikticus (Grula and Hopfer, 1972; Grula and King, 1971). Pantoyl lactone also prevents the changes in membrane phospholipid composition observed in non-dividing M. lysodeikticus (Johnson and Grula, 1980).

On the basis of the data which have been summarized in the previous few paragraphs, Grula and coworkers have hypothesized that damage to the cell membrane is responsible for the inhibition of cell division. In most cases, membrane damage would be thought to occur as a secondary effect resulting from the loss of cell wall integrity, which is induced by many of the division inhibitors (Grula and Grula, 1964). More recent data, obtained by Horne et al. (1977), indicate that release of membrane lipids occurs upon addition of a number of different inhibitors of mucopeptide synthesis. This occurs in several species of Bacillus, Staphylococcus, and Streptococcus. These data support the

theory that cell wall integrity is required for cell membrane integrity. The fact that hyperosmotic conditions (ie. sodium chloride) can prevent or reverse both cell division inhibition and the accompanying membrane effects, argues in favor of the theory that maintenance of cell membrane integrity is closely related to the organism's ability to divide. Data have been published which suggest that pantoyl lactone exerts a physically stabilizing effect on the cell membrane surface, resulting in restoration of normal cell division activity in inhibited cells (Johnson and Grula, 1980). It is important that pantoyl lactone is able to prevent or reverse inhibition of cell division induced by all inhibitors tested (Johnson and Grula, 1980).

Electron micrographs provide further evidence for the involvement of the cell membrane in cell division. Studies conducted by Grula and Smith (1965) show quite clearly that the cell membrane and cell wall invaginate concomitantly during division. It has been suggested by these authors that the cell membrane initiates and sustains the division process. Jacob et al. (1963) have proposed an attachment of the nuclear body to the cell membrane as an integral part of cell division and later provided evidence which implicated the cell membrane as being involved in chromosome replication (Shapiro et al., 1970). Inouye and Pardee (1970) have backed these findings with similar results obtained in their own laboratory. Lastly, it has been suggested, by Helmstetter (1974), that the cell membrane

controls the timing of chromosome replication by way of attachment of the DNA to the cell membrane.

The original purpose of this endeavor was to study proteins involved in cell division, on the premise that it might be possible to somehow demonstrate direct involvment of "division proteins" in the cell division process. There are numerous reports in which inhibition of cell division is accompanied by both quantitative and qualitative changes of membrane proteins (Grula and King, 1971; Inouye and Pardee, 1970; Shapiro et al., 1970). This is to be expected, especially when one considers the data implicating the involvement of the cell membrane in cell division and the fact that "activities" of cells are generally the result of protein actions and interactions.

It is significant, then, that the amino acid analog p-fluorophenylalanine (p-FPA) is able to inhibit cell division as well as cell growth in Escherichia coli (Previc and Binkley, 1964; Hardy and Binkley, 1967; Carpenter and Binkley, 1968). It has been postulated by these workers that this compound acts to inhibit cell division by replacing phenylalanine in "cell division proteins", thereby inactivating or altering their function. However, their results indicate that the phenomenon is probably not that simple. First of all, the inhibitions of cell growth and cell division are separable. An E. coli mutant which requires all three aromatic amino acids is not made filamentous by p-FPA, nor is an E. coli auxotroph which

requires phenylalanine only, although both mutants show inhibition of cell growth. They suggest, as an alternative to the hypothesis discussed above, that tyrosine deprivation induced by the presence of p-FPA might result in cell division inhibition. There are two enzymes in these E. coli mutants which catalyze the condensation of phosphoenol-pyruvate with erythrose 4-phosphate to form 3-deoxy-D-arabino-heptulosonic acid 7-phosphate, which is an early precursor in the pathway leading to the aromatic amino acids via shikimic acid. Since these enzymes are under complex end-product inhibition and repressive control by the aromatic amino acids, they believe that p-FPA might somehow lead to deprivation of tyrosine by upsetting the complex control of aromatic amino acid synthesis and that this altered metabolism leads to inhibition of cell division, although they do not define a mechanism by which cell division is inhibited.

The incorporation of p-FPA into cell division proteins is plausible because its incorporation into other proteins has been shown. Yoshida (1960) has demonstrated incorporation of p-FPA into the ∝-amylase of Bacillus subtilis. He found that p-FPA had replaced phenylalanine and as a result the enzyme had a lowered activity, but was unaltered in any other way. Richmond (1963) has shown that p-FPA randomly replaces phenylalanine in the phosphatase(s) of E. coli. Further, Kang and Markovitz (1967) have found that p-FPA causes a derepression of the alkaline

phosphatase(s) of E. coli. They have shown that p-FPA is incorporated into the regulatory protein for the alkaline phophatase(s). The regulatory protein thus formed is inactive at 30°C and cannot bind the promoter-operator region of the alkaline phosphatase gene(s), causing derepression of alkaline phosphatase, but the regulatory protein retains activity at 22°C. This indicates that alterations of enzymatic activity caused by incorporation of p-FPA can be quite subtle.

Experiments in this laboratory indicate that growth in the presence of p-FPA results in the inhibition of cell division of Erwinia carotovora (M. M. Grula, unpublished observation) and Bacillus sp. #4 (T. Conway, unpublished data), a soil isolate of Bacillus commonly used in this lab. Early experiments concerning the effect of p-FPA on growth and division of Bacillus sp. #4 led to the observation that older cultures of the organism made filamentous by p-FPA contain a preponderance of cell wall ghosts. This suggested that the autolytic system of Bacillus sp. #4 is somehow inhibited by growth in the presence of p-FPA.

Subsequent attempts to characterize the effects of and to correlate the inhibitions of cell division and autolysis induced in Bacillus sp. #4 by growth in the presence of p-FPA are the basis for the research presented in this manuscript. The study of the effect of growth in the presence of p-FPA on autolysis is attractive from several standpoints. First of all, this inhibition of autolysis

occurs upon addition of the cell division inhibitor p-FPA, a compound which may act to form fraudulent proteins. Secondly, autolysis is the result of the activity of the autolytic enzymes (autolysins), proteins which possess the ability to hydrolyze specific bonds within the cell wall mucopeptide. There are reports in the literature concerning the involvement of autolytic enzymes during cell division, which will be discussed below. Next, the location of the autolytic enzymes is thought to be the inner face of the cell wall and/or the outer face of the cell membrane, again discussed below. Lastly, the importance of cell wall integrity for stability of the cell membrane and normal cell division has already been discussed. It should be apparent that the study of this effect of p-FPA ties in with the basic premises of the research problem; the addition of this cell division inhibitor results in an alteration in non-dividing cells which is presumably mediated by its effect on the proteins of the autolytic system which, in turn, are thought to be located on or near the outer face of the membrane, and also thought to be involved in cell division.

A brief description of the activities of the autolytic enzymes is germane to this discussion. There are two autolysins known to be present in Bacillus subtilis: an N-acetylmuramyl-L-alanine amidase and an endo-β-N-acetyl-glucosaminidase (Fan and Beckman, 1972). The amidase cleaves the L-alanine residue linked to N-acetylmuramic acid and the glucosaminidase (glycosidase) cleaves the β 1,4 bond

between N-acetylmuramic acid and N-acetylglucosamine at the reducing end of the glycan chain. There are numerous reports which demonstrate the presence of these enzymes, and no others, in many other species of Bacillus and in several other genera (Higgins and Shockman, 1971; Strominger and Ghuysen, 1967). Although the Bacilli contain only the two autolytic activities mentioned above, numerous other organisms contain endopeptidases and/or exopeptidases which cleave within the peptide crosslinkage units of mucopeptide (Strominger and Ghuysen, 1967). However, there appears to be only amidase activity present in Streptococcus pneumoniae (Holtje and Tomasz, 1976).

Most workers will agree that autolysins are important for growth, but on this point, the data are widely different (Rogers, 1970). There are many reports in the literature concerned with the consequence on growth and morphology of mutants which show deficiencies in their autolytic system. However, not all workers have found that deficiency of autolytic activity results in detrimental effects to the cells. For example, Fiedler and Glaser (1973) have isolated an autolytic deficient mutant of Bacillus subtilis which grows normally in the apparent absence of autolytic activity. Coronett et al. (1978) have isolated an autolytic-defective mutant of Streptococcus faecalis which is thermosensitive for cellular autolysis. Their data indicate that the amidase of this organism is altered slightly in its substrate specificity. However, this

mutation has no effect on growth rate. In fact, under conditions of inhibited autolysis (non-permissive temperature) the mutant grows faster than the wild type. Workers in Rogers's laboratory have found that autolytic-deficient (deficient in total autolytic activity) strains of Bacillus subtilis and Bacillus lichenformis do not show any alteration of growth rate (Fein and Rogers, 1976; Forsberg and Rogers, 1974).

Autolytic defective mutants of Streptococcus faecium have been isolated (Shungu et al., 1979). These strains have a lowered rate of cellular autolysis, accompanied by a fifty percent slower growth rate. These mutants also contain increased levels of lipoteichoic acid, the significance of which will be discussed below. Fan and Beckman (1971) have isolated an autolytic defective strain of Bacillus subtilis which shows a greatly decreased doubling time and altered morphology (the cells show a twisted and knotted morphology). Exogenous addition of lysozyme or B. subtilis autolysins was able to restore the growth rate back to normal. The degree of restoration of growth rate to normal was a function of the concentration of autolytic enzyme added. They interpreted their data as indicating that a normally functioning autolytic system is a necessity for normal growth. Ultrastructural studies of this mutant have shown that addition of autolytic enzyme to cultures growing slowly at the non-permissive temperature also results in restoration of normal morphology (Fan and

Beckman, 1972).

Fiedler and Glaser (1973) have examined the various reports in the literature and discussed the discrepancies concerning the requirement for autolysis during bacterial growth. They suggest that the Bacillus subtilis mutant (described above) of Fan and Beckman (1971) has an autolytic system which is more completely inhibited than that of the other mutants. It may be that only a minimal amount of autolytic activity is required for normal growth and would explain why many of the less completely inhibited autolytic-deficient mutants have a normal growth rate (such as the B. subtilis mutant of Fiedler and Glaser (1973), described above). Unfortunately, studies correlating the degree of inhibition of the autolytic system with the degree of change in growth rate are lacking.

It was Young (1966), who first advanced the hypothesis that autolytic enzymes are necessary for enlargement of the cell surface, which occurs as a result of cell wall growth. The autolytic enzymes are thought to cleave the cell wall in a controlled manner, leaving room for insertion of new wall material. This intercalary insertion of newly synthesized monomers during cell wall growth has been demonstrated by several workers (Rogers, 1970; Shockman et al., 1974; Pooley, 1976a). Numerous other authors think that autolytic enzymes participate in cellular functions related to growth and division (Rogers, 1970; Shockman et al., 1974). The dynamic relationship between mucopeptide synthesis and

degradation by autolytic enzymes results in turnover of the cell wall (Mauck and Glaser, 1970, 1971; Pooley, 1976a; deBoer et al., 1982) and remodeling of the cell wall (Pooley, 1976b), which are thought to be related to expansion of the wall during growth (Rogers, 1970).

The cell wall mucopeptide of many Gram-positive organisms turns over during logarithmic growth (Mauck and Glaser, 1970, 1971). This turnover results in loss of selected portions of the cell wall and this loss can be measured as a loss of radiolabelled cell wall from intact bacteria or as an increase in radiolabelled cell wall units released into the medium (Mauck and Glaser, 1970, 1971; Pooley, 1976a; deBoer et al., 1982; Glaser and Lindsay, 1977). Furthermore, loss of cell wall teichoic acids occurs along with mucopeptide turnover and can be measured in a similar manner (deBoer et al., 1981). Turnover of both types of cell wall polymers has been shown to be the result of the hydrolytic action of wall-bound autolytic enzymes on the mucopeptide in the outer layer of B. subtilis (Pooley, 1976a, 1976b).

The concentration of cell wall turnover products in the medium of logarithmically growing B. subtilis is 8-10% of the total cell wall per generation (Pooley, 1976a). However, studies show that when bacteria with cell walls labelled under steady-state conditions are transferred to non-radioactive medium, the rate of release of labelled turnover products increases exponentially over a period of

0.5 to 1.0 generations (Lindsay and Glaser, 1977) or up to 2.0 generations (Pooley, 1976a). A second phase of turnover is attained after this first phase, at an apparent first-order rate of 50% per generation (Pooley, 1976a; Lindsay and Glaser, 1977). These kinetics are consistent with, but not direct evidence for, turnover occurring randomly over the bacterial surface; the rate of release of label is proportional to the concentration of labelled wall remaining on the surface.

When pulse labelled cultures of B. subtilis are utilized, there is a distinct lag before turnover of labelled products occurs (Pooley, 1976a). Pooley has concluded that new cell wall is not subject to turnover and implies that there is a non-random distribution of new and old cell wall. In a second series of experiments, Pooley (1976b) has confirmed this hypothesis. The method of pulse-chase labelling, followed by sampling at short intervals, has revealed that wall newly synthesized at the outer face of the cell membrane passes out through the thickness of the cell wall layer during subsequent growth and only becomes susceptible to turnover as it reaches the outer cell wall surface. This pulse-labelled cell wall is largely in the form of a layer and takes more than one generation to reach the surface.

Experiments of Lindsay and Glaser (1977) have shown that turnover of cell wall is directly related to growth of the cell wall and thus to the rate of expansion of the cell

wall. These data have been confirmed by the elegant work of deBoer, et al. (1982), in which they followed cell wall turnover and cell wall synthesis concomitantly, using a double label system. However, other data presented in this same report by deBoer et al. indicate that the rate of turnover is not always directly related to the rate of cell wall growth. They have created situations in which cell wall growth (synthesis of mucopeptide) was inhibited by limiting glucose in a chemostat culture, but autolysis was allowed to continue at a normal rate, as monitored by release of labelled cell wall. The continued cell wall degradation resulted in lysis of the cells. The result was the same when they added various cell wall synthesis inhibitors, such as penicillin.

On the other hand, situations could be created in which autolysis and, therefore, cell wall degradation was inhibited, but cell wall synthesis was allowed to continue. Addition of chloramphenicol to a logarithmic culture of Bacillus cereus does just that; the result was a thickening of the cell wall to two or three times the normal thickness (Chung, 1971). This experiment has been repeated and the conclusion confirmed, using B. subtilis (deBoer et al., 1982). Growth of a trp^- mutant of B. subtilis on minimal medium plus tryptophan and subsequent transfer during logarithmic growth to medium which lacks tryptophan resulted in cells with walls approximately two to three times thicker than normal (Hughes et al., 1970). These workers

demonstrated that under tryptophan starved conditions the organism continued to synthesize cell wall, but degradation of the cell wall did not occur.

The importance to the cell of regulating the rates of cell wall synthesis and turnover is obvious. The fate of a cell in which degradation occurs without wall synthesis is lysis; the fate of a cell in which wall synthesis occurs without cell wall turnover is a thicker cell wall. Thus, for normal growth, the cell must balance turnover and growth of its cell wall (deBoer et al., 1982).

Every enzyme, at least theoretically, has the ability to catalyze a reaction in both directions and that is one way in which a cell could regulate the growth and turnover of its cell wall. There is some indication that this may be a viable possibility. Sharon and Seifter (1964) have noted that after long incubation of cell wall monomeric units in the presence of egg white lysozyme, some small amount of polymerization of subunits occurs. This did not occur in the absence of lysozyme. Holtje et al. (1975) have isolated a murein: murein transglycosylase from E. coli which has both lytic properties and the ability to catalyze the formation of an intramolecular 1, 6 anhydro-N-acetylmuramyl bond. They speculate that its in vivo activity might be different (more like a traditional transglycosylase), since the reaction they have demonstrated required triton X-100 when performed in vitro. However, the situation is probably not quite so simple, as other mechanisms of autolytic

control are beginning to be understood.

Experiments with E. coli implicate the cation magnesium as being involved in control of its autolytic system (Leduc et al., 1982). Leduc and coworkers were able to induce autolysis by several methods. Regardless of the induction method used, addition of magnesium ion efficiently inhibited autolysis. They speculate that magnesium ion could play a direct role as an effector of the autolytic enzymes, keeping their activity in check. Removal of magnesium ion by the inducing methods would result in derepression of autolytic activity. On the other hand, activity of the autolysins might depend upon the state of the cell envelope as a whole and removal of the cation could result in disorganization of the cell envelope, thus activating the autolytic system.

The results of Mosser and Tomasz (1970) indicate that the primary structure of cell wall teichoic acid might play a role in control of the autolytic system of Streptococcus pneumoniae. By utilizing a strain of pneumococcus which cannot produce choline or ethanolamine, they were able to grow cultures of cells which contained either ethanolamine or choline, only, in their teichoic acid. They found that substitution of ethanolamine for choline in the teichoic acid renders the cell wall resistant to degradation by its autolytic enzyme (pneumococci contain amidase only). They concluded that a precise relationship between the teichoic acid and mucopeptide components is required for normal autolytic function. Whether ethanolamine alters some

feature of enzymatic recognition by the amidase or causes a distortion in the organization of the cell wall, or both, was not determined.

In searching for a mechanism by which autolysis is controlled, an autolysis inhibitory factor in Streptococcus faecalis was discovered (Shockman et al., 1974). A powerful inhibitor of pneumococcal autolysin (amidase) has been identified as the Forssman antigen, a choline-containing lipoteichoic acid (Holtje and Tomasz, 1975). When Forssman antigen is added to the medium of logarithmically growing pneumococci, the cells are made resistant to lysis by penicillin and vancomycin. These results strongly suggested that lipoteichoic acids play a physiological role in the regulation of autolytic activity. Lipoteichoic acids have a primary structure analogous to that of choline and ribitol containing wall teichoic acids, but also contain covalently bound lipids. These compounds are localized in the cell membranes in several Gram-positive bacteria (Cleveland et al., 1975). Cleveland and coworkers tested the effects of several lipoteichoic acids on four different bacterial autolytic systems. The bacteria tested were: Streptococcus faecalis, Lactobacillus acidophilus, Bacillus subtilis, and Streptococcus pneumoniae. They found that each of these autolytic systems could be inhibited by one or more, but not all of the lipoteichoic acids tested. Their results confirm the general occurrence of inhibition of autolysis by lipoteichoic acid and also demonstrate a type of specificity for

each bacterial system tested.

Herbold and Glaser (1975) have succeeded in purifying the amidase of Bacillus subtilis, along with a modifier protein which binds stoichiometrically with the amidase and stimulates its activity three-fold. A detailed analysis of the wall cleavage products obtained in the presence and absence of the modifier protein indicates that the major effect of the modifier is not to change the rate of amidase activity, but rather to change the pattern of hydrolysis to a more sequential pattern, as opposed to the random pattern of cleaveage which occurs in the absence of the modifier. Tight binding of the amidase to the cell wall and functional interaction of the amidase with the modifier protein occur only when the substrate cell walls contain teichoic acid. They conclude that a general function of cell wall teichoic acids is to act as a specific allosteric ligand for bacterial autolytic enzymes.

Lindsay and Glaser (1977) have proposed a highly speculative model in which they suggest that the cell membrane is actually interdigitated into the cell wall mucopeptide layer, thus preventing cell wall degradation. The cell membrane, of course, would contain the lipoteichoic acids which have been shown to inhibit autolytic activity. Interdigitation of membrane into wall is thought to occur as a result of nascent mucopeptide chains being attached both to the membrane and the wall mucopeptide during wall growth. deBoer et al. (1982) have expanded this model to include the

findings of Pooley (1976a, 1976b), which indicate that cell wall turnover occurs in the older, outer layer of cell wall. Inhibition of the autolytic enzymes by cell membrane associated lipoteichoic acids would occur in the deeper, membrane associated layer of the wall. Autolysin activity which results in cell wall turnover would occur in the outer wall layers and might be regulated only by the amount and activity of autolytic enzymes produced by the cells and translocated to the outer wall layer. It is of more than passing interest to note that deficiency of autolytic activity is associated with a decrease in permeability of the wall in several Gram-positive bacteria (Williamson and Ward, 1981). It may be that autolytic enzymes are simply not allowed access to the outer wall layer as a result of this decreased permeability.

As for the involvement of autolysins in bacterial cell division, there is little doubt that these enzymes play a major role in the separation of daughter cells following septum formation. Bacillus subtilis grows as long filamentous chains at 48°C. Addition of lysozyme or B. subtilis autolysin causes separation of the chained cells, resulting in cells of normal length (Fan, 1970). Fiedler and Glaser (1973) find that mutants which are autolytic-defective grow as chains. The parental cells, with a normal autolytic system, grow as non-chained, short cells.

The involvement of autolytic enzymes in actual cell division, that is, formation of a septum which divides the

daughter cells, is more obscure (Schwarz et al., 1969; Shockman et al., 1974). As mentioned previously, autolytic activity is thought to be required to allow cell wall growth, by opening the wall just enough to allow intercalation of new wall material (Young, 1966). In normal situations, cell wall synthesis and turnover (degradation) are in dynamic balance (deBoer et al., 1982). It can be assumed that this same is true for synthesis of the division septum (Shockman et al., 1974). These workers have advanced the theory that the activity of autolysins at the division site is strictly controlled and timed such that the initiation of septum formation (for which they assume a requirement for autolysins, which open the wall to allow insertion of newly synthesized wall precursors) occurs at the completion of a round of DNA synthesis and nuclear body separation. One should recall that Helmstetter (1974) has shown a direct correlation between the timing of DNA replication and the timing of cell division.

Data used to support this theory were obtained by Schwarz et al. (1969) who took advantage of the fact that penicillin has two different effects on cell wall synthesis, depending on the concentration of penicillin used. That is, synthesis of mucopeptide at the septum is more sensitive to inhibition by penicillin than is synthesis of mucopeptide which results in cell elongation, thus cell division (septum formation) can be inhibited by a relatively low concentration of penicillin which allows elongation of the cell to

continue. At this low concentration of penicillin, synthesis of mucopeptide at the division center (septum site) was inhibited, but autolysin activity was not. The result was a localized degradation of the cell wall at the division center. This result indicates that there are autolysins localized at the division center. Addition of various agents which inhibit DNA synthesis, when added in conjunction with a low concentration of penicillin, prevented the localized degradation of cell wall at the division center. The autolysins which are involved in cell elongation were not affected by inhibition of DNA synthesis. Thus, it appears that the timing of action of the autolysins which are localized at the division center is correlated with the timing of DNA replication. Proper compartmentalization of the autolysins and timing of their action could be an important part of the control of cell division.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in this study has been identified as a member of the genus Bacillus. This particular Bacillus sp. was isolated from soil by Dr. E. A. Grula and has been carried in this laboratory for about twenty-five years. None of the descriptions in either the 7th or 8th editions of Bergey's Manual of Determinative Bacteriology fit this organism (Buchanan and Gibbons, 1974). The bacterium, referred to as Bacillus sp. #4, most closely resembles Bacillus sphaericus or Bacillus brevis, on the basis of its morphological and biochemical characteristics, which have been published (Champlin and Grula, 1979). Although the organism remains unidentified, it proved to be ideal for the experimentation described herein; division of the organism could be inhibited by growth in the presence of p-fluoro-phenylalanine. Also, the large size of the organism (normally 3 μm long by 1 μm wide) made manipulations of its cell wall a fairly easy task.

Bacillus sp. #4 stock cultures were maintained on nutrient agar slants and grown at room temperature. The culture was transferred daily and reserve cultures were

stored in a refrigerated cold room. Inocula for growth of the organism in batch culture were prepared from 16-20 hour old slants. The cells were washed once with sterile physiological saline solution (0.85 % NaCl) and resuspended in saline to an absorbance of ca. 0.100 (Bausch and Lomb Spectronic 20, 540 nm). This cell suspension was used as inoculum, added to growth medium at a level of 1 ml of inoculum per 100 ml of medium.

Media

The aspartic acid-glucose defined medium of Grula (1960b) was used for growth of Bacillus sp. #4 in all situations, except where noted otherwise. The aspartic acid-glucose defined medium contained the following per 100 ml: D-glucose, 150 mg; L-aspartic acid, 280 mg; K_2HPO_4, 174 mg; KH_2PO_4, 136 mg; $MgSO_4 \cdot 7\ H_2O$, 3.0 mg; and trace minerals. Trace minerals were kept as a concentrated stock solution and added to the medium such that the following amounts were present (per 100 ml of medium): H_3BO_3, 0.5 µg; $CaCO_3$, 10.0 µg; $CuSO_4 \cdot 5\ H_2O$, 1.0 µg; $FeSO_4(NH_4)_2SO_4 \cdot 6\ H_2O$, 50 µg; KI, 1.0 µg; $MnSO_4 \cdot H_2O$, 2.0 µg; MoO_3, 1.0 µg; and $ZnSO_4 \cdot 7\ H_2O$, 5.0 µg. Aspartic acid, phosphates, magnesium sulfate, and trace minerals were all autoclaved in the culture flask, together. The pH of the medium before autoclaving was pH 6.8. Glucose was autoclaved separately and added to the medium aseptically. Any other compounds which were added to this medium were either autoclaved separately or filter-

sterilized (as was prudent) and added aseptically to the medium.

A solid medium was used to detect hydrolysis of starch by extracellular amylase. Aspartic acid-glucose defined medium was used as the basal medium, to which was added 1.5% agar and 2.0% starch. p-Fluorophenylalanine (p-FPA) was added to some of the plates at a final concentration of 6.7 µg/ml.

Chemicals

Amino acids and their analogs were all purchased from Sigma Chemical. The fluorophenylalanines used in this study were shown to be chromatographically pure by chromatography in the solvent system of Heathcote and Jones (1965). All chromatography standards were the purest available, purchased from Sigma Chemical. The chromatography standards utilized were L-alanine, L-lysine, muramic acid, glucosamine, D, L-glutamic acid, and meso-diaminopimelic acid. The bovine serum albumin used as protein standard was purchased from Bio-Rad. All radioisotopes utilized in these studies were purchased from New England Nuclear.

Growth and Harvest of Bacteria

Cultures of Bacillus sp. #4 were normally grown in 100 ml of aspartic acid-glucose defined medium in 250 ml Erlenmeyer flasks and incubated at 25°C in a shaking water bath (180 rpm). Additions of phenylalanine analogs (D, L-

isomers; 10.0 μg/ml, final concentration) were routinely made as the cultures entered logarithmic growth phase (absorbance at 540 nm of ca. 0.100; a Bausch and Lomb Spectronic 20 was used for monitoring growth). Amino acids (D, L- isomers), where added, were added to reach a final molar concentration one-half that of p-FPA, unless otherwise specified. Additions of pantoyl lactone (PL) were from a filter-sterilized stock solution of 5.0 M, to produce the final concentration specified.

It was determined experimentally that the optimum time for harvest of a control culture which would ensure isolation of maximal autolytic activity (as measured by any of the methods used to determine autolytic activity, discussed below) was when the control culture was in the middle of logarithmic growth phase (mid-log). Thus, control cultures were typically harvested while in mid-log phase (ca. 9-11 hours of growth; absorbance at 540 nm of ca. 0.25-0.35). Stationary phase (stationary control) cultures, on the other hand, contained cells which showed little measurable autolytic activity. Stationary control cultures were typically harvested after 24 hours of growth (absorbance at 540 nm of ca. 0.90-1.10). The fluorophenylalanines (FPAs) were found to exhibit a high degree of toxicity; cultures to which a fluorophenylalanine (such as p-FPA) was added at the time of inoculation showed very little visible turbidity and contained insufficient biomass for any subsequent experimentation. However, p-FPA could be

added after growth had been initiated, without being so very toxic. It was for this reason that the fluorophenylalanines were routinely added during early-log phase and the cultures harvested after 12 hours growth in the presence of the FPA (the exact timing of addition and concentration of FPA used were determined experimentally). Absorbance of these cultures at the time of harvest varied, depending upon the absorbance at the time of addition of the FPA. A slight deviation from the normal absorbance at 540 nm of 0.100 at the time of addition would be translated into a fairly large difference at the time of harvest. (See Chapter III for rationale, especially Figures 1 and 2.)

Cell lengths were measured using a Nikon phase contrast microscope which was fixed with a calibrated ocular micrometer. The cell lengths reported are the average of the lengths of at least 100 different cells, chosen randomly in 10 microscope fields. The values reported also give the length of the longest cell included for the average, such that the range in length is also reported, except where there is very little variation in length (as for control cells).

For many experiments, it was necessary to isolate the cell walls of Bacillus sp. #4. The cultures were grown as described previously and their cell walls were isolated by the method of Fein and Rogers (1976). This basically involved the following steps: Cells, grown in the usual manner, were harvested by centrifugation and washed once

with glass distilled water. The cells were then suspended in ca. 10-15 ml glass distilled water (ca. 100-250 mg cell dry weight per ml of water). Breakage of the cells was by repeated (usually five times) sonication (Branson sonifier; 15 second bursts with one minute cooling on ice between bursts). The cell walls were separated from other cell constituents by centrifugation at 20,000 xg. A Pasteur pipet was used to remove the cell walls which formed a pellet on top of any cells which remained unbroken after sonication. The cell wall preparation was washed at least four times with glass distilled water. All steps were performed on ice. Cell walls could be stored, frozen in water, at -20°C for periods of up to six or eight months.

For assays of activity of isolated autolytic enzyme preparations, cell walls were used as a substrate. In order to use cell walls as a substrate, all native autolysins had to be removed or inactivated such that these would not interfere with the assay. Inactivated cell walls were prepared in the following manner: A 10% stock solution of sodium dodecyl sulfate (SDS) was added to a cell wall suspension such that the final concentration of SDS was 1% and the final concentration of cell walls was ca. 1-2 mg/ml. This step was performed on ice and the SDS-cell wall suspension was allowed to stand on ice for 15 minutes. The suspension was then placed in a boiling water bath for 15 minutes. After cooling to room temperature, the cell walls were washed at least six times with glass distilled water.

Inactivated cell walls prepared in this manner were stored at -20°C in 10 mM Tris-HCl buffer, pH 7.0.

Standard curves (absorbance vs. dry weight) for both cell and cell wall dry weights were prepared according to the procedure of Gerhardt (1981). The samples were dried at 105 C to constant weight. For cells, absorbance was measured at 540 nm on a Spectronic 20. Separate curves were prepared for cells made filamentous by growth in the presence of p-FPA and for short cells (mid-log control cells, stationary control cells, and cells grown in the presence of o-FPA or m-FPA). Absorbance of cell walls was measured at 450 nm on a Spectronic 100. It was found that dry weight curves for cell walls varied significantly depending upon the method of cell wall breakage. Basically, the smaller the cell wall fragments produced, the lower the absorbance for a given amount of cell wall dry weight. Therefore, the sonication of cells for cell wall isolation was standardized with respect to the time and number of bursts employed (15 second bursts, five times).

Antibiotic-Induced Cell Lysis

Antibiotics were added to mid-log control cultures or to cultures grown for ca. 12 hours in the presence of p-FPA, o-FPA, or m-FPA. Polymyxin B sulfate was added to growing cells to produce a final concentration of 20.0 µg/ml. Penicillin G was added to growing cells at a final concentration of 0.64 mg/ml (1050 units/ml). Both

antibiotics were filter-sterilized and added to the medium aseptically. Lysis of cells was measured as a loss of absorbance at 540 nm on a Spectronic 20.

Assay of Autolysis Using Whole Cells

Control cultures in mid-log phase or cultures grown in the presence of fluorophenylalanine for 12 hours were harvested at 4°C, washed once with physiological saline, and resuspended to the desired initial absorbance in 0.01 M potassium phosphate buffer, pH 6.8, at room temperature. The percent reduction in absorbance at 540 nm was not dependent upon the initial absorbance, so long as the initial absorbance at 540 nm was between 0.10 and 0.50. Lysis of these cells was measured spectrophotometrically at 540 nm using a Spectronic 20. Autolytic activity was expressed as the percentage reduction from the original absorbance at 540 nm during an eight hour period.

Assay of *In Situ* Autolytic Activity Using Isolated Cell Walls

Native autolysins present in cell wall preparations which had not been inactivated were able to degrade the cell walls to which they were bound. Degradation of cell walls by their native autolysins is a type of *in situ* autolytic activity, since the orientation of the autolysins to the cell walls was not changed during isolation of the walls (the autolysins are bound very tightly to the cell

walls). Native autolytic activity present in cell wall preparations (not inactivated) was measured as a loss of absorbance at 450 nm during incubation in 10 mM Tris-HCl buffer, pH 7.0. Absorbance was measured in 2 ml volumes with a 1 cm pathlength, at 25°C (Bausch and Lomb Spectronic 100). One unit of enzyme activity is defined as that amount which will give a decrease in absorbance at 450 nm of 0.001 per hour. Thus, this type of measurement of autolytic activity (following degradation of cell walls spectrophotometrically at 450 nm) was a more precise method than that of whole cell autolysis.

The effect of pH on the rate of degradation of cell walls by native autolysins was used as a preliminary comparison of the autolysins present in cells grown in the presence of p-FPA and in mid-log control cells. Cell walls were suspended in Tris-Maleate-Borate buffer (TMB; 50 mM each of Tris, maleic acid, and boric acid) at 0.5 pH unit steps over the range of pH 4.5 to pH 10.0. Degradation of the cell walls was followed spectrophotometrically, as before. The rate of degradation was expressed as the percentage decrease in absorbance at 450 nm per hour (Spectronic 100).

In Vitro Assay of Autolytic Activity Using Isolated Enzyme Preparations

Autolytic enzymes are bound very tightly to cell walls by ionic interactions (non-covalent interactions).

Therefore, removal of autolysins from the cell walls, a necessary step in the isolation of crude autolytic enzyme preparations, must take place in the presence of a compound which will break up the ionic interaction of the enzymes with the cell walls, with out damaging the enzymes themselves (Coyette and Shockman, 1973). One such method is the NaOH method of Coyette and Shockman (1973). Basically, this method involved removal of the autolytic enzymes from cell wall preparations by treatment of the cell wall preparation (ca. 1 mg/ml cell wall) for 10 minutes with 0.01 N NaOH. The dilute NaOH was able to interfere with the ionic interaction of the enzymes with their native cell walls. While still in the presence of dilute NaOH, the cell walls were separated from the autolysins by centrifugation at 25,000 xg for 10 minutes. (Removal of the cell walls had to be complete, since the enzymes would otherwise "reattach" to the walls during the subsequent neutralization step and be lost). The enzyme preparation (autolysins remained in the supernatant) was decanted into a separate container and quickly neutralized with HCl and buffered with 10 mM Tris-HCl (pH 7.0). These steps were carried out as quickly as possible, since the NaOH could inactivate the enzymes.

For some experiments, the rapid method of Brown (1973) was used for enzyme isolation. This simple method involved extraction of washed, lyophilized whole cells with 5 M LiCl at 4°C, pH 7.0. As with the previous method, the treatment disrupted the ionic interaction of the autolysins with the

cell walls, without damaging the enzymes. Again, removal of the cell walls by centrifugation left the autolytic enzymes in the supernatant.

Regardless of the method of isolation of autolytic enzymes used, the intent was to compare the autolytic activity present in cells grown in the presence of one of the fluorophenylalanines with that present in mid-log control cells or in stationary control cells. In order to allow direct comparison, the following precautions were taken: For any given experiment, cultures grown under the various conditions were all prepared at the same time, the same amount of cell wall (on a dry weight basis) was subjected to isolation for autolytic enzymes, and the assays were performed on the same day as the enzymes were prepared. Enzymes could be stored frozen at -20°C in Tris-HCl buffer, but lost activity very quickly after just three days, even when frozen.

Assay of the autolytic activity present in the isolated autolysin extracts was performed by following degradation of SDS-inactivated cell walls (by autolysin action) spectrophotometrically. The substrate cell walls (inactivated) were suspended in 2 ml TMB buffer, at pH 7.0, to an initial absorbance at 450 nm of 0.200-0.600 (Spectronic 100). 50 µl of isolated autolytic enzyme preparation was added to begin the assay. One unit of autolytic enzyme activity is defined as that amount which will give a decrease in absorbance at 450 nm of 0.001 per hour. Both this method and the one

described for the assay of native autolysins in isolated cell wall preparations are commonly employed for studying degradation of cell walls by autolytic enzymes.

The *in vitro* effect of p-FPA on isolated autolytic enzyme preparations was tested by addition of p-FPA to the substrate cell wall preparation prior to addition of the autolytic enzyme extract. p-FPA was added to the reaction mixture at a final concentration of 25 µg/ml. Otherwise, the assay was performed just as described previously.

Tests for susceptibility of SDS-inactivated cell walls to degradation by egg white lysozyme were performed as described above. Substrate walls were suspended in 10 mM Tris-HCl buffer, pH 7.0, and lysozyme was added to a final concentration of 40 µg/ml. Loss of absorbance at 450 nm was measured as before.

Characterization of Individual Autolytic Enzyme Activities

Thin-layer chromatography was used to detect the presence of N-acetylmuramyl-L-alanine amidase (amidase), the enzyme which hydrolyzes the peptide (amide) linkage between L-alanine and N-acetylmuramic acid within the peptide cross link groups of cell wall mucopeptide. As a result of amidase activity, free amino groups from alanine are liberated. These can be detected by dansylation; dansyl chloride forms bonds specifically with free amino groups. Dansylated amino acids can be separated and identified on

the basis of their migration, using thin-layer chromatography.

Cell wall preparations were allowed to autolyze not at all (control) or for seven hours (as described for assay of native autolysins, above). Exactly 100 µl of cell wall suspension, containing 0.5-1.5 mg of cell walls, was added to 100 µl of 0.4 M sodium carbonate plus 200 µl of dansyl chloride (5 mg/ml in acetone). This mixture was allowed to stand for one hour at 37°C. At this time, HCl was added to a final concentration of 6 N and the mixtures were hydrolyzed for 17 hours in-vacuo at 105°C. Following hydrolysis, the HCl was removed by evaporation over NaOH pellets, in-vacuo. The dansylated samples were dissolved in acetone and an amount equivalent to 75 µg of cell wall was chromatographed on heat activated (105°C for one hour) silica G thin-layer plates using a benzene: pyridine: acetic acid (80: 20: 2) solvent system. The chromatograms were normally run for 90 minutes. Dansylated compounds were detected using ultraviolet light; the derivatives could be seen as yellow fluorescing spots (Morse and Horecker, 1966). Dansylated alanine and other dansylated amino acids were run as standards.

Endo-β-N-acetylglucosaminidase (glycosidase) activity results in the release of a reducing group as the bond between N-acetylglucosamine and N-acetyl-muramic acid is hydrolyzed. For this reason, quantitation of the amount of reducing sugar released by glycosidase activity can be used

as a measure of that activity. The method of Ghuysen et al. (1966), used in this study, employs a reducing sugar assay for the detection of glycosidase activity during autoylsis of isolated cell wall preparations by native autolysins.

The method of Ghuysen et al. (1966) was followed without modification. N-Acetylglucosamine was used as the standard.

Analysis of Cell and Cell Wall Composition

Thin-layer chromatography was used to analyze the components present in Bacillus sp. #4 cell wall mucopeptide. For determination of amino acid components, 1-5 mg of SDS-inactivated cell walls were hydrolyzed in 6 N HCl at 105°C, in-vacuo, for 24 hours. When analyzing for amino sugar constituents, the cell walls were hydrolyzed for four hours in 4 N HCl, in-vacuo, at room temperature. Normally, hydrolysates equivalent to 175 µg of cell wall were applied to each cellulose thin-layer plate. The chromatograms were developed in the two-dimensional solvent system of Heathcote and Jones (1965). Standards were run in the same system; these consisted of 0.1 µMole each of muramic acid, D-glucosamine, meso-diaminopimelic acid (DAP), D, L-glutamic acid, L-lysine and D, L-alanine. Both amino acids and amino sugars were detected by spraying the chromatograms with a solution of 0.5% ninhydrin in acetone, followed by heating for five minutes at 105°C.

One poorly migrating compound detected while running cell wall hydrolysates in the Heathcote-Jones system was tentatively identified as DAP, by comparison with known standards. In order to confirm this, the solvent system of Rhuland et al. (1955) was employed. With this system, DAP could be distinguished from L-lysine and the D, D- and meso-DAP isomers could be distinguished from L, L-DAP on the basis of their migration on the paper chromatograms.

The method of Lowry et al. (1951) was used to assay protein content of the isolated autolytic enzyme preparations extracted from cells or cell walls. Bovine serum albumin (BSA) was used as the protein standard.

For assay of the total amount of protein present in cell walls, a modified version of the Lowry method was employed (Hanson and Phillips, 1981). The modification, which entails heating of the protein sample in 1 N NaOH for 10 minutes at 90°C, is necessary to ensure that any protein which is tightly bound to the cell wall will not escape detection. The procedure had to be further modified, in that the cell wall samples were kept on ice up to the time of addition of 1 N NaOH (to avoid autolysis) and the samples had to be centrifuged at 4000 xg for 10 minutes during the 30 minute stand time of the Lowry protocol in order to remove residual wall material which would otherwise interfere with the assay. Again, the protein standard used was BSA.

Fractionation of radiolabelled cells was performed

according to the method of Park and Hancock (1960). Cells were cultured in the usual manner, except that uniformly labelled ^{14}C-L-aspartic acid was added to the cultures (prior to inoculation) to obtain a specific activity of 1.19 μCi/mMole (2.5 μCi total radioactivity in 100 ml of medium). The various fractions of the cells obtained by the Park-Hancock procedure were assayed for radioactivity (1 ml samples) in Aquasol scintillation cocktail, using a Beckman LS-7500 liquid scintillation counter.

The thickness of cell walls was determined by transmission electron microscopy. The cells were grown as described previously. Washed cells were fixed with 1% osmium tetroxide in cacodylate buffer, pH 7.2, for 30 minutes. The samples were then post-fixed with 2% glutaraldehyde in cacodylate buffer, pH 7.2, for one hour. Following an ethanol dehydration series, the cells were embedded in Polybed 812. The cells were sectioned with a glass knife on a Sorvall Poter-Blum ultramicrotome and electron micrographs taken on an RCA EMU-3T transmission electron microscope. Cross and longitudinal sections of cells were obtained randomly as a result of the chance orientation of the cells in the embedding material during sectioning.

Lipoteichoic acids (LTA) were extracted from Bacillus sp. #4 and the LTA quantitated by the method of Kessler and Shockman (1979). Mid-log control cells, stationary control cells, and cells grown in the presence of p-FPA were

cultured in 25 ml of aspartic acid-glucose defined medium which contained uniformly labelled ^{14}C-glycerol, with a specific activity of 9.11 µCi/µMole (total radioactivity in 25 ml of medium was 10 µCi). The labelled glycerol was added to the medium prior to inoculation of the cultures. At the time of harvest of the cultures, absorbance of the cultures at 540 nm was measured and the dry weight of cells in the cultures determined from dry weight standard curves. These values were used later when analyzing the data of this experiment; the amounts of LTA present in the various cultures were compared on the basis of cpm incorporated per µg cell dry weight.

Total radioactivity in the cultures was determined by the method of Kessler and Shockman (1979). Exactly 0.5 ml culture samples were mixed with 4.5 ml of 10% trichloroacetic acid (TCA), on ice, and allowed to stand on ice for one hour. The TCA precipitated material was retained by filtering the sample through Whatman GF/C glass fiber filters which had been pre-washed twice with 1 ml of water. The filters were then washed six times with 1 ml of cold 10% TCA and, next, washed twice with 1 ml of 100% ethanol. Radioactivity trapped in the filters was assayed by liquid scintillation in Aquasol scintillation cocktail, using a Beckman LS-7500 liquid scintillation counter.

For quantitation of the amounts of both intracellular and extracellular LTA, the cells were separated from the culture medium by centrifugation at 10,500 xg for 10

minutes. The culture medium contained the extracellular LTA and was dialyzed against distilled water (4 1 of water, changed three times; using cellulose dialysis tubing, 3787-D-10, A. H. Thomas Co.) for 24 hours. The dialyzate was then concentrated by lyophilization. The cell pellet (from above) was resuspended in 2 ml of water (1-4 mg cell dry weight per ml). To this was added 2 ml of 90% aqueous phenol (w/v). The intracellular LTA was extracted from the cells by vigorous stirring of this mixture for 30 minutes at 68°C. The mixture was then chilled to 4°C and the phases separated by centrifugation at 4000 xg for 10 minutes. The upper aqueous phase was removed and the phenol phase re-extracted, as before, by adding 2 ml of water and repeating the vigorous stirring at 68°C for 30 minutes. The phases were again separated by centrifugation, after chilling, and the upper aqueous phase removed and pooled with the first aqueous extract. The pooled extracts were dialyzed against water, as described above, and lyophilized. Radioactivity present in both LTA extracts was assayed by resuspending the concentrated extracts in 1 ml of water and counting 0.5 ml of this in Aquasol scintillation cocktail, using a Beckman LS-7500 liquid scintillation counter.

Assay of Aspartase Activity

Aspartase catalyzes the deamination of aspartic acid, producing fumarate and releasing free ammonia. The reaction can be assayed by quantitative detection of the freed

ammonia, using Nessler's reagent (Hanson and Phillips, 1981). Cultures were grown in the usual manner, washed twice with cold physiological saline, and resuspended in cold 0.1 M potassium phosphate buffer, pH 6.8, such that a 1: 50 dilution of this suspension gave an absorbance at 540 nm of ca. 0.45 (Spectronic 20). Cells were then freeze-thawed three times to allow breakage of cells and the consequent access of aspartase to the substrate. 1 ml of freeze-thawed cell suspension was incubated for 30 minutes, at 25°C, in the presence of 0.56 millimoles of aspartic acid (pH 6.8) and 33 mM potassium phosphate buffer, pH 6.8. Total reaction volume was 3 ml. Following incubation, the cells were removed by centrifugation at 4000 xg for 10 minutes. Exactly 1 ml of supernatant from the reaction mixture was added to 1 ml of Nessler's reagent plus 8 ml of water. This mixture was allowed to stand for 20 minutes at 25°C, at which time absorbance was read at 450 nm on a Spectronic 100, using 1 cm pathlength. Ammonium chloride was used as the standard. A control, used to subtract intracellular ammonia from that released by aspartase activity, consisted of the same assay, using freeze-thawed cells (prepared in the same manner as above) which had been boiled for 10 minutes prior to the assay. Results are reported in terms of nanomoles of ammonia released per µg cell dry weight.

Rates of Synthesis of Macromolecules

Rates of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis were measured in the following manner: DNA synthesis was measured by incorporation of ^{3}H-thymidine in the presence of 2.0 mg/ml of 2-deoxyadenosine (Rana and Halvorson, 1972). The 2-deoxyadenosine was added to prevent incorporation of ^{3}H-thymidine into RNA, since 2-deoxyadenosine inhibits the conversion of thymidine to precursors of RNA synthesis. This method has been employed to successfully investigate DNA replication in Bacillus subtilis (Sandler and Keynan, 1981). RNA synthesis was measured by incorporation of ^{3}H-uracil into RNA (Steinberg and Halvorson, 1968b). Protein synthesis was measured by incorporation of ^{14}C-aspartic acid into protein (Steinberg and Halvorson, 1968a).

^{3}H-thymidine stock solution, containing 2-deoxyadenosine, was added to 35 ml cultures (protocol described below) to reach a specific activity of 121.10 mCi/mMole of ^{3}H-thymidine (2.5 µCi total radioactivity per ml; 5.0 µg total thymidine per ml, 20.6 µM) and 2.0 mg/ml 2-deoxyadenosine (7.4 mM) in the growth medium. ^{3}H-uracil was added to a specific activity of 33.60 mCi/mMole (1.5 µCi total radioactivity per ml; 5.0 µg total uracil per ml, 44.6 µM) in the growth medium. ^{14}C-aspartic acid was added to a specific activity of 0.109 mCi/mMole (2.5 µCi total radioactivity per ml; 3.042 mg total aspartic acid per ml, 22.8 mM) in the growth medium.

Control and p-FPA containing cultures were grown as usual, except that the volumes were 35 ml each. The labelling experiment was started when the control culture reached early-log phase (absorbance at 540 nm of 0.107) and when the p-FPA culture had been grown for 11.25 hours in the presence of 10 µg/ml p-FPA (absorbance at 540 nm of 0.282). At this time the cultures were split into four portions: 10 ml for determination of the rate of DNA synthesis, 10 ml for RNA synthesis and 5 ml for protein synthesis. To each flask was added the levels of labelling compound as described previously. The remaining 10 ml of each culture was saved to follow growth rates of the cultures. The cultures were incubated, from this point forward, in separate 125 ml Erlenmeyer flasks on a Burrell wrist-action shaker, setting 2.3, at room temperature (24° C).

Samples (1 ml) were removed at 30 minute intervals for the next 2.5 hours (samples removed at t=0.5 hour, t=1 hour, t=1.5 hours, t=2 hours, and t=2.5 hours). The 1 ml samples were added to 1 ml of 20% cold TCA, on ice, which contained 200 µg/ml of unlabelled compound (thymidine, uracil, or aspartic acid), referred to as "cold carrier". All glassware had been silanized to prevent any precipitates from adhering to the glass. The TCA containing samples for DNA and RNA synthesis were kept on ice for one hour and the protein samples were heated for 30 minutes at 93° C, followed by cooling to room temperature.

Before filtering the samples on Whatman GF/C glass

fiber filters, the flters were pre-washed once with 2 ml water, then with 2 ml 10% TCA which contained 1 mg/ml of cold carrier (again, thymidine, uracil, or aspartic acid). Next, 1 ml of each of the TCA containing samples was filtered, using vacuum suction; separate positions on the "uptake train" were used for each of the six situations examined. The filters were then washed twice with 3 ml of 10% TCA, containing 50 µg/ml of cold carrier, followed by two washes with 3 ml of 95% ethanol. The filters were dried on plastic Petri dishes using a heat lamp. Radioactivity on the filters was assayed by counting the filters in Aquasol scintillation cocktail, after standing overnight to dissolve the filters, using a Beckman LS-7500 liquid scintillation counter.

Growth of the cultures was followed spectrophotometrically, at 540 nm, using a Spectronic 20. The Absorbances were converted to µg cell dry weight for calculation of the rates of macromolecular synthesis. Since the two cultures grew at different rates (p-FPA culture in its second log phase grows at a rate which is 20% that of the control culture; see chapter III, Figure 2), the rates of incorporation of the labels had to be normalized with respect to the difference in growth rate. Therefore, a first derivative plot was made: The logarithm of cpm per µg cell dry weight per ml was plotted vs. time (in hours that the culture had spent in the presence of label).

CHAPTER III

RESULTS

Studies on Filamentation

Effect of p-FPA on Cell Division and Growth

A number of agents have been shown to inhibit cell division in Gram-negative bacteria (Grula and Grula, 1962a, 1962b, 1964; Inouye and Pardee, 1970; Shapiro et al., 1970). Addition of these agents to growing cultures of bacteria results in the formation of filaments: The cells continue to grow in length, but do not divide. In most cases, the rate of growth is lowered in the presence of the division inhibiting agents, but some growth does continue; this is an obvious requirement for filamentation.

para-Fluorophenylalanine is able to induce filament formation in Escherichia coli, as well as slow its rate of growth (Previc and Binkley, 1964). Experiments in this laboratory, conducted by Dr. Mary Grula, have shown that p-FPA is also able to inhibit cell division of Erwinia carotovora, as well as slow its rate of growth (data not published).

During investigation of the properties of p-FPA, it was

found that if Bacillus sp. #4 was inoculated into a culture which contained as little as 0.5 µg/ml of p-FPA in aspartic acid-glucose defined medium, the result was formation of filamentous bacteria. Growth of this organism under these conditions was severely limited, in that after 36 hours of growth the absorbance at 540 nm of the culture was less than 0.10 (Figure 1). Data in Table I show that after nearly 14 hours growth in the presence of p-FPA, the cells averaged 10 µm in length, as compared with 4 µm cell length of untreated cells and that after 19 hours of growth in the presence of p-FPA, the filaments, now averaging better than 15 µm in length, began to lyse; this probably is the reason for the low absorbance of the culture.

Addition of p-FPA to cultures which were entering logarithmic growth phase (p-FPA added in early-log) still resulted in inhibition of cell division, but the effects on growth were not nearly so great as addition of pFPA at the time of inoculation. Data in Figure 1 show the effects of increasing concentrations of p-FPA on growth rate when added to the cultures while in early-log phase. Table I shows the effects on filament length of the differing concentrations of p-FPA during the same experiment. It is apparent that increasing concentrations of p-FPA resulted in decreased growth and that above a level of 10 µg/ml, filament formation induced by p-FPA decreased slightly. For this reason, and because growth in the presence of 10 µg/ml p-FPA allowed sufficient growth to harvest the filaments for

Figure 1. Growth of Bacillus sp. #4 in the Presence of Various Concentrations of p-FPA. Control culture, no additions (●); 0.5 µg/ml p-FPA added at time of inoculation, t=0 hours (×); 0.5 µg/ml p-FPA added in early-log, t=13.7 hours (o); 10 µg/ml p-FPA added at t=13.7 hours (□); 25 µg/ml p-FPA added at t=13.7 hours (o); 50 µg/ml p-FPA added at t=13.7 hours (△). Absorbance at 540 nm is plotted on a linear scale in order to fit all curves in one figure. Standard deviations range from 0.003 to 0.032 absorbance units (at 540 nm).

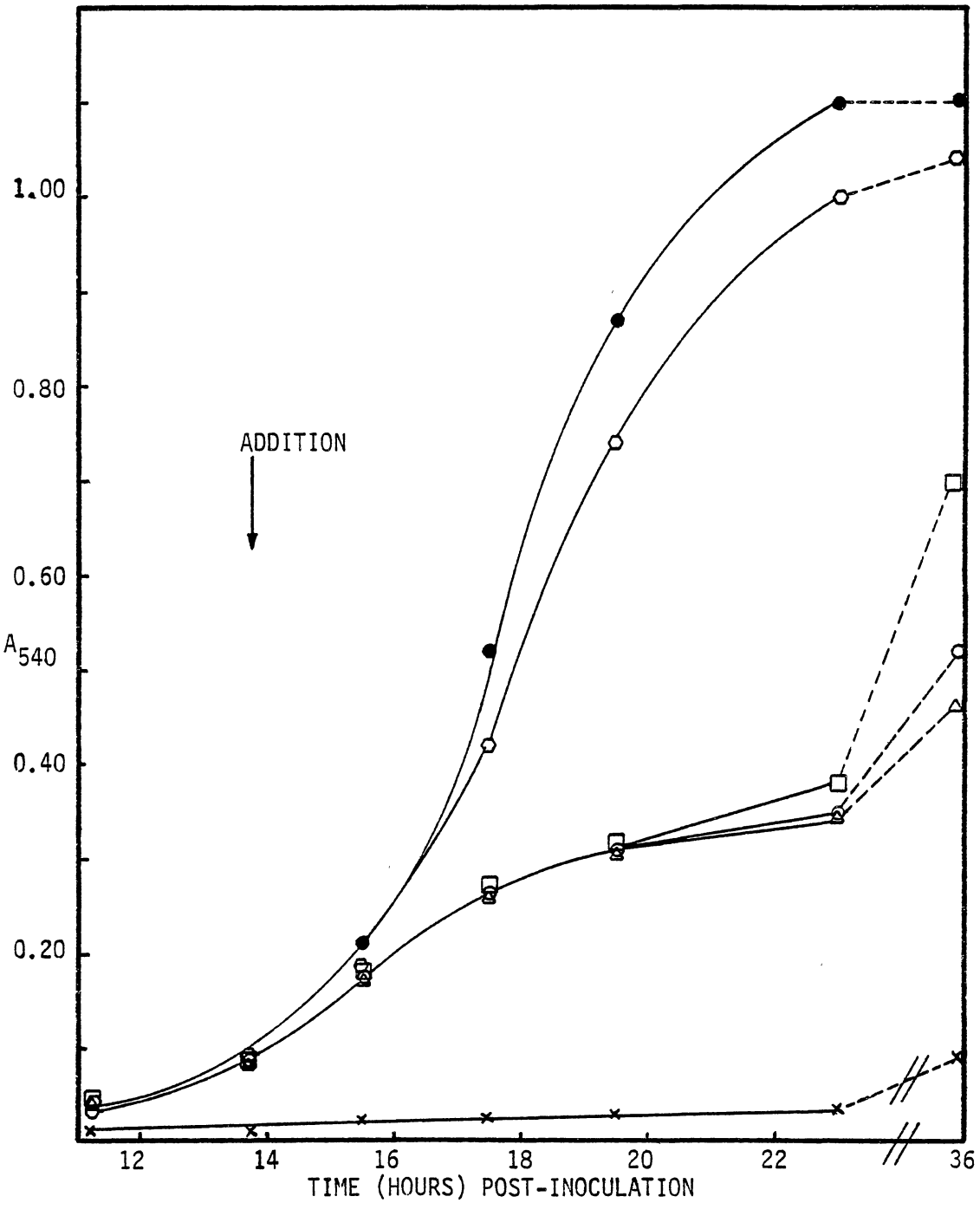

1.00
0.80
0.60
A_{540}
0.40
0.20
ADDITION
12
14
16
18
20
22
36
TIME (HOURS) POST-INOCULATION

TABLE I

LENGTH OF CELLS GROWN IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF p-FPA

Concentration of p-FPA (μg/ml)	Time of Addition[a]	Cell Lengths (μm) at Various Times Post-Inoculation		
		13.7 Hours	19.5 Hours	36.0 Hours
——	——	4±0.5*	4±0.5	3±0.5
0.5	t=0	10±1.1 (15)	15±1.7 lysis	5±0.6 ext. lysis[b]
0.5	early-log	4±0.5	5±0.6 (15)	7±0.8 (18)
10.0	early-log	4±0.5	7±0.8	20±2.3 (50)
25.0	early-log	4±0.5	7±0.8	10±1.1 (25)
50.0	early-log	4±0.5	6±0.6	6±0.6 (15)

[a] p-FPA added at time of inoculation, t=0 hours; or p-FPA added at early-log phase, t=13.7 hours after inoculation.

[b] Extensive lysis.

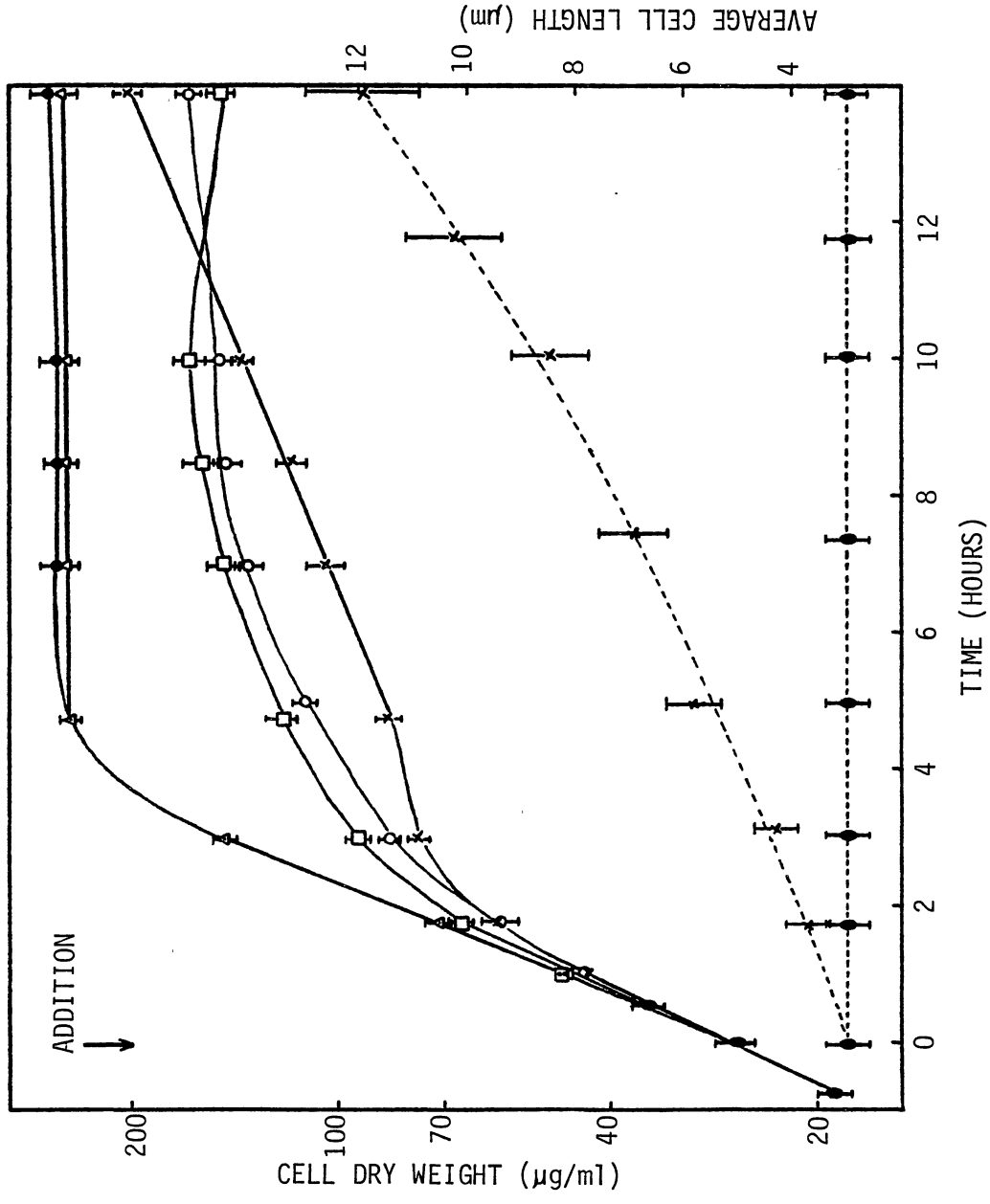
* Average cell length (μm), plus and minus standard deviation. (Maximum filament length)

further investigation, a level of 10 µg/ml p-FPA, added as the culture entered log phase, was chosen for all subsequent studies.

It should be mentioned here that addition of sodium fluoride (NaF) to cultures of Bacillus sp. #4, under no condition resulted in formation of filaments (data not shown), although this compound does induce filamentation in E. carotovora (Grula and Grula, 1976). Therefore, it is unlikely that loss of the fluoride moiety from p-FPA, within the cells or in the medium, is the reason for the effects of p-FPA. A check for purity of the p-FPA used in these experiments, performed by thin-layer chromatography in the Heathcote-Jones system (1965), showed the compound to be chromatographically pure. It was also found that penicillin G was unable to induce filament formation in Bacillus sp. #4 (data not shown).

Data in Figure 2 provide a more complete characterization of the effects of addition of 10 µg/ml p-FPA to an early-log culture of Bacillus sp. #4. Growth of the organism began to slow down shortly after the addition of p-FPA. By five hours after addition of p-FPA, the culture had resumed log growth, but at a rate which was only 20% that of the control culture (generation time of 6 hours, 20 minutes as opposed to 1 hour, 10 minutes for the untreated control culture). An increase in cell length was first observed at approximately 90 minutes after addition of p-FPA. Thereafter, cell length continued to increase to an average

Figure 2. Growth and Cell Length of Bacillus sp. #4 as Influenced by the Addition of Various Phenylalanine Analogs. Cell length determinations (dashed lines): p-FPA culture (×); other cultures (●). Growth determinations (solid lines): p-FPA culture (×); m-FPA (□); o-FPA (○); p-ClPA (△); control culture (●). Phenylalanine analogs added in early-log phase, t=0 hours; final concentrations of each analog, 54.6 μM. Lines and bars at each point indicate standard deviation.


AVERAGE CELL LENGTH (μm)
12
10
8
6
4
ADDITION
200
100
70
40
20
CELL DRY WEIGHT (μg/ml)
0
2
4
6
8
10
12
TIME (HOURS)

length of ca. 12 μm at 14 hours after addition of p-FPA, with some cells as long as 40-50 μm in length.

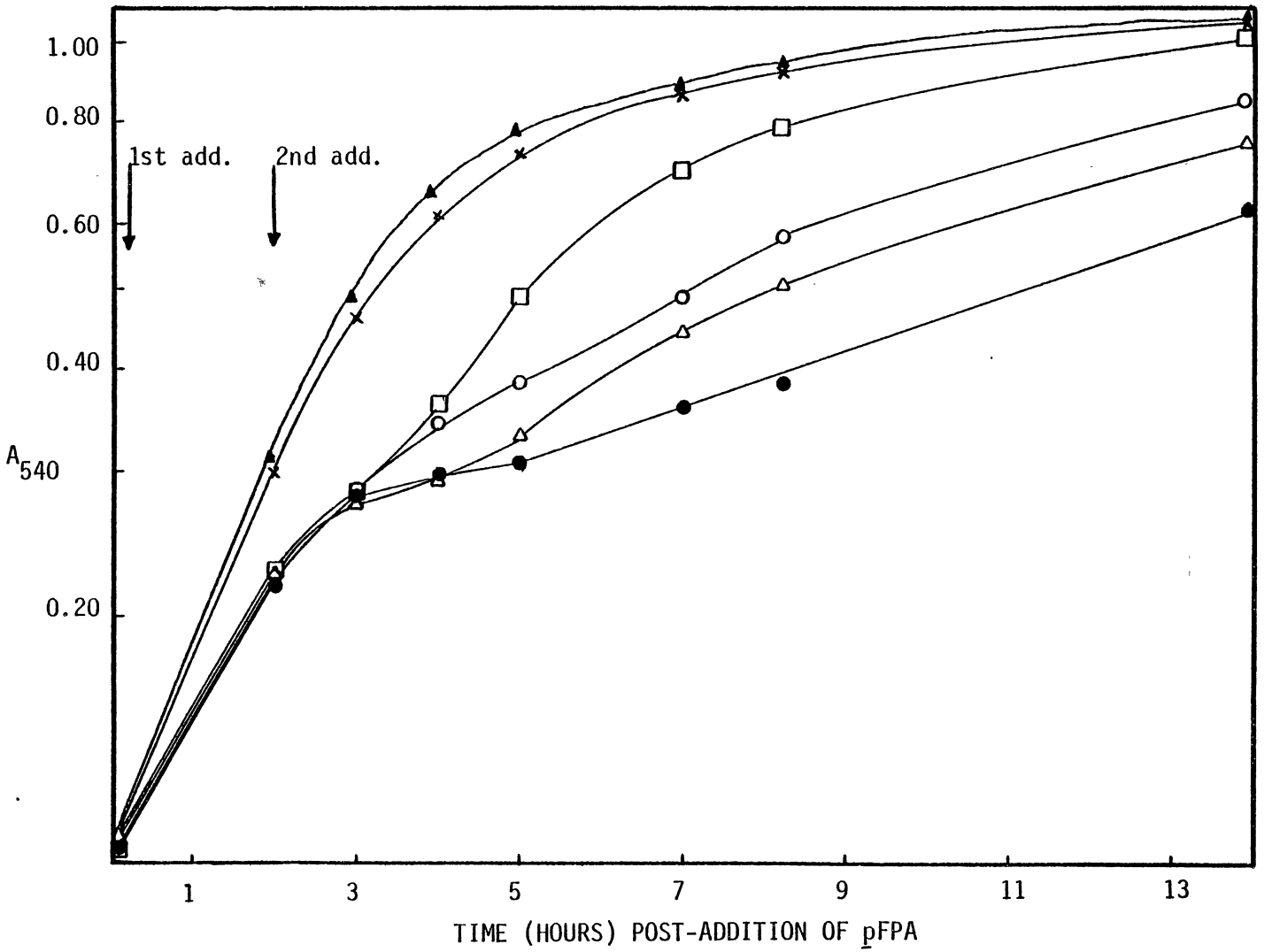
Effects of Other Phenylalanine Analogs

As the effectiveness of other phenylalanine analogs as inhibitors of cell division was tested, it became obvious that inhibition of cell division was a rather specific effect of p-FPA. As an example, the fluoride moiety of p-FPA could not be replaced with chloride, as in the compound p-chlorophenylalanine (Figure 2). p-Chlorophenylalanine (p-ClPA) did not inhibit cell division or growth. It was also found that the fluoride moiety of fluorophenylalanine must be in the para position to inhibit cell division; ortho- and meta-fluorophenylalanie (o-FPA and m-FPA, respectively) did not inhibit cell division. However, o-FPA and m-FPA did inhibit growth, although they did so without inducing the second log phase of growth which is characteristic of p-FPA inhibition.

Influence of Phenylalanine and Tyrosine on Effects of p-FPA

When phenylalanine was added to an early-log culture at the same time as addition of p-FPA (molar concentration of phenylalanine one-half that of p-FPA), growth of the culture continued at a rate similar to that of a control culture (Figure 3). Phenylalanine added at the same time as p-FPA was able to completely prevent growth inhibition caused by

Figure 3. Growth of *Bacillus sp.* #4 as Influenced by Addition of Phenylalanine or Tyrosine to Cultures Growing in the Presence of p-FPA. Control culture, no additions (▲). Additions made at t=0 hours (1st addition), or at t=2 hours (2nd addition). p-FPA added to all but control culture at t=0 hours (1st addition) at a concentration of 54.6 µM. p-FPA added only (●); plus phenylalanine, added at t=0 hours (×); plus phenylalanine, added at t=2 hours (□); plus tyrosine, added at t=0 hours (○); plus tyrosine, added at t=2 hours (△). Standard deviations range from 0.002 to 0.031 absorbance units (at 540 nm).


1.00
0.80
0.60
0.40
0.20
A_{540}
1st add.
2nd add.
1
3
5
7
9
11
13
TIME (HOURS) POST-ADDITION OF pFPA

p-FPA, but, if the phenylalanine was added two hours after addition of p-FPA, growth inhibition by p-FPA was not prevented. Growth was, however, increased somewhat over that of a culture containing p-FPA only, by the presence of phenylalanine. Addition of tyrosine (molar concentration one-half that of p-FPA) either at the same time as addition of p-FPA, or two hours after, did not prevent growth inhibition caused by p-FPA. However, addition of tyrosine at either time did stimulate growth somewhat over that of a culture which contained p-FPA only, as did phenylalanine when added two hours after addition of p-FPA.

The effects of phenylalanine when added at the same time as p-FPA were even more pronounced when the cultures were examined for filamentation. The data of Table II indicate that addition of phenylalanine at the same time as addition of p-FPA completely prevented the filamentation which normally occurred in the presence of p-FPA. Moreover, addition of phenylalanine two hours after addition of p-FPA did not prevent filamentation caused by p-FPA, nor did additions of tyrosine prevent filamentation when added at either time. In fact, the filaments formed in the three latter situations were longer than filaments normally formed in the presence of p-FPA, probably as a result of the stimulation of growth caused by addition of the amino acids, which has already been discussed.

The data presented here, concerning the addition of phenylalanine, suggest that the commitment to filamentation

TABLE II

CELL LENGTH OF BACILLUS SP. #4 AS INFLUENCED BY ADDITION OF PHENYLALANINE OR TYROSINE TO CULTURES GROWING IN THE PRESENCE OF p-FPA

Additions[a]	Time of Addition[b] of Amino Acid (Hours)	Average Cell Length at Various Times[c] Post-Addition of p-FPA (μm)		
		t=5 Hours	t=8 Hours	t=14 Hours
None	——	4±0.5*	4±0.5	4±0.5
p-FPA only	——	6±0.6	8±0.9 (15)	15±1.7 (50)
p-FPA + Phe	t=0	2±0.5	3±0.5	4±0.5
p-FPA + Tyr	t=0	7±0.8	10±1.1 (20)	20±2.3 (50)
p-FPA + Phe	t=2	8±0.9	15±1.7 lysis	15±1.7 ext. lysis
p-FPA + Tyr	t=2	6±0.6	10±1.1 (20)	20±2.3 (50)

[a] Phe = Phenylalanine; Tyr = Tyrosine. Concentration of p-FPA = 54.6 μM and amino acids = 27.3 μM. *Plus and minus standard deviation provided.

[b] p-FPA added at t=0 hours, cultures in early-log. Amino acids added at same time as p-FPA (t=0) or two hours after addition of p-FPA (t=2 hours).

[c] ext. lysis = extensive lysis. Maximum filament length provided in parentheses.

TABLE III

ABILITY OF VARIOUS CONCENTRATIONS OF PHENYLALANINE TO PREVENT EFFECTS OF GROWTH IN THE PRESENCE OF p-FPA

Additions[a]	Concentration of[b] Phenylalanine (μM)	Cell Length (μm) 14 Hours Post-Additions	A_{540} 14 Hours Post-Addition
None	—	4±0.5*	1.05±0.030
p-FPA only	—	12±1.3 (40)	0.57±0.021
p-FPA + Phe	54.60 (100%)	4±0.5	1.00±0.026
p-FPA + Phe	27.30 (50%)	4±0.5	1.02±0.028
p-FPA + Phe	5.46 (10%)	15±1.7 (50)	0.85±0.023
p-FPA + Phe	0.55 (1.0%)	18±1.9 (50)	0.65±0.023

[a] Phe = Phenylalanine; p-FPA and phe both added to early-log cultures; concentration of p-FPA = 54.6 μM.

[b] Ratio of phenylalanine to p-FPA, on a molar basis, is provided in parentheses.

* Plus and minus standard deviation provided.

caused by the presence of p-FPA was made early, since addition of phenylalanine just two hours after addition of p-FPA did not prevent filamentation. This was to be expected, however, since an increase in cell length was observed just 90 minutes after addition of p-FPA. These data also suggest that phenylalanine, at least when added at the same time as addition of p-FPA, competed with the p-FPA for incorporation into protein. Tyrosine did not appear to give this same type of competition, as it did not prevent filamentation caused by p-FPA.

In order to determine the ratio of phenylalanine to p-FPA required to prevent the effects of p-FPA from occurring, an experiment similar to that discussed previously was run. In this experiment, various concentrations of phenylalanine (expressed as the percentage of molarity of the concentration of p-FPA) were added, along with p-FPA, to early-log cultures (Table III). It was found that concentrations of phenylalanine equimolar to or 50% equimolar to the concentration of p-FPA were able to inhibit the formation of filaments. Phenylalanine, when added at concentrations which were 10% equimolar to the concentration of p-FPA, or less, were unable to prevent filamentation induced by growth in the presence of p-FPA.

Influence of Pantoyl Lactone on Effects of p-FPA

Addition of pantoyl lactone (PL) to filaments of E.

TABLE IV

EFFECT OF PANTOYL LACTONE ON FILAMENTATION INDUCED BY p-FPA

Additions[a]	Time of Addition[b] of PL (Hours)	A_{540} at[c] t=24	Cell Length at Various Times Post-Addition of p-FPA (μm)			
			t=5 Hours	t=9 Hours	t=14 Hours	t=24 Hours
——	——	1.05±0.030	3±0.5	3±0.5	3±0.5	3±0.5
p-FPA only	——	0.69±0.023	6±0.6	9±0.8 (20)	12±1.3 (40)	20±2.3 (60)
p-FPA + 0.20 M PL	t=0	0.27±0.011	3±0.5	4±0.5	4±0.5	5±0.6
p-FPA + 0.05 M PL	t=0	0.41±0.020	3±0.5	5±0.6	6±0.6	7±0.8 (15)
p-FPA + 0.20 M PL	t=9	0.54±0.021	——	9±1.0 (20)	12±1.3 (30)	13±1.5 (30)
p-FPA + 0.05 M PL	t=9	0.60±0.023	——	9±1.0 (20)	12±1.3 (30)	18±1.9 (40)

[a] p-FPA added as cells entered log phase (p-FPA = 54.6 μM). Pantoyl lactone (PL) added to final concentration indicated.

[b] PL added at time of addition of p-FPA (t=0) or 9 hours post-addition of p-FPA (t=9).

[c] A_{540} measurements made 24 hours post-addition of p-FPA. Plus and minus standard deviation provided. Maximum filament length provided in parentheses.

carotovora results in reversion of the filaments (Grula and Grula, 1964). PL is able to prevent inhibition of cell division in Micrococcus lysodeikticus, when added at the same time as the division inhibitor, but is unable to reverse division inhibition, when added to non-dividing M. lysodeikticus (Grula and King, 1970).

The data of Table IV indicate that PL was able to prevent filamentation of Bacillus sp. #4 induced by growth in the presence of p-FPA, but was unable to reverse the inhibition of cell division. When PL, at a concentration of 0.2 M, was added at the same time as p-FPA, the cells did not filament, although growth of the culture was significantly lowered. Addition of 0.05 M PL, in a similar manner, was not quite as effective as the higher concentration in preventing filamentation. Addition of PL to filaments of Bacillus sp. #4 (grown for nine hours in the presence of p-FPA) did not result in reversal of filamentation at any concentration of PL tested. These data, and those from the experiment in which phenylalanine was unable to prevent filamentation when added after just two hours growth in the presence of p-FPA (Figure 3 and Table II), indicate that once the commitment to filamentation is made, the effect of growth in the presence of p-FPA on cell division is irreversible.

Studies on the Autolytic System

The Effects of Growth in the Presence of p-FPA on Cell Lysis Induced by Antibiotics

Polymyxin B sulfate is a membrane-directed antibiotic which, upon addition to a suspension of bacterial cells (growing or non-growing culture), results in a rapid loss of phase density (as observed using phase contrast microscopy) of the culture, the consequence of the loss of membrane integrity (Newton, 1956). Addition of this antibiotic to an autolytically competent control culture (culture capable of undergoing autolysis -- not inhibited) of Bacillus sp. #4 resulted in a rapid loss of phase density, followed by degradation of the cell wall. Degradation of the cell walls of these bacteria resulted in a loss of turbidity of the culture, as shown in Figure 4. Data in Figure 4 also indicate that growth in the presence of p-FPA rendered the cells resistant to lysis induced by polymyxin B, although some small amount of lysis did occur. Since cell wall degradation is a result of autolytic activity, resistance to polymyxin B-induced lysis, by cells grown in the presence of p-FPA, would serve as an indicator of inhibited autolytic activity. This result suggests that cells grown in the presence of p-FPA are not autolytically competent.

Penicillin is a cell wall-directed antibiotic which causes lysis of growing cells. Tomasz (1979) has shown that cells must have an active autolytic system in order to be

Figure 4. Response of Cells Grown in the Presence of p-FPA to Polymyxin B Treatment. Control culture (circles, solid lines); culture grown in p-FPA (triangles, dashed lines). Polymyxin B treated cultures (open circles, open triangles); growth control cultures (closed circles, closed triangles). Polymyxin B sulfate, where added, was at a final concentration of 20 µg/ml. Lines and bars at each point indicate standard deviation.

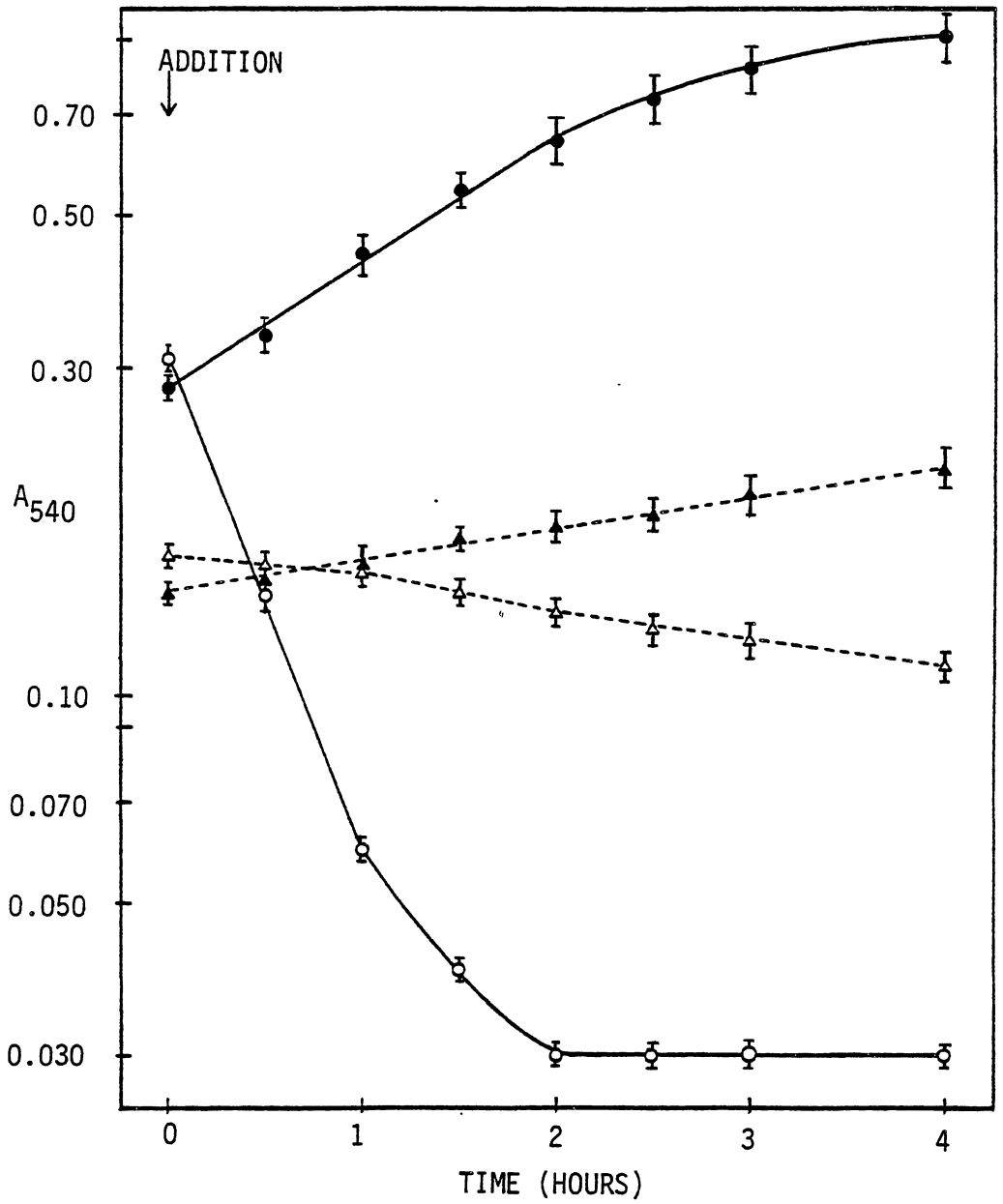
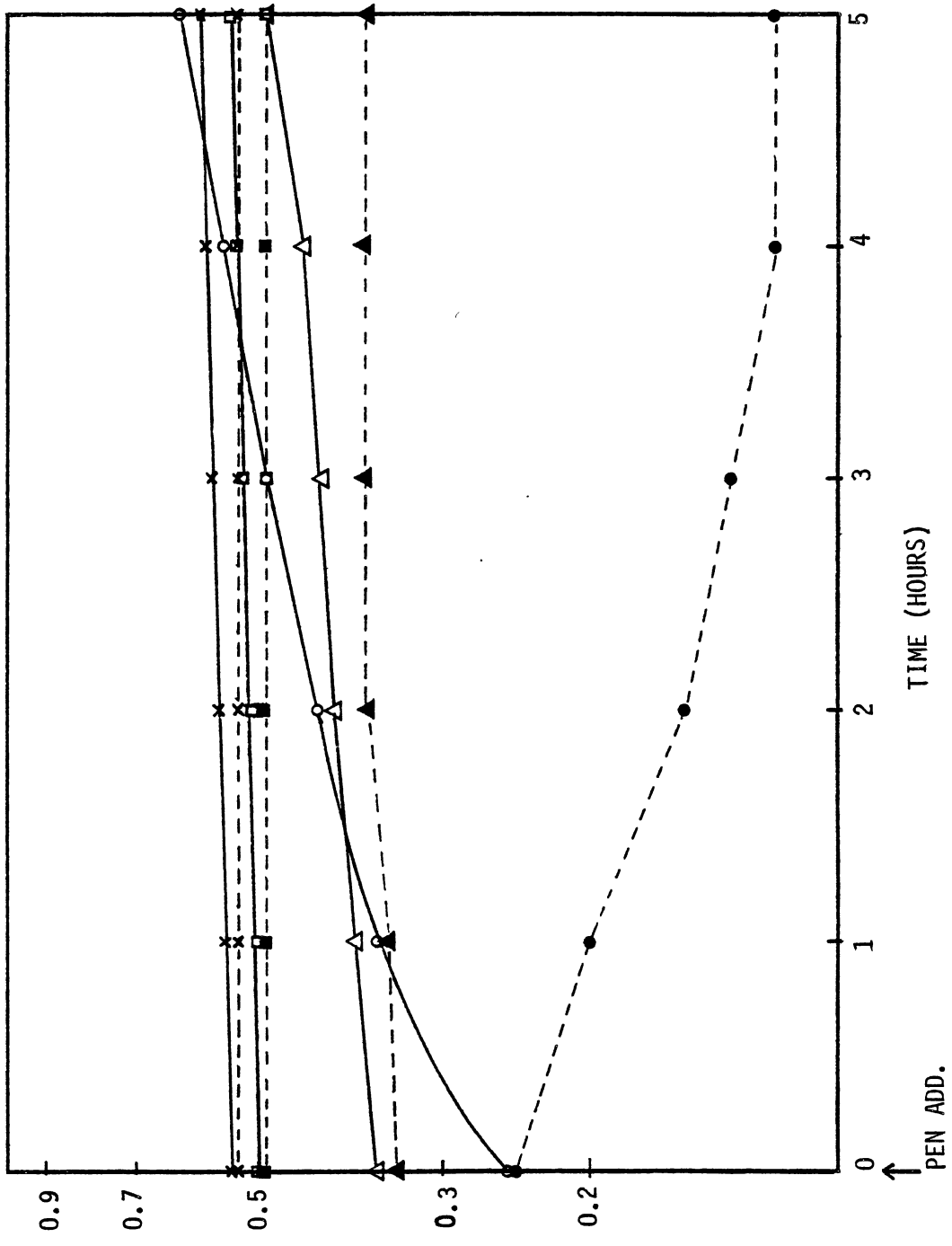

ADDITION
0.70
0.50
0.30
A_{540}
0.10
0.070
0.050
0.030
0
1
2
3
4
TIME (HOURS)

Figure 5. Response of Cells Grown in the Presence of p-FPA, o-FPA, or m-FPA to Treatment with Penicillin. Control culture (o); p-FPA (Δ); o-FPA (□); m-FPA (×). Penicillin treated cultures (closed figures, dashed lines); growth control cultures (open figures, solid lines). Penicillin G, where added was at a final concentration of 0.64 mg/ml (1050 units/ml). Standard deviations range from 0.002 to 0.024 absorbance units (at 540 nm) for experimental curves.


0.9
0.7
0.5
0.3
0.2
0
1
2
3
4
5
TIME (HOURS)
PEN ADD.

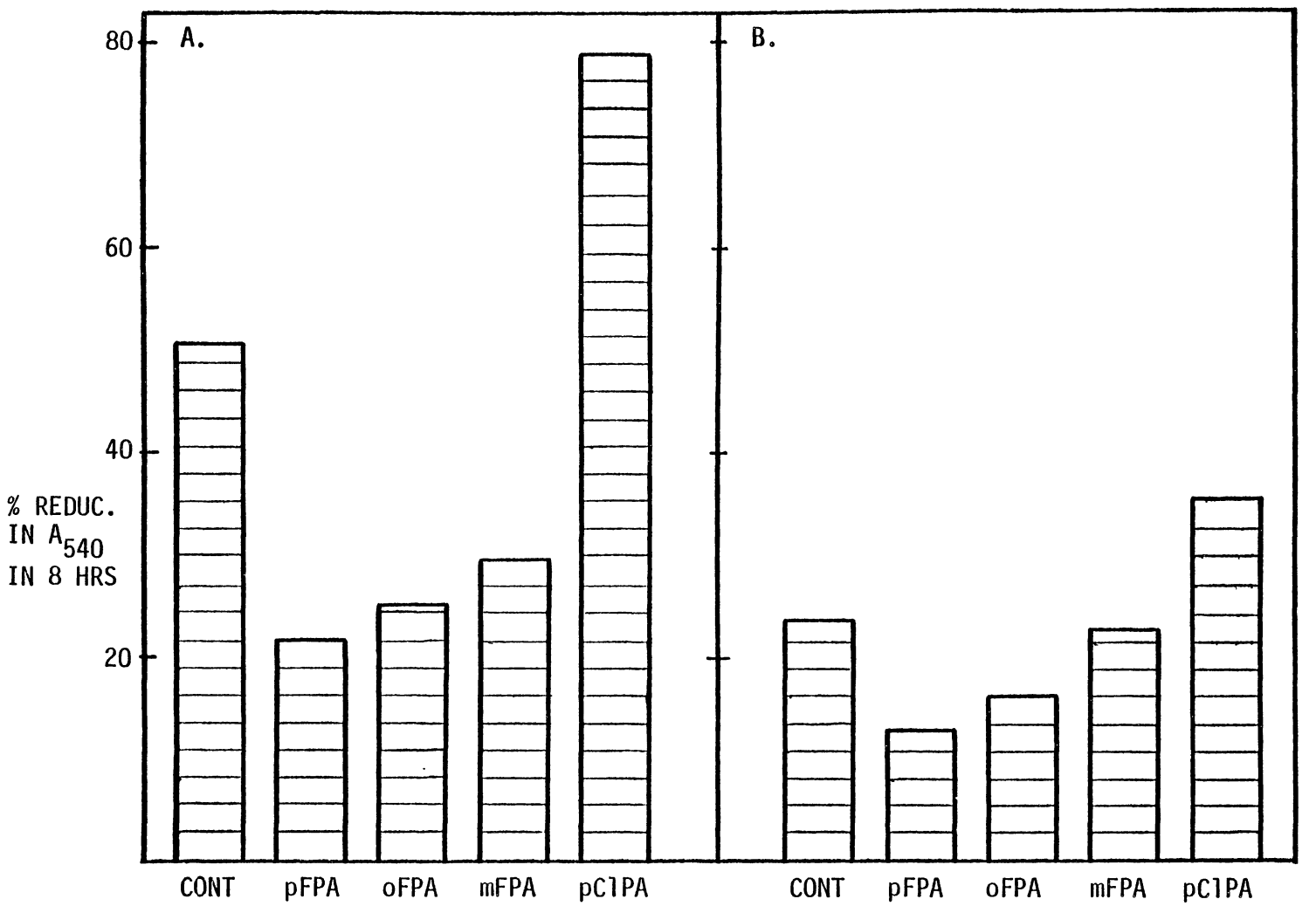
lysed by penicillin. Therefore, resistance to lysis by penicillin would serve as an indicator of a non-functional autolytic system. Data in Figure 5 show that a growing control culture was lysed by addition of penicillin. Figure 5 also presents data indicating that growth in the presence of p-FPA caused a resistance to lysis induced by penicillin. This observation again suggests that cells grown in the presence of p-FPA do not have a functional autolytic system. Other data in Figure 5 show that growth in the presence of o-FPA or m-FPA resulted in a similar resistance to lysis induced by penicillin.

Assay of Autolysis, Using Whole Cells

A more direct measurement of in vivo autolytic activity could be performed by monitoring autolysis of washed whole cells which have been suspended in potassium phosphate buffer, pH 6.8. Figure 6 shows the effects of growth in the presence of various phenylalanine analogs on in vivo autolytic activity. Just 1.75 hours after addition of p-FPA, o-FPA, or m-FPA to early-log cultures, autolysis of the cells was significantly inhibited when compared with autolysis of cells from a control culture which was in mid-log phase (Figure 6-A). Growth in the presence of p-ClPA, which did not inhibit cell division or growth, caused a stimulation of autolytic activity; the significance of this stimulation is not understood (Figure 6-A).

After growth in the presence of fluorophenylalanine for

Figure 6. Autolysis of Whole Cells as Influenced by Growth in the Presence of Various Phenylalanine Analogs. A. Autolysis of cells grown in the presence of the three isomers of fluorophenylalanine, labelled below their respective bar (1.75 hours after addition of the FPA); control culture in mid-log phase. B. Autolysis of cells grown in the presence of the various phenylalanine analogs for seven hours; control culture in stationary phase. Standard deviations, for % reduction in absorbance at 540 nm in 8 hours, range from 0.71 to 6.31 percent.

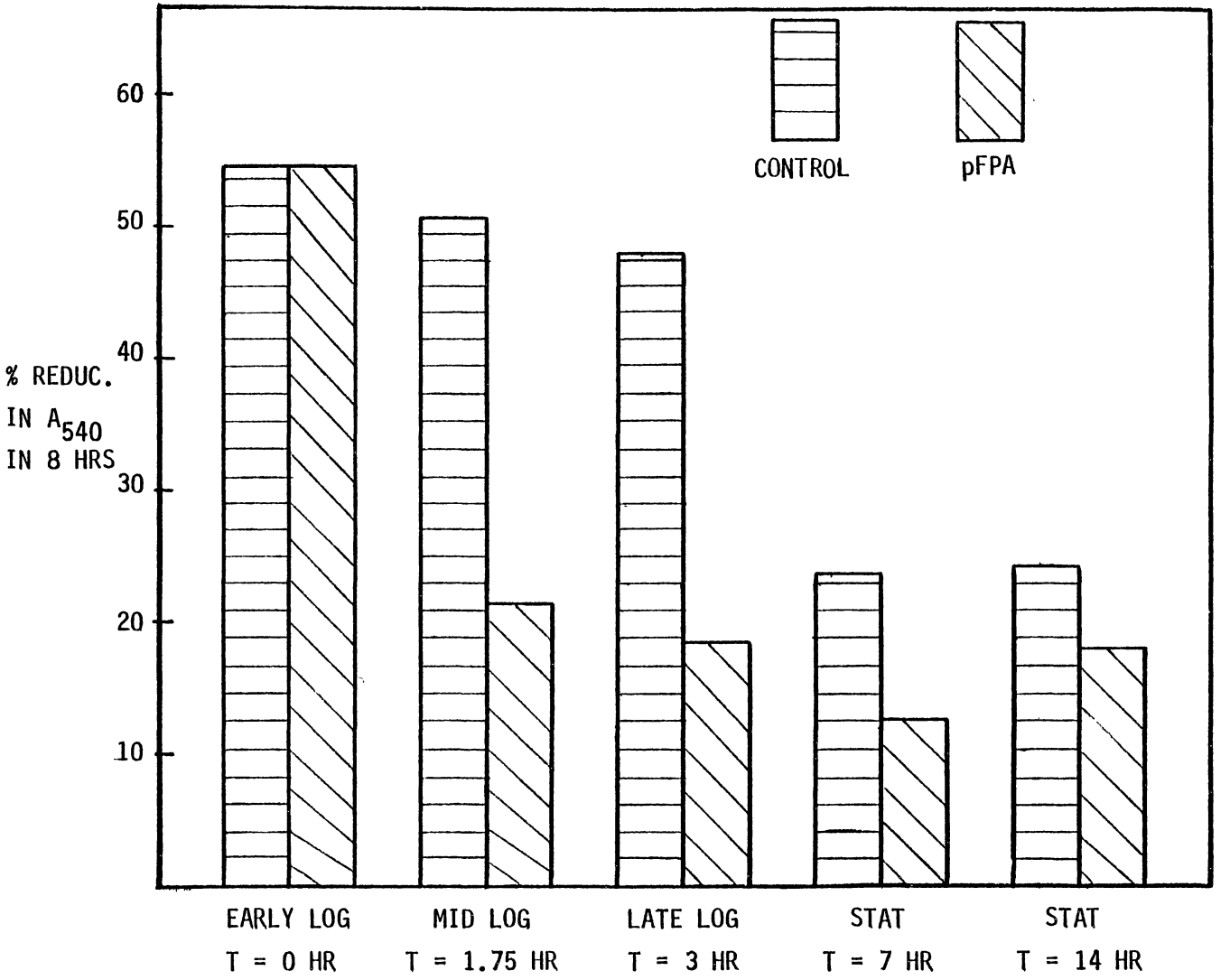

A.
B.
80
60
40
20
% REDUC.
IN A_{540}
IN 8 HRS
CONT
pFPA
oFPA
mFPA
pClPA
CONT
pFPA
oFPA
mFPA
pClPA

seven hours, autolytic activity remained lowered. The control culture, then in stationary phase, also showed decreased autolytic activity, as did the culture grown in the presence of p-ClPA, which was also in stationary phase (Figure 6-B).

It is important to note that only the phenylalanine analogs which slowed growth caused a loss of autolytic activity within 1.75 hours. It is also significant that a natural slowing of growth rate, as in the case of the control culture entering stationary phase, also was accompanied by a loss of autolytic activity. Also of great significance is the fact that o-FPA and m-FPA were able to inhibit autolytic activity without inhibiting cell division. These data indicate that the inhibitions of cell division and of autolytic function caused by growth in the presence of p-FPA are not necessarily related.

In order to examine more closely the effects on autolysis resulting from a slowed growth rate of the control culture as it entered stationary phase, cells were measured for in vivo autolytic activity while in various phases of culture growth. The data of Figure 7 show the results of such an experiment. Addition of p-FPA to an early-log culture resulted in loss of autolytic activity as quickly as 1.75 hours after addition and activity remained inhibited even after the culture had resumed log growth (see Figure 2), by five hours after addition of p-FPA. The control culture retained autolytic activity throughout the

Figure 7. Autolysis of Whole Cells at Various Phases of Growth of the Control Culture and at Various Times After Addition of p-FPA. Control culture, horizontally slashed bars; p-FPA, bars slashed at an angle (as shown in figure). Phase of growth refers to the control culture; times refer to the time (in hours) after addition of p-FPA to an early-log culture. Age of the control culture in early-log phase was 8.5 hours, in mid-log the age was 10.25 hours, in late log the age was 11.5 hours, in the first stationary phase bar the culture was 15.5 hours old, and in the second stationary phase bar the culture was 22.75 hours old. Standard deviations, for % reduction in absorbance at 540 nm in 8 hours, range from 0.42 to 3.52 percent.


CONTROL
pFPA
60
50
40
30
20
10
% REDUC.
IN A_{540}
IN 8 HRS
EARLY LOG
T = 0 HR
MID LOG
T = 1.75 HR
LATE LOG
T = 3 HR
STAT
T = 7 HR
STAT
T = 14 HR
PHASE OF GROWTH OF CONTROL / HOURS POST-ADDITION pFPA

logarithmic growth phase, but abruptly lost the activity as it entered stationary phase. It is interesting to note the similarity in reduced autolytic activity of a control culture entering stationary phase and that of a culture grown in the presence of p-FPA.

It has already been shown that PL is able to prevent filamentation caused by growth in the presence of p-FPA (Table IV). Autolysis of whole cells was used to examine the autolytic activity of short cells which had been grown in the presence of both p-FPA and PL (Table V). As a control, PL was added to an early-log culture. The control culture, with no additions, showed normal autolytic activity. Addition of PL caused a significant reduction in autolytic activity of the control culture. Filamentous cells, grown in the presence of p-FPA, showed a significant inhibition of autolytic activity, as expected. Addition of PL at the same time as addition of p-FPA prevented filamentation, but did not restore autolytic activity. If anything, PL further reduced autolytic activity of the culture grown in the presence of p-FPA.

These data reinforce the conclusion that the inhibitions of cell division and autolytic activity resulting from growth in the presence of p-FPA are not related: Short cells grown in the presence of both p-FPA and PL showed lowered autolytic activity. The data do, however, support the conclusion that lowering the growth rate of an autolytically competent culture, for example, by

TABLE V

COMPARISON OF THE EFFECTS OF PANTOYL LACTONE ON GROWTH, CELL DIVISION, AND AUTOLYTIC ACTIVITY OF CELLS GROWN IN THE PRESENCE OF p-FPA

Additions[a]	A_{540}[b] 2/12 Hours Post-Addition	Cell Length (μm)[b] 2/12 Hours Post-Addition	Autolytic Activity[b] (% Reduction A_{540}/Hour)*
——	0.28±0.008	4±0.5	38.3±0.34[c]
PL only	0.16±0.010	4±0.5	10.7±0.07
p-FPA only	0.45±0.021	13±1.4	4.5±0.10
p-FPA + PL	0.23±0.019	5±0.6	3.5±0.09

[a] PL, where added, was to a final concentration of 0.10 M. PL was added to early-log cells, for the PL only situation; PL was added at the same time as addition of p-FPA to cultures grown in the presence of p-FPA.

[b] Control cultures were examined two hours after addition of PL (control in mid-log); cultures grown in the presence of p-FPA were examined 12 hours after addition of p-FPA and PL. Plus and minus standard deviation provided.

[c] The value of 38.3 % reduction in A_{540} per hour is the autolytic activity exhibited by the mid-log control culture.

* The loss of A_{540} refers to lysis of the cell suspension, that is, the percentage reduction in A_{540} per hour is used as a quantitative value of autolytic activity.

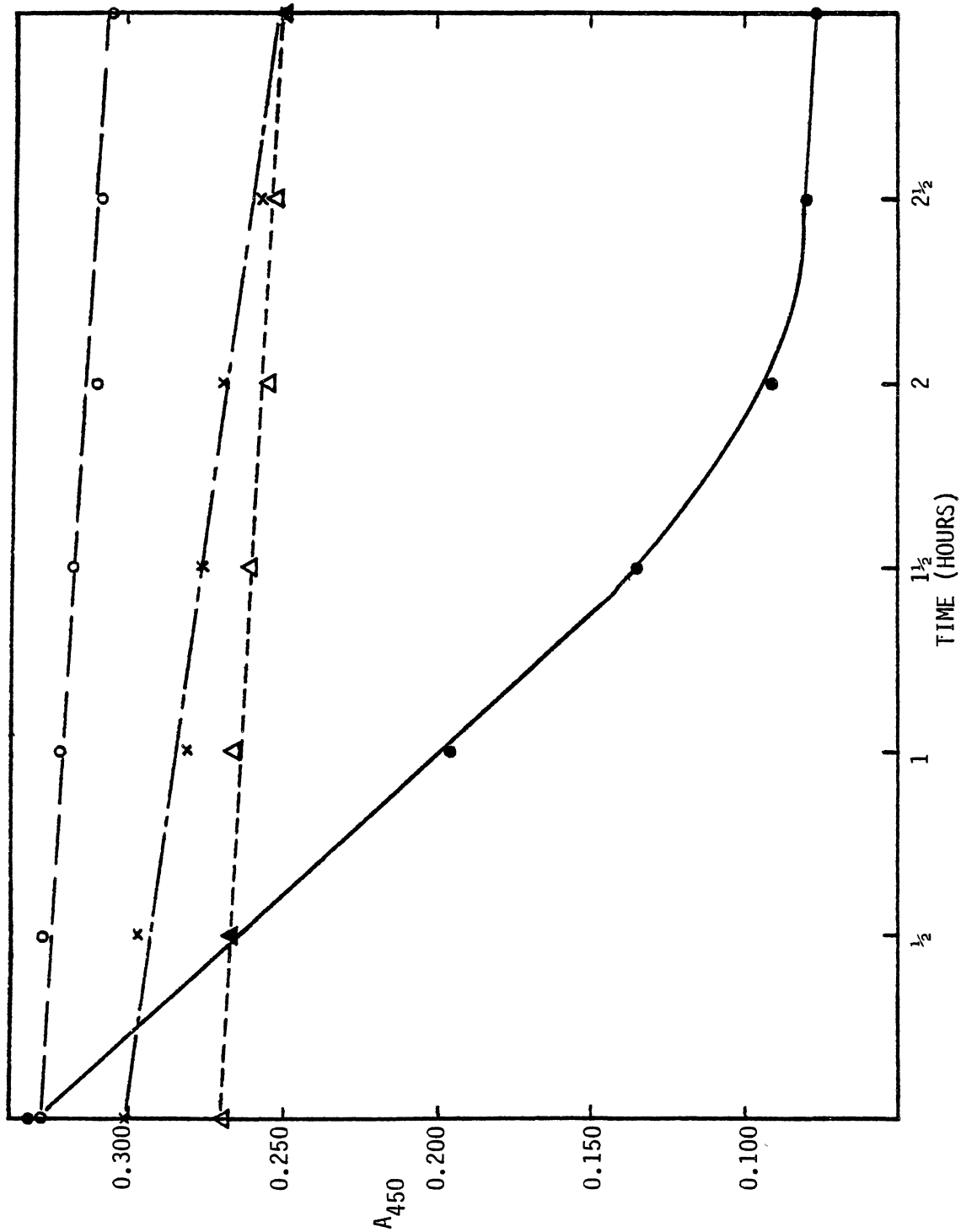
addition of PL to an early-log control culture, resulted in loss of autolytic activity.

Autolysis of Cell Walls by Native Autolysins

In order to confirm the observation that autolysis of cells was inhibited by growth in the presence of any of the three isomers of fluorophenylalanine, autolysis of cell walls, prepared from cells grown in the presence of either p-FPA, or o-FPA, or m-FPA, by the native autolysins present in isolated cell wall preparations was examined. The data presented in Figure 8 show that cell walls prepared from control cultures which were in mid-log phase completely degraded within two hours. The data also indicate that cell walls prepared from cells grown in the presence of p-FPA, o-FPA, or m-FPA did not degrade to any great extent. Thus, these data are in agreement with the earlier observation that growth in the presence of any of the fluorophenylalanines resulted in an inhibition of autolytic activity.

It is possible that p-FPA inhibits autolysis by direct action on the preformed autolytic system, that is, it acts as a negative effector of the autolytic enzymes. This seemed unlikely, since whole cells grown in the presence of p-FPA were washed before assay and cell wall preparations were assumed to be completely free of non-incorporated p-FPA, but the possibility remained to be tested. Addition of p-FPA, at a level of 10 µg/ml (same level as used to induce

Figure 8. Autolysis of Isolated Cell Walls by Native Autolysins, as Influenced by Growth in the Presence of the Various Phenylalanine Analogs. Control culture (●); p-FPA (x); oFPA (o); m-FPA (Δ). Phenylalanine analogs were added in early-log phase, cell walls isolated after growth for 12 hours in the presence of the analog. Control culture was in mid-log phase. For each curve in figure, correlation coefficient (r) equals 0.993, or better.


0.300
0.250
0.200
0.150
0.100
A_{450}
½
1
1½
2
2½
TIME (HOURS)

filamentation of growing cultures), to the buffer of the control cell wall assay had absolutely no effect on the rate or extent of cell wall degradation by the native autolysins (data not shown).

The data of Figure 8 also reinforce the conclusion that the inhibitions of cell division and autolysis caused by growth in the presence of p-FPA are not necessarily related. Just as growth in the presence of o-FPA or m-FPA resulted in a great reduction of cellular autolysis, cell walls prepared from cells grown in the presence of o-FPA or m-FPA also showed inhibition of degradation by their native autolysins. It should be remembered that neither of these fluorophenylalanines caused filamentation of Bacillus sp. #4.

Activity of Isolated Autolytic Enzyme Preparations

The observed decrease in autolytic activity of whole cells, and of cell walls, grown in the presence of p-FPA, is a complex phenomenon, and may be the end result of one, or several changes in Bacillus sp. #4 which occur during growth. Since it has been shown that p-FPA (in vitro) does not inhibit autolysis of washed whole cells, or of isolated cell walls, some change or changes in the autolytic enzymes, or in their substrate, must have occurred while cells were growing in the presence of p-FPA.

One of the possibilities for change is alteration in the synthesis, or in the catalytic activities, of enzymes

(presumably an amidase and a glycosidase) involved in autolysis. In order to test this possibility, the autolytic enzymes were removed by extracting them from isolated cell walls by use of dilute NaOH. The autolytic enzyme preparation obtained by this treatment is a crude extract of proteins which are associated with the cell wall, including the enzymes of the autolytic system, but surely not limited to these proteins.

Substrates for the assay consisted of cell walls, from the same organism, which had been inactivated by treatment with SDS to remove all native autolytic activity. The data of Figure 9 show the amounts of activity of crude extracts of autolytic enzyme systems prepared from both mid-log and stationary phase cultures, as well as enzymes prepared from a culture grown for 12 hours in the presence of p-FPA. The amounts of activity described by the data of Figure 9 can be compared directly, as exactly 50 µl of enzyme, extracted from equal amounts of the various cell wall preparations, were used in this assay (see page 33 for standardization procedure). The substrate for this particular assay was an inactivated cell wall preparation from a culture grown in the presence of p-FPA for 12 hours. The data clearly show that autolytic enzyme extracts prepared from stationary phase control cultures or from cultures grown in the presence of p-FPA have greatly lowered autolytic activity when compared with the enzyme extract prepared from a mid-log control culture.

Figure 9. Assay of Isolated Autolytic Enzyme Systems. Autolysins extracted from: mid-log control culture (●); stationary phase control culture (■); p-FPA (×). Substrate cell walls prepared from a culture grown in the presence of p-FPA, inactivated with SDS. Enzymes isolated from cell walls by extraction with dilute NaOH. For each curve in figure, r=0.998, or better.

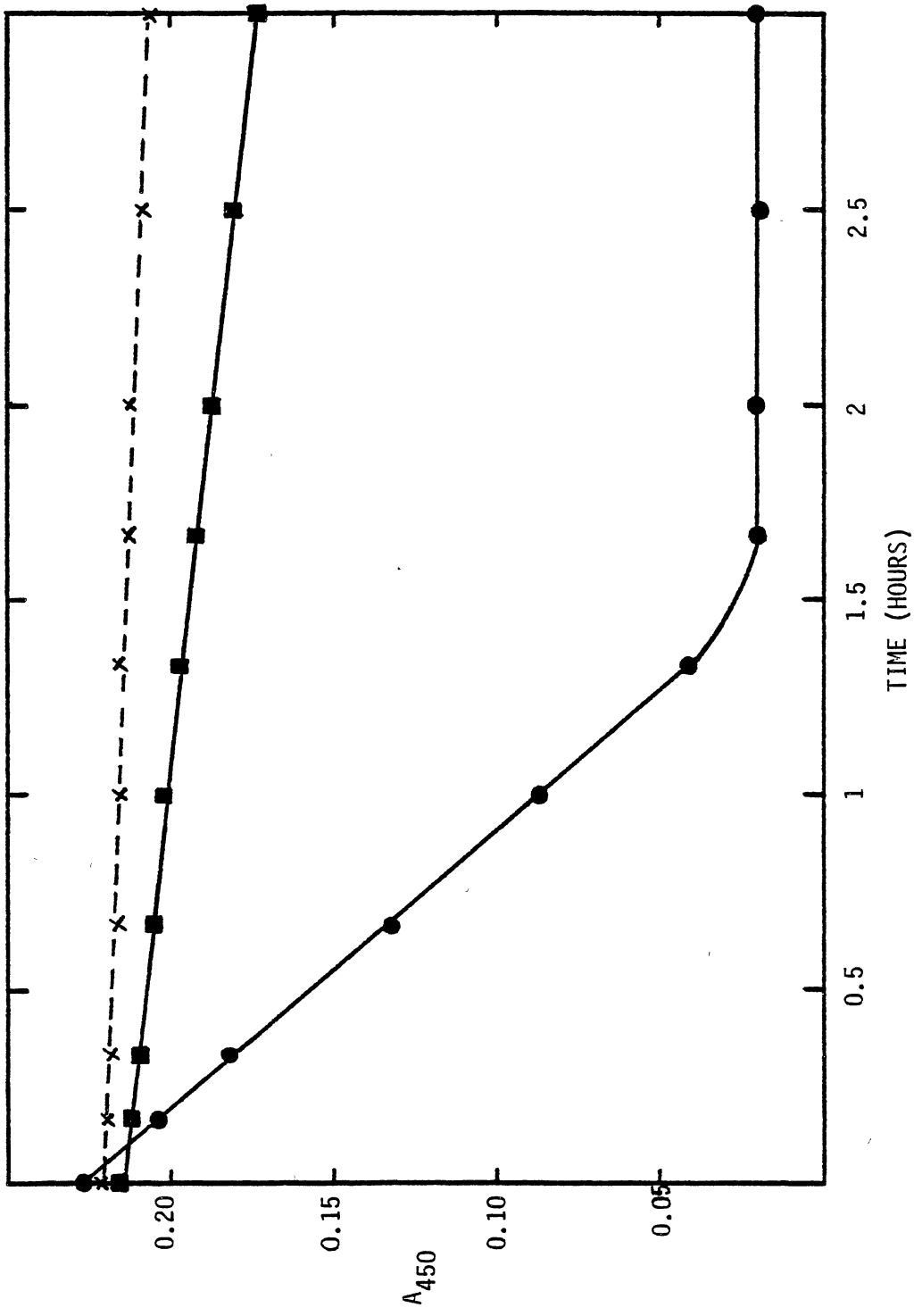
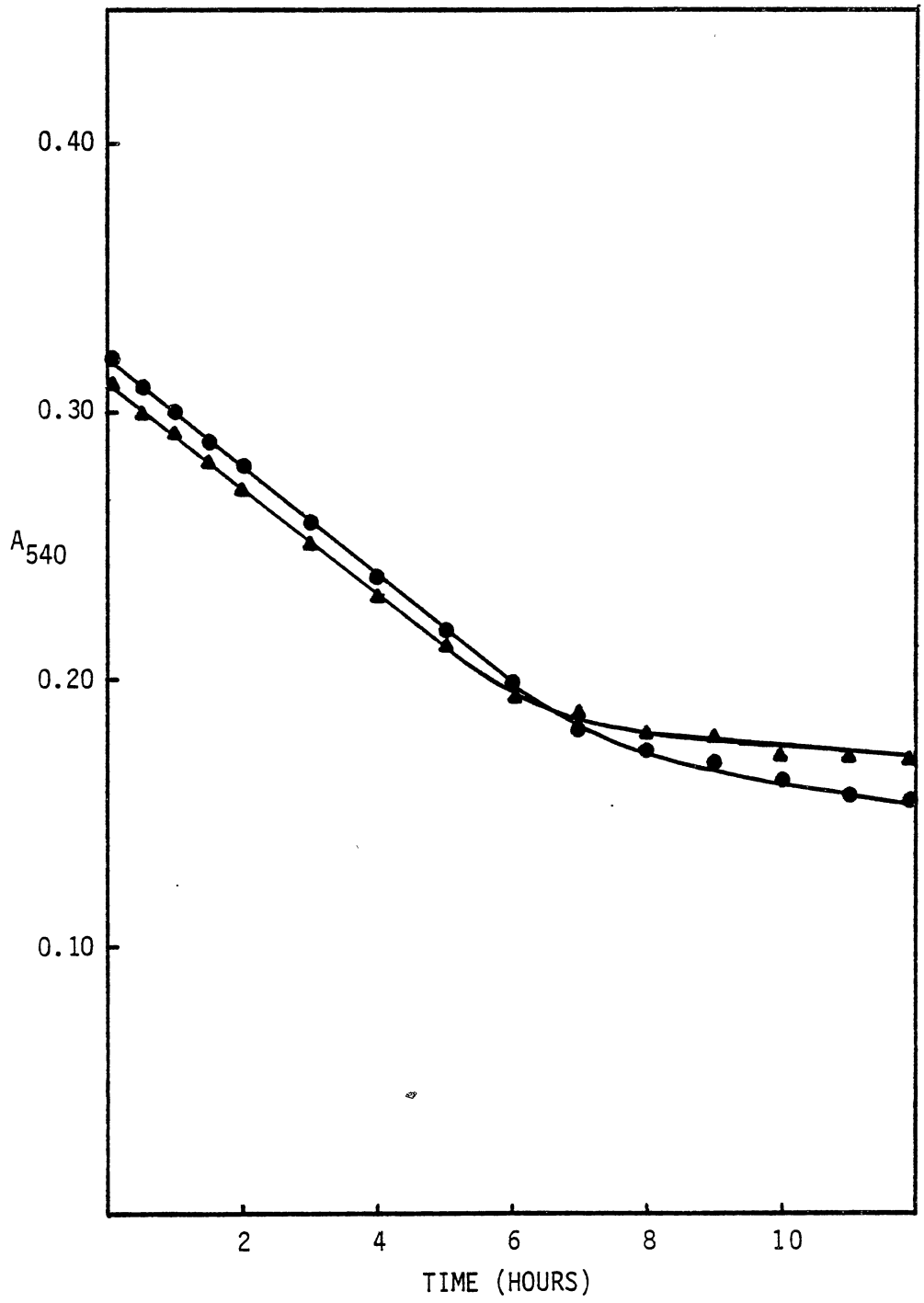

0.20
0.15
0.10
0.05
A_{450}
0.5
1
1.5
2
2.5
TIME (HOURS)

Figure 10. Assay of Isolated Autolytic Enzyme Systems as Influenced by Addition of p-FPA to the Reaction Mixture. Mid-log control autolysins in the presence (▲) or absence (●) of p-FPA. Cell wall substrate prepared from a culture grown in the presence of p-FPA. p-FPA added to the reaction mixture at a level of 136.5 μM. For each curve in figure, r=0.986, or better.


0.40
0.30
A_{540}
0.20
0.10
2
4
6
8
10
TIME (HOURS)

Data identical to those presented in Figure 9 were obtained using inactivated cell wall substrates prepared from any of the culture situations examined and for this reason, the other data are not presented. Thus, these results indicate that it was not an alteration in the substrates of the enzymes, but rather in the autolytic system itself which resulted in lowered autolytic activity of cultures grown in the presence of p-FPA or in stationary phase cultures. Since the autolysins prepared from a mid-log control culture could degrade cell wall substrates prepared either from control cultures or cultures grown in the presence of p-FPA, it is evident that growth in the presence of p-FPA does not alter the cell wall with respect to its ability to serve as a substrate for the autolytic enzymes. The possibility that growth in the presence of p-FPA altered some other aspect of the cell wall itself is considered further in a later section.

One experiment, described previously, suggested that p-FPA was not acting as a negative effector of the autolysins, inhibiting autolytic activity directly. In order to confirm this, p-FPA was added to an assay of the activity of autolytic enzymes extracted from a mid-log control culture, at a level of 25 µg/ml. The results, presented in Figure 10, show that p-FPA did not inhibit autolytic activity, *in vitro*. It was confirmed that p-FPA was not acting as a negative effector of the autolysins.

Further Characterization of Autolytic Enzyme Activities

For a preliminary comparison of the autolysins present in mid-log control cultures and in cultures grown for 12 hours in the presence of p-FPA, the effect of pH on autolytic activity of these cultures was examined. Curves showing the rates of autolytic activity at various pH values were prepared. These curves consisted of the rates of degradation of cell walls by native autolysins which occurred in buffers of several different pH values.

The data presented in Figure 11 show that the effects of the various pH values on the rate of degradation of cell walls by native autolysins of the two types of cell walls were remarkably similar. The results indicate an overall reduction in the rate of autolysis of cell walls prepared from cells grown in the presence of p-FPA, but no change in the complement of activities present, as measured at the different pH values. The general shapes of the pH-activity curves for both control and p-FPA cells are very similar, even to a shoulder between pH 5.5 and 6.0 for the control cells, and between pH 5.0 and 6.0 for cells grown in the presence of p-FPA. The range of activity for both preparations is about pH 4.5 to 9.0, with a clear optimum at pH 7.0.

The data of Figure 12 show that both mid-log control cultures and cultures grown in the presence of p-FPA are positive for the presence of amidase activity (N-acetyl-

Figure 11. Comparison of Rates of Degradation of Cell Walls by Native Autolysins at Various pH's. Mid-log control culture cell walls (▲, solid line); cell walls prepared from cells grown in the presence of p-FPA (●, dashed line). Rate of degradation expessed as the percentage reduction in absorbance at 540 nm per hour. Lines and bars at each point indicate standard deviation.

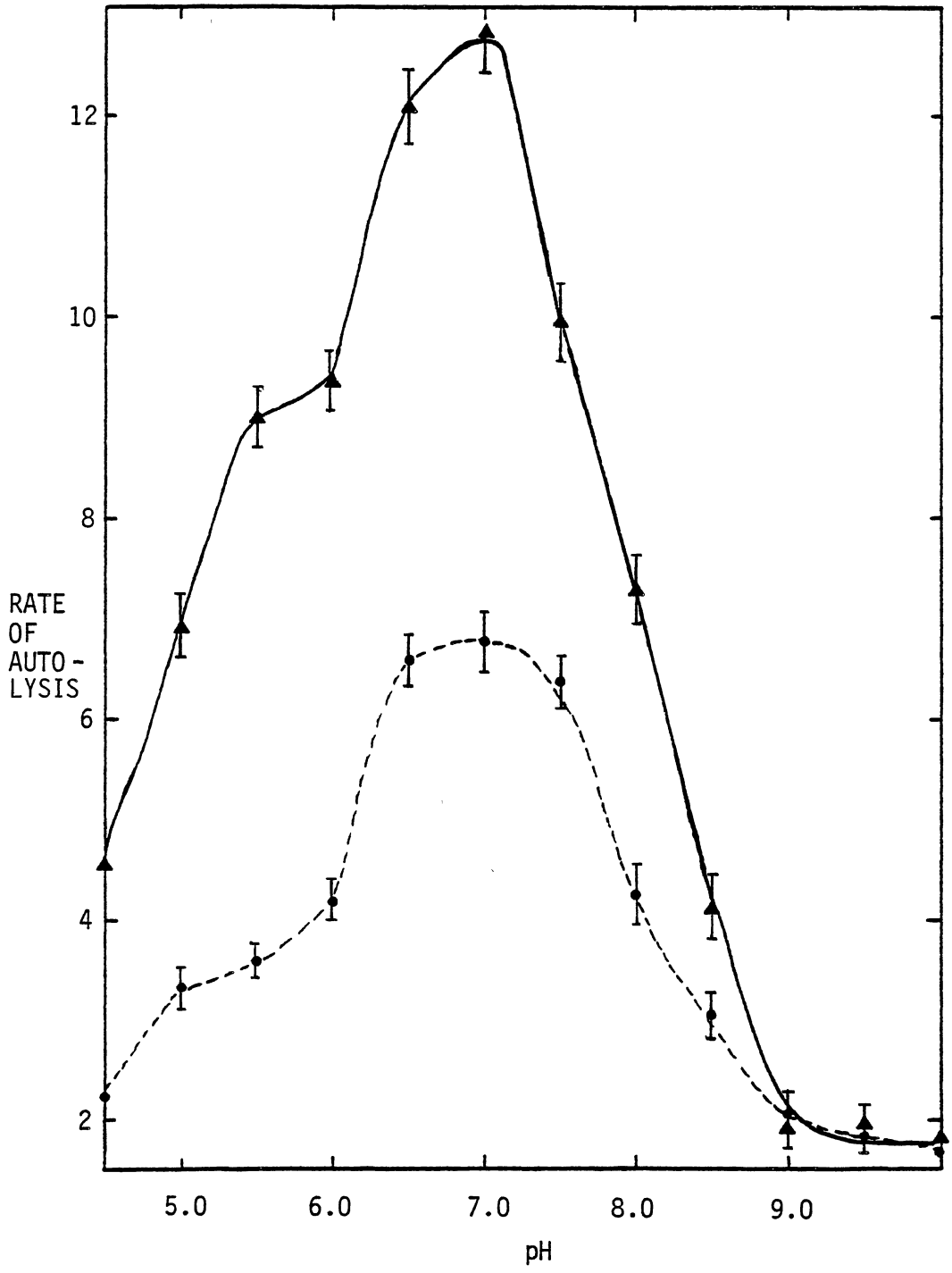
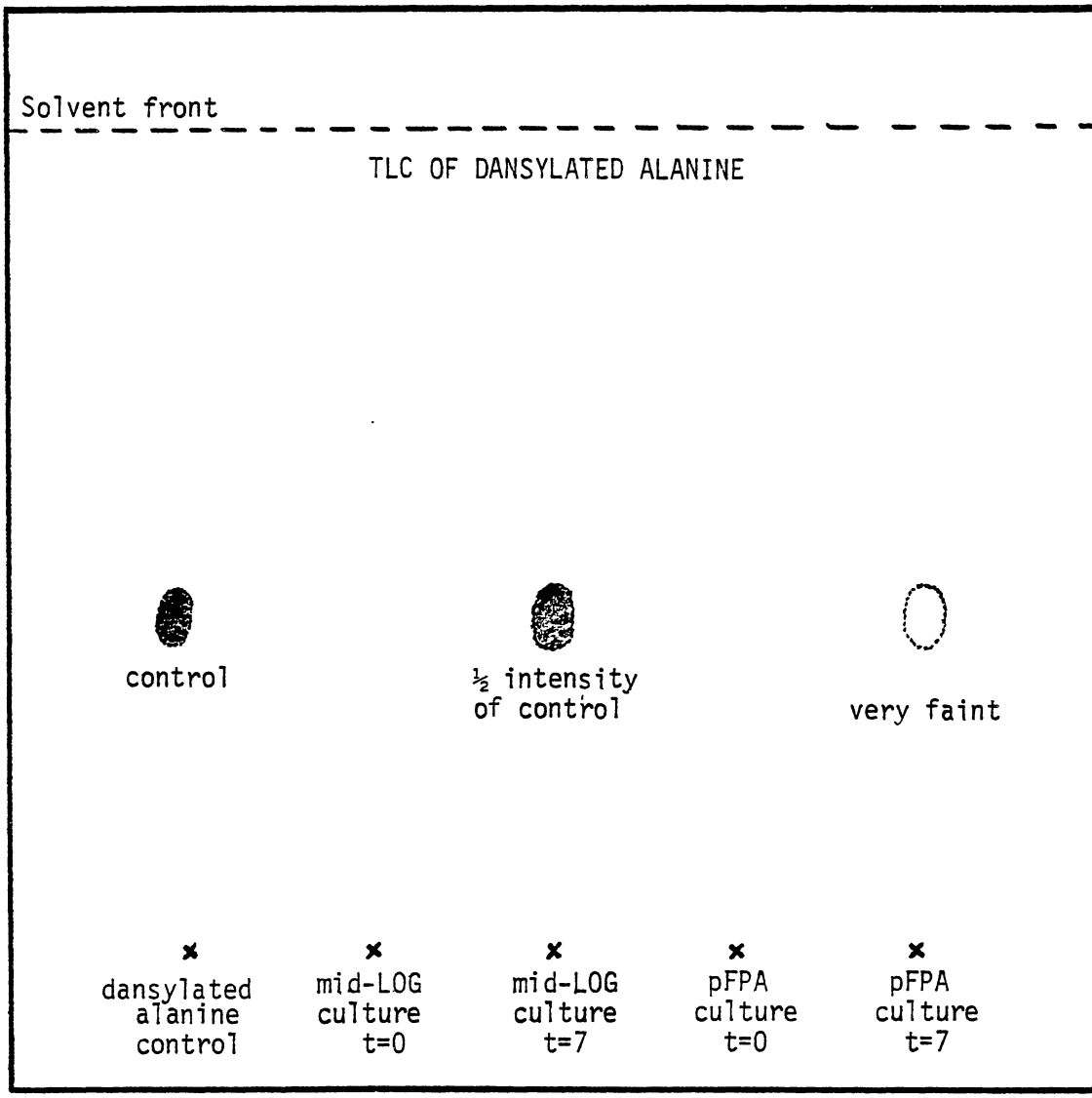

RATE
OF
AUTO-
LYSIS
12
10
8
6
4
2
5.0
6.0
7.0
8.0
9.0
pH

Figure 12. Determination of Amidase Activity. Liberation of free amino groups from alanine, as a result of hydrolysis of the peptide bond between L-alanine and N-acetylmuramic acid, served as an indication of the presence of amidase activity. The freed amino groups were detected by dansylation and analyzed by TLC (chromatogram shown here). Control is dansylated alanine (50 μg alanine). Amount of free amino groups from alanine was detected in mid-log control culture cell walls and cell walls prepared from cells grown in the presence of p-FPA, after seven hours of cell wall autolysis. Amounts of free amino groups from alanine present before cell wall autolysis are also shown (t=0 hours).


Solvent front
TLC OF DANSYLATED ALANINE
control
½ intensity of control
very faint
dansylated alanine control
mid-LOG culture t=0
mid-LOG culture t=7
pFPA culture t=0
pFPA culture t=7

muramyl-L-alanine amidase). Although these data are primarily qualitative in nature, it was estimated (by visual examination of the thin-layer chromatograms) that the amidase activity was at least ten-fold lower in cultures grown in the presence of p-FPA as compared to a mid-log control culture.

The presence of glycosidase activity (endo-β-N-acetyl-glucosaminidase) in cell walls of Bacillus sp. #4 was detected by assaying for liberation of reducing groups during cell wall degradation. Indeed, the assay showed an increase in detectable reducing groups following cell wall autolysis (Table VI). Again, these data are primarily qualitative in nature, but do indicate the presence of glycosidase in all cells examined. The stationary phase control culture and the culture grown in the presence of p-FPA show significantly less activity than the mid-log control culture. It is interesting that in each of the two former situations the onset of glycosidase activity is slowed, when compared with that of the mid-log control. It is also important to note the further similarity in results obtained using cell walls prepared from stationary phase control cells and from cells grown in the presence of p-FPA.

Taken together, the data presented in Figure 12 and in Table VI indicate that growth in the presence of p-FPA did not result in specific loss of either type of autolytic activity (amidase or glycosidase). Rather, the data indicate an overall loss of total autolytic activity,

TABLE VI

MEASUREMENT OF GLYCOSIDASE ACTIVITY AS DETECTED BY APPEARANCE OF REDUCING SUGARS

Growth Situation[a]	nanomoles NAG/mg CW[b]	% of Control
Control		
t=2	6.4±0.28	100.0
t=8	7.8±0.31	100.0
p-FPA		
t=2	1.3±0.19	20.5
t=8	3.2±0.25	41.6
STAT		
t=2	1.4±0.25	22.2
t=8	2.5±0.25	31.5

[a] Control = control culture in mid-log phase; p-FPA = culture grown in the presence of p-FPA for 12 hours; STAT = stationary phase culture. Times are given in hours of cell wall hydrolysis.

[b] N-acetyl glucosamine used as standard: results given as reducing equivalents released per mg of cell wall dry weight. Plus and minus standard deviation provided.

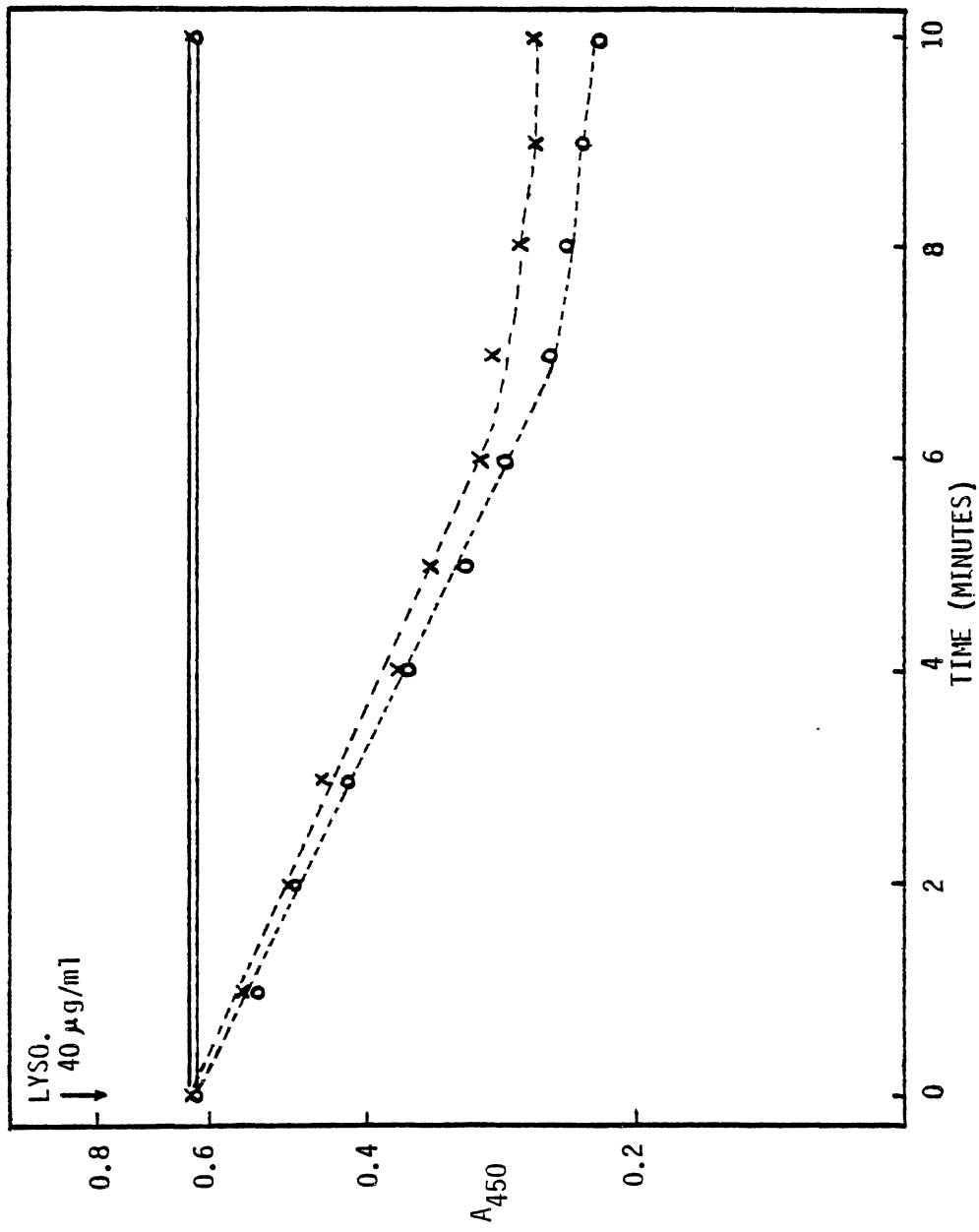
presumably the result of a lesser rate of both types of activity, or the result of lesser amounts of both amidase and glycosidase present in the cell walls.

Susceptibility of Cell Walls to Degradation by Lysozyme

One effect of growth in the presence of p-FPA might be to somehow alter the cell wall such that it could no longer serve as a substrate for the autolytic enzymes, thereby limiting their activity. Data have already been presented which suggest that this was not the case (Figure 9). When assaying the activity of isolated autolytic enzyme extracts, it was found that the enzyme preparation from mid-log control cultures could degrade inactivated cell walls prepared from cultures grown in the presence of p-FPA at the same rate and to the same extent as it could degrade cell walls prepared from a control culture.

In order to confirm this finding, inactivated cell walls prepared from a culture grown in the presence of p-FPA and from a mid-log control culture were compared for their susceptibility to degradation by egg white lysozyme, an enzyme similar in action to glycosidase. The data of Figure 13 indicate that both types of cell walls were degraded at the same rate and to about the same extent by lysozyme. Therefore, it was concluded that growth in the presence of p-FPA did not alter the cell wall with respect to its serving as an autolytic enzyme substrate.

Figure 13. Susceptibility of Cell Walls to Degradation by Lysozyme. Cell walls prepared from mid-log control cells (o); cell walls prepared from cells grown in the presence of p-FPA (×). Controls, with no lysozyme added (solid lines); experimental, with 40 µg/ml lysozyme added (dashed lines). For each curve in figure, r=0.993, or better.


LYSO.
40 µg/ml
0.8
0.6
0.4
0.2
A_{450}
0
2
4
6
8
10
TIME (MINUTES)

Studies on Cell and Cell Wall Composition

Composition of the Cell Wall Mucopeptide

In order to determine if growth in the presence of p-FPA had any effect on the basic composition of the cell wall mucopeptide, cell walls, from which protein had been removed by treatment with SDS, were hydrolyzed and chromatographed in the solvent system of Heathcote and Jones (1965), as described in Chapter II. Figure 14 shows a typical thin-layer chromatogram of hydrolyzed cell walls prepared from a mid-log control culture. The mucopeptide was composed of muramic acid, glucosamine, alanine, glutamic acid, and diaminopimelic acid (DAP). The presence of each component was confirmed by use of known standards, chromatographed in the same solvent system. Identical data (both qualitative and quantitative) were obtained whether the cell walls were prepared from mid-log phase control cultures, stationary phase control cultures, or cultures grown in the presence of p-FPA.

DAP showed poor migration in the Heathcote-Jones system. In order to confirm the presence of DAP, a second solvent system was employed (Rhuland et al., 1955). In this system, meso- and D, D-DAP could be distinguished from L, L-DAP on the basis of their migration. The data of Figure 15 confirm the presence of DAP in the cell wall mucopeptide. The DAPs present in the cell wall mucopeptide of cells grown

Figure 14. Typical Thin-Layer Chromatogram Showing the Five Basic Components of the Cell Wall Mucopeptide. Hydrolysates equivalent to 175 µg of cell wall were applied at the origin (marked x). Chromatograms were developed in the solvent system of Heathcote and Jones (1965). Data were identical for cell walls prepared from mid-log control cells, stationary control cells, or cells grown in the presence of p-FPA. Data shown here are for cell walls prepared from mid-log control cells.

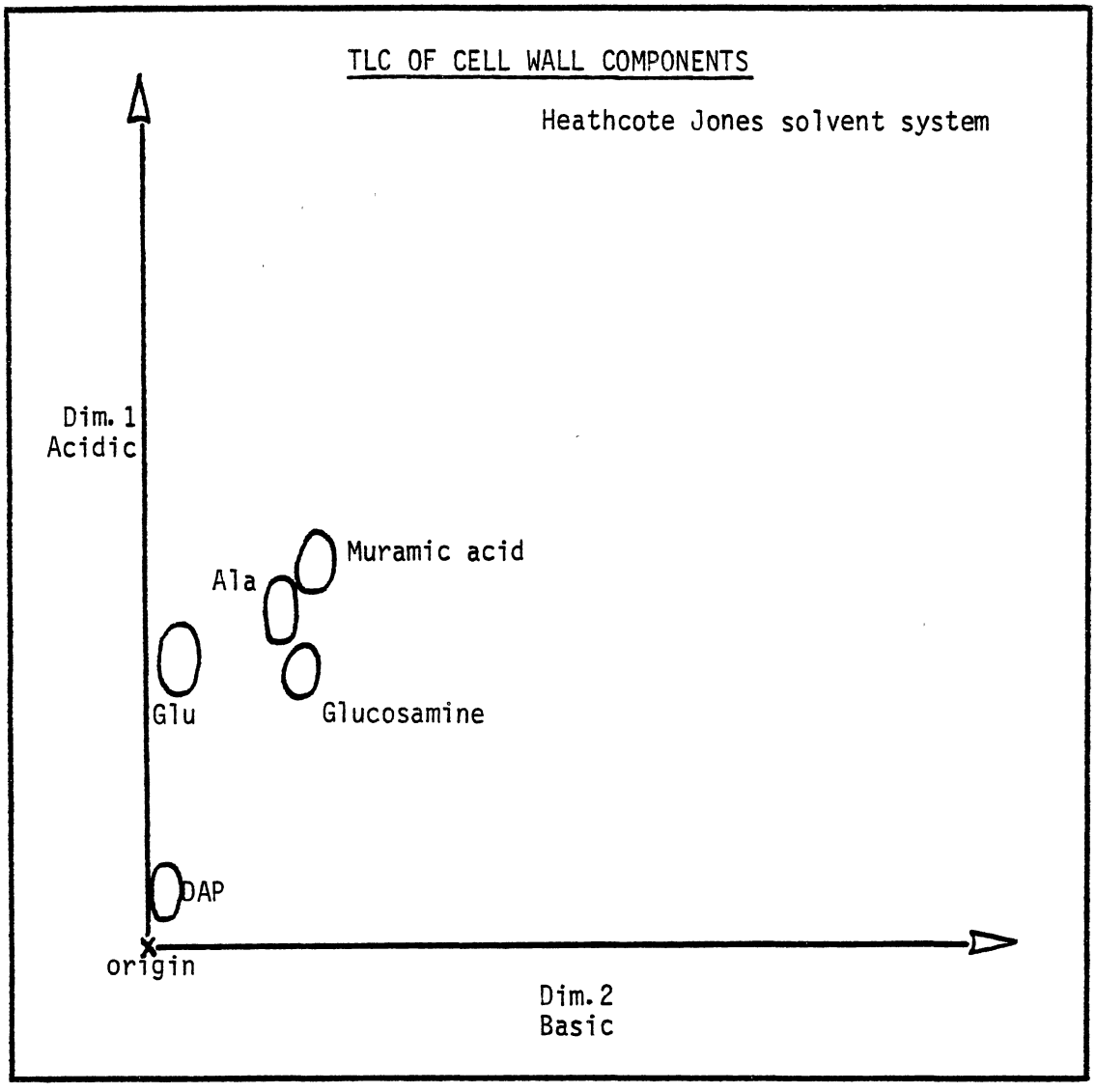
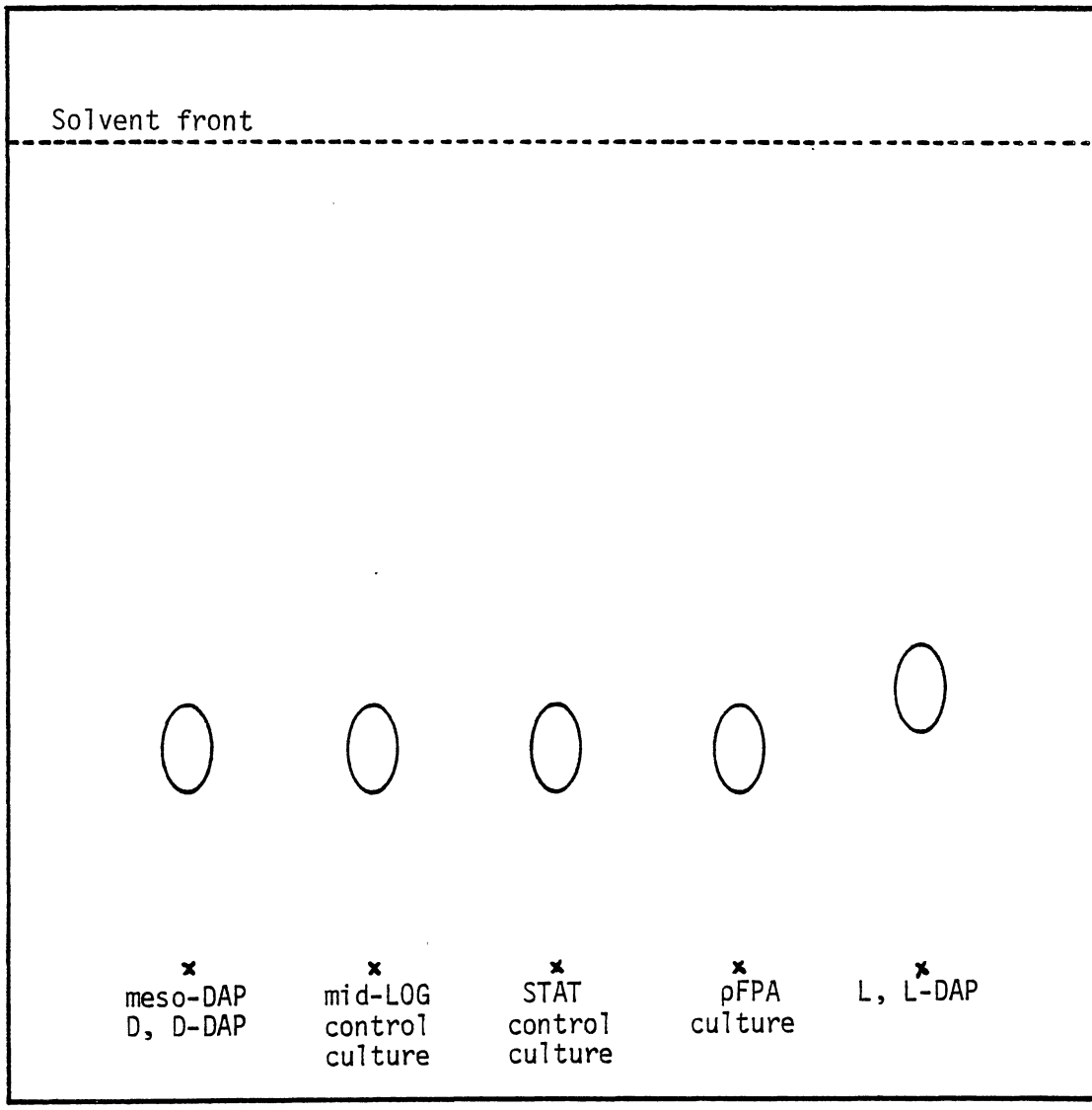

TLC OF CELL WALL COMPONENTS
Heathcote Jones solvent system
Dim. 1
Acidic
Muramic acid
Ala
Glu
Glucosamine
DAP
origin
Dim. 2
Basic

Figure 15. Paper Chromatogram of the Diaminopimelic Acid Present in Cell Wall Mucopeptide. Hydrolysates of cell walls, equivalent to 125 µg of cell wall, prepared from a mid-log control culture, a stationary control culture, and a culture grown in the presence of p-FPA were applied at the origins (marked x) where indicated in the figure. DAP standards (0.1 µMole, each) were applied where indicated in the figure. The chromatogram was developed in the solvent system of Rhuland et al. (1955).


Solvent front
meso-DAP
D, D-DAP
mid-LOG
control
culture
STAT
control
culture
ρFPA
culture
L, L-DAP

in the presence of p-FPA, mid-log control cells, or stationary phase control cells all showed the same migration in this system. On the basis of their migration, the DAPs from each of these growth situations was shown to be either meso- or D, D-DAP; the two could not be distinguished in this system.

The data presented in Figures 14 and 15 provide evidence that growth in the presence of p-FPA did not alter the basic composition of the cell wall mucopeptide as compared with that of control cell walls. It was concluded that the effect of p-FPA on autolysis was not a result of a change in the composition of its cell wall mucopeptide.

Protein and Autolysin Content of Cell Walls

Williamson and Ward (1981) have shown that deficiency of autolytic activity in Bacillus subtilis and Streptococcus pneumoniae is associated with a decrease in the amount of total protein, as well as autolytic activity, present in the cell walls of these organisms. This effect was demonstrated both in autolytic-deficient mutants (which lack autolytic enzymes) and in cells which were made phenotypically deficient in autolytic activity (such as occurs when S. pneumoniae is grown in ethanolamine-containing medium). Examination of the cell wall protein extracts, which had been fractionated by gel filtration chromatography, using SDS-polyacrylamide gel electrophoresis showed that the major

difference between cell wall proteins of autolytic-deficient and non-deficient cells was in the total amount of protein present and did not result from marked changes in the molecular weight of extracted proteins.

The data presented in Table VII show that growth in the presence of p-FPA resulted in cells which contain less protein in their cell walls. Other data in Table VII indicate that growth in the presence of o-FPA or m-FPA also resulted in cells which contained less protein in their cell walls. Entry of a control culture into stationary phase resulted in a similar reduction in the total amount of protein per µg cell wall dry weight present in these cell walls. These four growth situations resulted in a reduction of total cell wall protein of between 65% (for a culture grown in the presence of o-FPA) and 48% (for a culture grown in the presence of m-FPA) as compared with the amount of protein present in cell walls of a mid-log control culture. Cell walls prepared from a culture grown in the presence of p-FPA and a stationary phase control culture contained 60% and 57% less protein, respectively.

Cells grown in the presence of p-FPA and stationary phase control cells, extracted for autolytic enzymes using dilute NaOH, contained significantly less protein in the crude extracts when compared with the extract of mid-log control cells (Table VII). The culture grown in the presence of p-FPA contained 88% less extractable protein and the stationary phase control culture had 64% less

TABLE VII

AMOUNTS OF PROTEIN AND AUTOLYTIC ACTIVITY PRESENT IN CELL WALLS

Growth Situation [a]	Total Protein in Cell Walls [b]		Extractable Protein in Cell Walls [c]		Extractable Activity per ug Protein [d]	
	ug Protein per ug Cell Wall	% Control	ug Protein per mg Cell Wall	% Control	Units per ug Protein	% Control
mid-log Control	0.303±0.011	100.0	43.4±0.27	100.0	2.88±0.091	100.0
p-FPA Culture	0.119±0.009	39.4	5.1±0.04	11.7	0.16±0.008	5.6
Stationary Culture	0.131±0.015	43.2	15.7±0.09	36.1	0.24±0.016	8.3
m-FPA Culture	0.160±0.012	52.8	—	—	—	—
o-FPA Culture	0.107±0.015	35.3	—	—	—	—

[a] Cultures as usual. Fluorophenylalanine cultures grown for 12 hours in the presence of FPA.

[b] Total protein present in cell wall preparations; expressed in terms of cell wall dry weight. Plus and minus standard deviation (S. D.) provided.

[c] Protein extracted using dilute NaOH, as for isolation of autolytic enzymes; expressed in terms of cell wall dry weight. m-FPA and o-FPA cultures not examined. Plus and minus S. D. provided.

[d] Autolytic activity of isolated enzyme preparations (as in [c]) measured as in Figure 5; expressed in terms of ug protein extracted. Plus and minus S. D. provided.

extractable protein in comparison with the amount of extractable protein present in the mid-log control cells. A similar reduction was also seen when extracts were prepared using the 5 M LiCl method (data not shown). When these extracts were assayed for autolytic activity, it was found that the extract prepared from cells grown in the presence of p-FPA contained 94% less activity per unit of protein extracted and that stationary phase control cell extracts contained 92% less activity per unit of protein extracted, when compared with that of a mid-log control cell extract (Table VII; data are for extracts prepared using the dilute NaOH method). Again, it is significant that cultures grown in the presence of p-FPA and stationary phase control cultures gave remarkably similar results.

Comparison of the data concerning the amount of extractable protein present and the amount of autolytic activity per unit of protein extracted revealed that the amount of autolytic activity per unit of protein extracted was reduced further than the total amount of protein extracted, when these determinations were compared with the mid-log control culture (cultures grown in the presence of p-FPA contained 88% less extractable protein per mg of cell wall and 94% less autolytic activity per µg of protein extracted; stationary control cultures contained 64% less extractable protein per mg of cell wall and 92% less autolytic activity per µg of protein extracted). These results suggest that the autolytic enzymes extracted from

cultures grown in the presence of p-FPA and from stationary phase control cultures were at least partially inhibited in autolytic activity, when compared with the mid-log control: The apparent specific activities of the autolytic enzymes, prepared from cells of these two autolytic-deficient growth situations, were lower than the reduction expected as a result of the lesser amount of extractable protein present.

Determination of Extracellular Amylase Production

It was thought that the lowered amounts of protein and autolytic activity present in the cell walls of the autolytic-deficient cells might be the result of an impaired ability of these cells to transport the proteins across their cell membrane to the cell wall. As a measure of this, production of extracellular α-amylase by cells made filamentous by growth in the presence of p-FPA was examined, and compared with α-amylase present extracellularly in control cultures.

Bacillus sp. #4 could be cultured on agar plates of aspartic acid-glucose defined medium which contained starch and p-FPA. The cells grew as filaments, indicating that the effects of p-FPA could occur on solid media. The cell division inhibited cells grown on plates containing p-FPA were able to hydrolyze starch to the same extent as control cultures grown on similar plates which did not contain p-FPA (data not shown), indicating that the filaments were able to

produce extracellular amylase. It should be pointed out that cell walls prepared from autolytic-deficient cultures have all contained at least some autolytic activity. Although the data are not conclusive, it is believed that inhibited autolysis is not the result of an inability to transport the autolytic enzymes across the cell membrane to the cell wall.

Determination of the Amounts of Lipid and Cell Wall of p-FPA Grown Cells

It has been shown that autolytic-deficient mutants of Streptococcus faecium have increased levels of cellular lipids when compared with the wild type parent strain (Shungu et al., 1979). Other workers have demonstrated that growth of Bacillus cereus or B. subtilis under conditions which inhibit autolytic activity, while allowing cell wall synthesis to continue, results in a thickening of the cell wall (Chung, 1971; deBoer et al., 1982; Hughes et al., 1970). This thickening of cell walls is manifested by an increase in the dry weight of cell wall per cell. In order to determine if growth of Bacillus sp. #4 in the presence of p-FPA resulted in an increase in the amounts of cellular lipids and/or cell wall components per cell, the cells were fractionated such that the amounts of these components could be assayed.

Cells were radiolabelled with ^{14}C-U-L-aspartic acid and fractionated according to the procedure of Park and Hancock

(1960). A comparison of the amounts of various macromolecular components of cells grown in the presence of p-FPA and of mid-log control cells was made (Table VIII). The data most relevant to this study are those for the amounts of lipids and cell wall. Cells grown in the presence of p-FPA contained 18% more lipid per unit of cell weight and 38% more cell wall per unit of cell weight, when compared with the results for mid-log control cells.

The increase in the amount of cell wall of cells grown in the presence of p-FPA was not unexpected. All cell wall preparations from cells grown in the presence of p-FPA were far more opaque than similar preparations from mid-log control cells, which were virtually transparent (estimated by visual examination of the cell wall preparations, data not shown). The same was true for each of the autolytic-deficient growth situations: Cell wall preparations from cells grown in the presence of o-FPA or m-FPA and from stationary phase control cells were all far more opaque than the mid-log control cell wall preparation (data not shown). It was thought possible that the opacity of the cell wall preparation was a result of their individually increased thickness. It was also found that cell walls prepared from cells of any of the growth situations which resulted in autolytic-deficiency appeared thicker than the mid-log control cell when examined using phase contrast microscopy. Light microscopy of cell walls stained with either alcian blue or crystal violet also showed a thickened cell wall

TABLE VIII

FRACTIONATION OF RADIOLABELLED CELLS

Culture[a] Examined	Nature of[b] Fraction	Radioactivity[b] in Fraction (cpm)	Radioactivity per[c] mg wet cell weight (cpm/mg)	Percentage Increase[d] or Decrease from Control
mid-log Control Culture	Cell Pool	790±28	9.19	———
	Lipids	270±16	3.14	———
	Nucleic Acids	1240±35	14.42	———
	Protein	3605±60	41.92	———
	Cell Wall	742±27	8.63	———
p-FPA Grown Culture	Cell Pool	1580±40	7.45	18.9 % <
	Lipids	810±29	3.82	17.8 % >
	Nucleic Acids	1090±33	5.14	64.4 % <
	Protein	5840±76	27.55	34.3 % <
	Cell Wall	2964±54	13.98	38.3 % >

[a] Mid-log control culture grown to A_{540}=0.24; culture grown in the presence of p-FPA (p-FPA grown culture) for 14 hours, A_{540}=0.32.

[b] Fractions contained the components indicated; fractionation of cells according to the procedure of Park and Hancock (1960). Cells labelled in steady-state with ^{14}C-U-L-aspartic acid (see protocol, Chapter II). Plus and minus standard deviation provided.

[c] Wet weight of cells measured at time of harvest of cultures: mid-log control culture, 86±4.3 mg total wet weight; p-FPA culture, 212±9.2 mg total wet weight.

[d] Percentage increase over control (>); percentage decrease from control (<): for data of radioactivity per mg wet cell weight.

(data not shown). These determinations were tentative, at best, and a firm conclusion could not be drawn from the data as described.

Electron Microscopy of Thin-Sections of Autolytic-Deficient Bacillus sp. #4

It has been shown that growth of Bacillus cereus or B. subtilis under conditions which inhibit autolytic activity, while allowing cell wall synthesis to continue, results in thickening of the cell wall, as shown by electron microscopy (Chung, 1971; deBoer et al., 1982; Hughes et al., 1970). Results have already been presented which indicate that growth of Bacillus sp. #4 in the presence of p-FPA resulted in an increase in the amount of mucopeptide per mg wet cell weight (Table VIII). In order to obtain more supporting evidence, and to determine if the cell walls of cells grown in the presence of p-FPA are actually thicker, p-FPA cells were sectioned and examined using transmission electron microscopy.

The electron micrographs presented in Figure 16 show the thickness of the cell walls of cells grown in the presence of p-FPA and of mid-log control cells. These micrographs are examples of typical results obtained during this study. It is quite obvious that growth in the presence of p-FPA resulted in thickening of the cell wall. Longitudinal and cross sections (and all degrees of orientation of the cells in between these two) were obtained

Figure 16. Thin-Sections of Bacillus sp. #4 Showing the Thickness of the Cell Walls. A. Longitudinal thin-section of mid-log control cells with an average thickness of 31 nm (33,167x). B. Cross-section of cell grown in the presence of p-FPA with average thickness of 105 nm (33,167x).

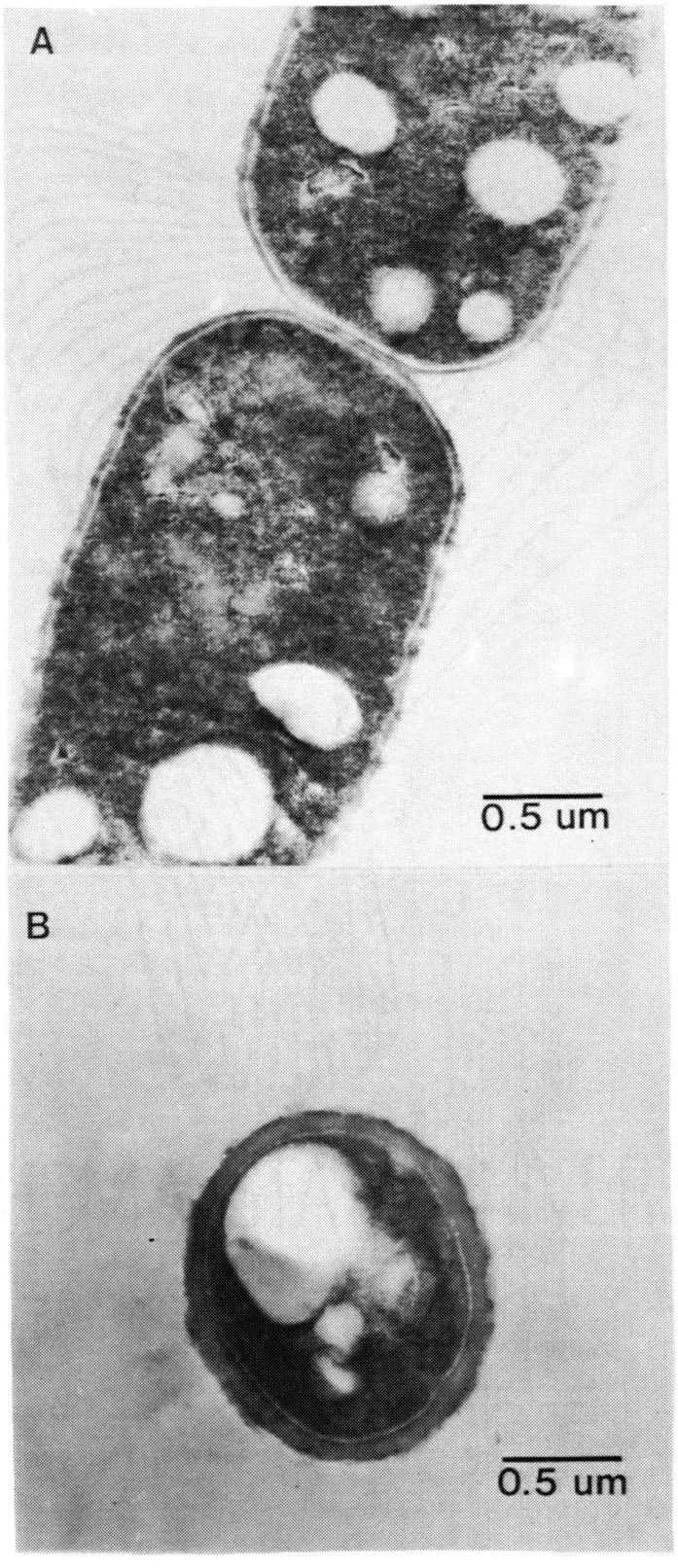

A
0.5 um
B
0.5 um

TABLE IX

CELL WALL THICKNESS OF BACILLUS SP. #4: ANALYSIS OF THE RESULTS OF THIN-SECTION TRANSMISSION ELECTRON MICROSCOPY

Growth Situation[a]	% of Cell Walls Which are Thicker[b]	Range of Thickness[c] of Thicker Cell Walls	Average Thickness[c] of Thicker Cell Walls	Overall Avg.[c] Thickness of Cell Walls	Overall % Thicker
mid-log Control	0	25-43 nm*	———	33.6±2.7 nm	——
p-FPA Culture	36.0%	50-292 nm	97.7±6.1 nm	56.7±3.9 nm	40.7%

* Range is for all of mid-log control cell sections studied (in nanometers). These cells were not considered to be thick.

[a] Cultures grown as usual: Control = mid-log control cells; p-FPA culture = cells grown in the presence of p-FPA for 12 hours.

[b] Thicker walls were chosen as those which were 50 nm thick, or thicker.

[c] Cell wall thickness determined by measuring the thickness of the cell wall in electron micrographs of Bacillus sp. #4 thin-sections. Plus and minus standard deviation provided.

randomly, depending upon the chance orientation of the cells during sectioning, but the orientation of the cell did not affect the apparent thickness of the cell wall, as this thickness is constant all about the cell.

An analysis of the results obtained for the thickness of cell walls of cells grown in the presence of p-FPA and of mid-log control cells is presented in Table IX. The data given are the cumulative results from examination of 100 different thin-sliced cells of either growth situation. It was found that cell walls of mid-log control cells had an average thickness of 33.6 nm with a range of 25-43 nm. Cell walls prepared from cells grown in the presence of p-FPA showed marked variation in thickness. In fact, only 36% of the cells (36 of 100) had thicker cell walls, where a thicker cell wall was chosen as one which was 50 nm thick, or thicker. The range in thickness of these thicker cell walls was 50-292 nm, with an average thickness for these thicker walls of 97.7 nm. The average overall thickness (thicker and normal wall thickness combined) was 56.7 nm, a value which is 40.7% higher than the average thickness for cell walls of mid-log control cells. Electron micrographs presented in Figures 17 and 18 are typical of cells grown in the presence of p-FPA.

Thus, the data presented in Figures 16, 17, and 18 and in Table IX demonstrate that cells grown in the presence of p-FPA had thicker cell walls. On the average, these walls were 40.7% thicker than the walls of mid-log control cells.

Figure 17. Thin-Section of Bacillus sp. #4 Grown in the Presence of p-FPA. I. Longitudinal thin-section of cell grown in the presence of p-FPA with an average thickness of 56 nm (35,425x).

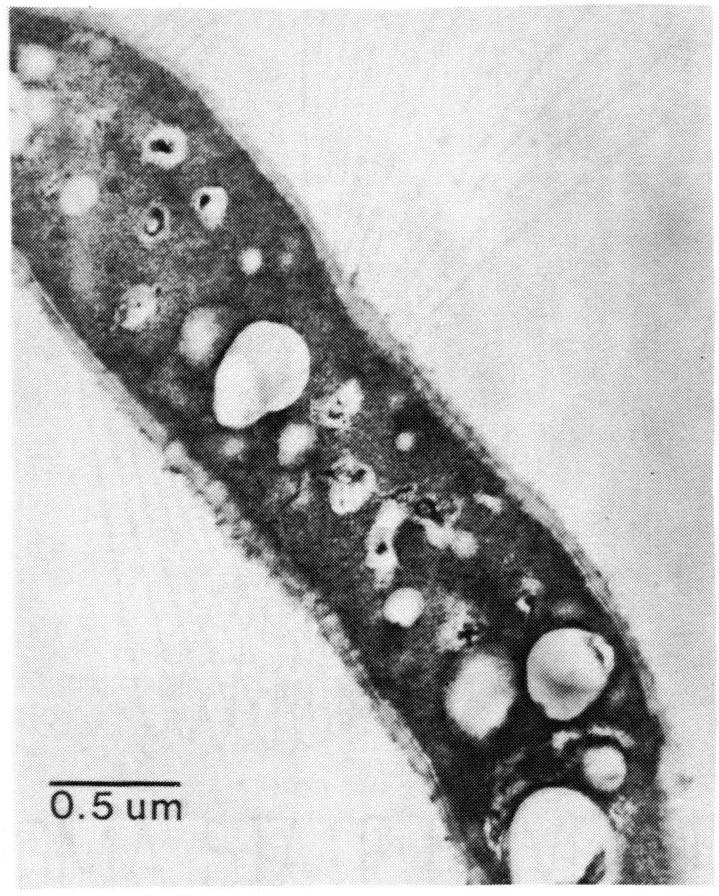
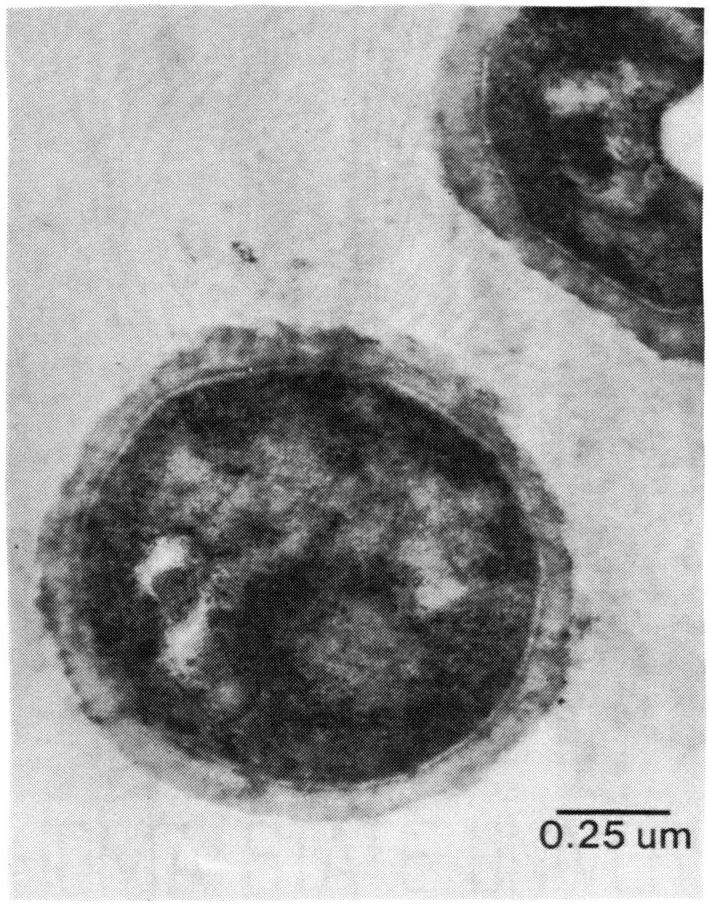

0.5 um

Figure 18. Thin-Section of Bacillus sp. #4 Grown in the Presence of p-FPA. II. Cross-section of cells grown in the presence of p-FPA with an average thickness of 72 nm (62,353x).


0.25 um

This result compares favorably with the result obtained by fractionation of the cells (Table VII) which indicates that cells grown in the presence of p-FPA contained 38.3% more cell wall than did mid-log control cells. The consequence of growth in the presence of p-FPA and the subsequent inhibition of autolytic-competence of these cells is typical, then, of what occurs in other species of Bacillus grown under conditions in which autolytic activity is inhibited, but cell wall synthesis is allowed to continue (Chung, 1971; deBoer et al., 1982; Hughes et al., 1970).

Determination of the Amounts of Lipoteichoic Acids Present in Autolytic-Deficient Bacillus sp. #4

Shungu et al. (1979) have shown that autolytic-deficient mutants of Streptococcus faecium have increased levels of both cellular lipids and lipoteichoic acids. Lipoteichoic acids have been shown to inhibit autolytic function both in vivo and in vitro (Shockman et al., 1974; Holtje and Tomasz, 1975; Cleveland et al., 1975). Fractionation of Bacillus sp. #4 has revealed that cells grown in the presence of p-FPA contained 17.8% more lipids than did mid-log control cells. In order to determine if growth of Bacillus sp. #4 in the presence of p-FPA resulted in a similar increase in the amount of cellular lipoteichoic acids, the amounts of intracellular and extracellular lipoteichoic acids produced by mid-log control cells,

stationary phase control cells, and cells grown in the presence of p-FPA were determined.

Table X presents the data of such an experiment. Cells grown in the presence of p-FPA and stationary phase control cells contained significantly less intracellular and extracellular lipoteichoic acid than did mid-log control cells. However, when the relative amounts of extracellular to intracellular lipoteichoic acids were examined, it was found that the ratio of extracellular to intracellular lipoteichoic acids was higher for stationary phase control cells and cells grown in the presence of p-FPA when compared with the ratio for mid-log control cells.

The finding that the two autolytic-deficient cultures (stationary phase control cells and cells grown in the presence of p-FPA) contained less lipoteichoic acids per μg cell dry weight was unexpected, especially when one considers that growth in the presence of p-FPA did result in an increase in cellular lipids. This could have been a problem which was the result of the labelling protocol used; in the experiment described here, label was added at the time of inoculation of the culture. In a similar experiment, performed by Shungu et al. (1979), where the amounts of lipoteichoic acids produced by several different strains of Streptococcus faecium were compared, the cultures were grown for six generations in the presence of label, in order to allow direct comparison of results from the various strains which had different generation times. However,

TABLE X

AMOUNTS OF INTRACELLULAR AND EXTRACELLULAR LIPOTEICHOIC ACIDS PRESENT IN BACILLUS SP. #4

Growth[a] Situation	Total[b] Incorporation (cpm/ug cell)	LTA_i[c] (cpm/ug cell)	LTA_x[c] (cpm/ug cell)	Total LTA (cpm/ug cell)	Ratio[d] LTA_x:LTA_i
mid-log Control	1142.9±34 (1)*	213.7±3.1 (1)	838.0±39 (1)	1051.7 (1)	3.92 (1)
p-FPA Culture	822.7±29 (0.72)	85.4±8.1 (0.40)	636.8±7.2 (0.76)	722.2 (0.69)	7.46 (1.90)
Stationary Culture	384.3±20 (0.34)	62.0±4.2 (0.29)	417.9±9.2 (0.50)	479.9 (0.46)	6.74 (1.72)

[a] Cultures as usual p-FPA culture grown for 12 hours in the presence of p-FPA.

[b] Total incorporation of ^{14}C-U-glycerol given in terms of cpm incorporated per ug cell dry weight.

[c] Incorporation of label into intracellular lipoteichoic acid (LTA_i) or extracellular LTA (LTA_x), in terms of cpm incorporated per ug cell dry weight. Plus and minus standard deviation provided.

[d] Ratio of LTA_x:LTA_i, using data of previous two data columns.

* Numbers in parentheses are relative to the mid-log control culture value.

since it was impossible to define a generation time for the stationary control culture, the cells could not be grown for a known number of generations in the presence of label. Rather, the label was present from the time of inoculation in order to circumvent this problem. It may be that the longer period of growth in the presence of label of the p-FPA grown and stationary phase control cultures (ca. 22 hours as opposed to ca. 12 hours for the mid-log control culture) did not allow direct comparison of the amounts of lipoteichoic acids present, even when compared on a dry cell weight basis.

At any rate, it may very well be significant that the ratios of extracellular to intracellular lipoteichoic acids of cells grown in the presence of p-FPA and of stationary phase control cells are higher than the ratio for mid-log control cells. Addition of lipoteichoic acid to cultures of Streptococcus pneumoniae conferred upon the cultures a resistance to penicillin-induced autolysis (Holtje and Tomasz, 1975). Although it is speculation, it may be possible that release of intracellular lipoteichoic acid to the culture medium (now extracellular) by cells grown in the presence of p-FPA and by stationary phase control cells could result in inhibition of autolytic activity. Again, it appears significant that cells grown in the presence of p-FPA gave results quite similar to those for stationary phase control cells.

Other Effects of Growth in the Presence of p-FPA

Assay of Aspartase Activity

One way in which p-FPA could act to inhibit growth would be to inhibit utilization of aspartic acid, a major carbon and energy source and sole nitrogen source present in the aspartic acid-glucose defined medium used in these studies. It has been concluded by Grula et al. (1968) that the ultimate basis of inhibition of cell division, induced by D-serine, in Erwinia carotovora lies at the metabolic level. Therefore, an inhibition of utilization of aspartic acid by cells grown in the presence of p-FPA could be involved in the effect of p-FPA on growth and cell division. For this reason, aspartase activity (this activity being an integral step in utilization of aspartic acid) was determined in mid-log control cells and in cells grown in the presence of p-FPA.

As seen in Figure 19, aspartase catalyzes the release of ammonia from aspartic acid, with fumarate as the end product. Detection and determination of the amount of ammonia released by the action of aspartase on aspartic acid was used as an assay of aspartase activity. Cells grown in the presence of p-FPA showed a 10.7% decrease in aspartase activity when compared with similar results for mid-log control cells (Figure 19).

It does not seem likely that a 10.7% decrease in

Figure 19. The Effect of Growth in the Presence of p-FPA on Aspartase Activity. A. Aspartase activity of mid-log control cells (control) and of cells grown in the presence of p-FPA (p-FPA). B. Reaction catalyzed by aspartase. Standard deviations, for nanomoles ammonia released per µg cell dry weight, range from 0.0052 to 0.0142.

A. ASPARTASE ACTIVITY: pFPA vs. CONTROL CELLS

$$\underline{\text{pFPA}} = 0.192 \frac{\text{nanomoles } NH_4^+ \text{ released}}{\mu\text{g cells (dry weight)}}$$

$$\underline{\text{CONTROL}} = 0.215 \frac{\text{nanomoles } NH_4^+ \text{ released}}{\mu\text{g cells (dry weight)}}$$

DIFFERENCE OF 10.7%

B.

ASPARTIC ACID FUMARIC ACID

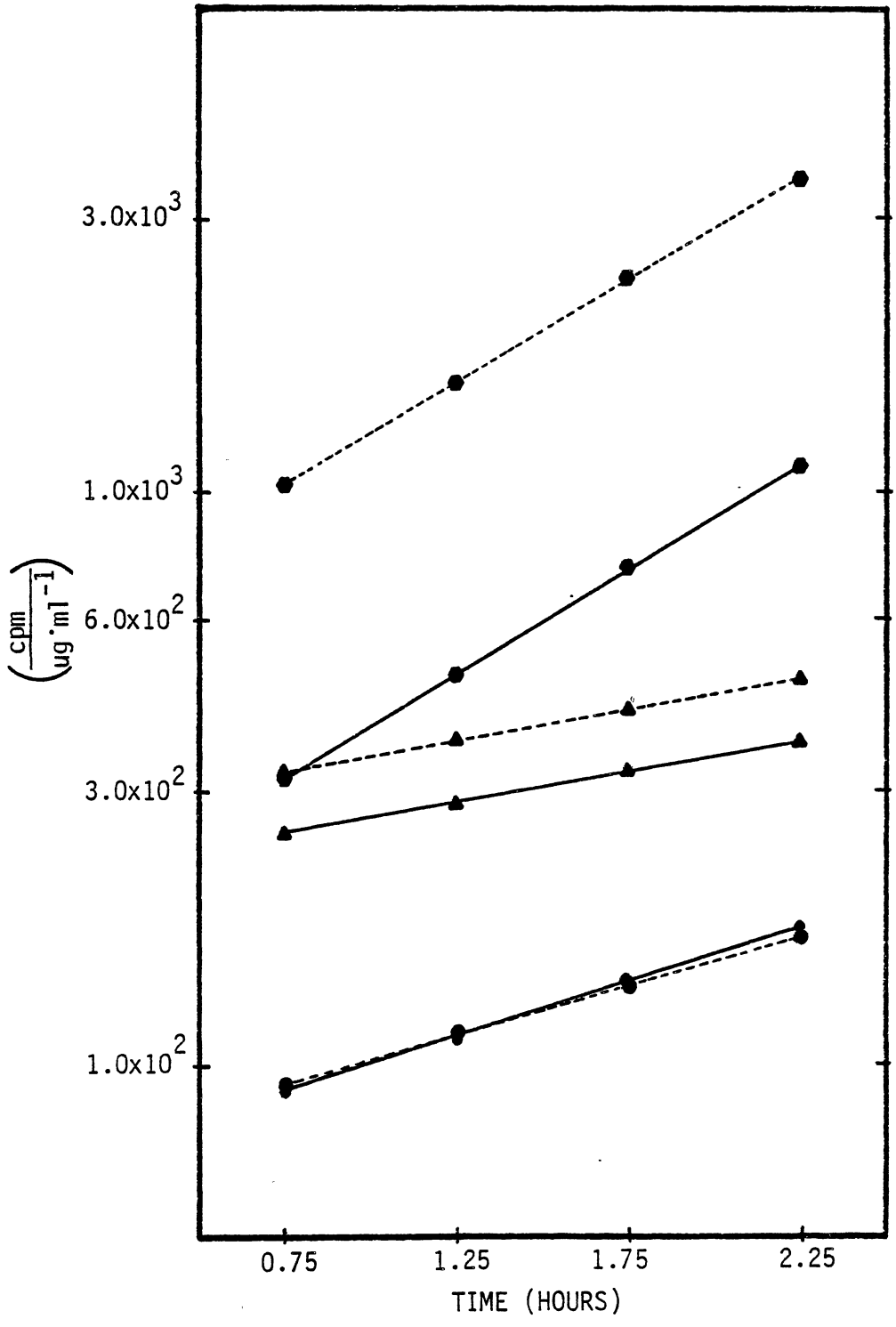
aspartase activity could result in the gross decrease in growth rate seen when cells were grown in the presence of p-FPA. Also, this minor decrease in aspartase activity did not appear to be sufficient to cause the marked changes in metabolism such as those that are observed when Erwinia carotovora is grown in the presence of D-serine.

Effects of Growth in the Presence of p-FPA on Synthesis of Macromolecules

It has been shown that DNA replication and cell division of bacteria are strictly regulated and the timing of these two processes correlated such that each daughter cell receives a copy of DNA following cell division (Jacob et al., 1963; Inouye and Pardee, 1970; Shapiro et al., 1970). Treatments which interfere with DNA replication have been found to inhibit cell division of E. coli (Inouye and Pardee, 1970) as well as Erwinia carotovora (Grula and Grula, 1962). Binkley and coworkers have found that p-FPA inhibits DNA replication in E. coli by inhibiting initiation of DNA synthesis (Carpenter and Binkley, 1968). p-FPA has also been shown to inhibit protein synthesis in E. coli (Previc and Binkley, 1964). Therefore, measurements of the rates of synthesis of macromolecules by Bacillus sp. #4 were made.

The data of Figure 20 show the effect of growth in the presence of p-FPA on the rates of synthesis of DNA, RNA, and protein. Comparison of the two curves for synthesis of each

Figure 20. Effect of Growth in the Presence of p-FPA on Synthesis of Macromolecules. Because the culture grown in the presence of p-FPA had a growth rate which was only 20% that of the control culture, a 1st derivative plot is presented. In this plot, the use of cpm/µg cell dry weight per ml, on the Y axis, normalizes the results in terms of the differing growth rates. Mid-log control culture (dashed lines); culture grown in the presence of p-FPA (solid lines). DNA synthesis (●); RNA synthesis (●); protein synthesis (▲). For each curve in figure, r=0.996, or better.


3.0x10^3
1.0x10^3
6.0x10^2
3.0x10^2
1.0x10^2
(cpm / ug·ml^-1)
0.75
1.25
1.75
2.25
TIME (HOURS)

of the macromolecules reveals the following: Cells grown in the presence of p-FPA synthesized DNA at the same rate, with respect to growth rate, as mid-log control cells (as indicated by the similarity of the slopes of the two curves). The fact that these two curves lie directly on top of one another indicates that cells from the two different growth situations contained the same amount of DNA per µg cell dry weight. Cells grown in the presence of p-FPA synthesized RNA at the same rate as mid-log cells, when the results were normalized for the difference in growth rates. The difference in height (along the Y axis) of the two curves for RNA synthesis indicates that mid-log control cells contained more RNA per µg cell dry weight than did cells grown in the presence of p-FPA (on the basis of radioacitvity incorporated per µg cell dry weight). Cells grown in the presence of p-FPA also synthesized protein at the same rate, with respect to growth rate, as did mid-log control cells. Again, the difference in the heights of the two curves indicates that mid-log control cells contained more protein per µg cell dry weight than did cells grown in the presence of p-FPA.

The difference in amounts of RNA and protein per µg cell dry weight for cells from the two different growth situations has already been demonstrated by fractionation of radiolabelled cells (Table VIII) and the data from this experiment confirm those results. The important finding of this experiment is that cells grown in the presence of p-FPA

synthesized DNA, RNA, and protein at the same rates as did mid-log control cells when normalized for the difference in growth rates. It can be concluded that cells grown in the presence of p-FPA for 12 hours are in a balanced state of growth with respect to synthesis of the macro-molecules examined. It can also be inferred that the inhibitions of growth, cell division, and autolytic activity induced by growth in the presence of p-FPA are not the result of an unbalanced state of growth in the presence of p-FPA.

The first-derivative plot (Figure 20) used to describe the rate of synthesis of each macromolecule was arrived at by Dr. Mary Grula (unpublished work). This plot, on semi-log paper, with cpm of label incorporated per µg cell dry weight per ml on the Y axis and time on the X axis, allows direct comparison of the rates of synthesis of macromolecules by cultures which are growing at different rates. The use of cpm per µg cell dry weight per ml on the Y axis normalizes the rate of incorporation with respect to the increase in culture biomass (growth rate). A similar method of plotting these types of data, arrived at independently by Friesen (1968) has been used to compare macromolecule synthesis by cultures growing at different rates. This plot places cpm on the Y axis and relative increase in culture biomass on the X axis, each plotted in scalar form. If the culture is in logarithmic phase and shows balanced growth, then both the incorporation of label and culture biomass increase exponentially, giving a straight line on a scalar plot.

CHAPTER IV

DISCUSSION

The purpose of this study has been to investigate the involvement of proteins in bacterial cell division. The phenylalanine analog para-fluorophenylalanine has been shown to be incorporated into protein in the place of phenylalanine (Yoshida, 1960; Richmond, 1963; Kang and Markovitz, 1967) and has been shown to inhibit cell division and growth of Escherichia coli (Previc and Binkley, 1964). It has been postulated that para-fluorophenylalanine acts to inhibit cell division by replacing phenylalanine in essential cell division proteins, thereby inactivating them, or altering their function (Hardy and Binkley, 1967). Therefore, this study has been focussed on detection and characterization of alterations in non-dividing cells made filamentous by growth in the presence of para-fluorophenylalanine.

para-Fluorophenylalanine has been shown to be a potent inhibitor of cell division and growth of Bacillus sp. #4. The phenylalanine analog is extremely toxic to growth and, if present from the time of inoculation of the culture, a concentration of as little as 0.5 μg/ml will limit growth of Bacillus sp. #4 to an absorbance at 540 nm of less than

0.100, even after 36 hours of growth. However, what few cells are present do show inhibited cell division, as these cells are filamentous.

Addition of para-fluorophenylalanine after growth has been initiated (early-log phase cultures) still result in inhibition of cell division, but the effects on growth are not nearly so great. Still, increasing concentrations of para-fluorophenylalanine, added in this manner, results in decreased growth, but not in increased filament lengths.

Growth of the organism begins to slow down shortly after addition of para-fluorophenylalanine and by five hours after the addition, the organism resumes logarithmic growth, although it does so at a rate which is only 20% that of the untreated control culture. An increase in cell length is first observed 90 minutes after addition of the analog. Thereafter, the organism continues to increase in length, with some cells as long as 50 μm after 14 hours growth in the presence of para-fluorophenylalanine.

Investigation of the effectiveness of some other phenylalanine analogs as inhibitors of cell division indicates that the effect of para-fluorophenylalanine on cell division is rather specific. For example, the fluoride moiety of para-fluorophenylalanine cannot be replaced with chloride; para-chlorophenylalanine has no effect either on cell division or growth. Further, use of ortho- and meta-fluorophenylalanines indicates that the fluoride moiety must be in the para position, as neither ortho- or meta-

fluorophenylalanine are able to inhibit cell division. These two analogs do, however, inhibit growth of Bacillus sp. #4, but do so without inducing the second log phase of growth which is characteristic of the inhibition induced by para-fluorophenylalanine.

Addition of phenylalanine to a culture at the same time as addition of para-fluorophenylalanine completely prevents the inhibitions of cell division and growth which normally are induced by the analog. Addition of phenylalanine just two hours after addition of para-fluorophenylalanine has no effect on the inhibition of cell division induced by the analog. Tyrosine is unable to prevent filamentation under any condition tested. The data suggest that phenylalanine, but not tyrosine, competes with para-fluorophenylalanine for incorporation into protein.

Pantoyl lactone is able to prevent filamentation induced by growth in the presence of para-fluorophenylalanine, but under no conditions is pantoyl lactone able to induce division of the filaments by reversing cell division inhibition. This result, and that of addition of phenylalanine two hours after addition of para-fluorophenylalanine (in which filamentation is not prevented), as well as the fact that cells begin to increase in length just 90 minutes after addition of para-fluorophenylalanine, indicate that the commitment to filamentation is made shortly after addition of the inhibitor. In addition, once the commitment to filamentation is made, the effect, on cell division, of

growth in the presence of para-fluorophenylalanine is irreversible.

Several experiments and observations indicate that growth of Bacillus sp. #4 in the presence of para-fluorophenylalanine causes an inhibition of the autolytic system of that organism. Older cultures of Bacillus sp. #4, grown in the presence of the inhibitor, contain a preponderance of cell wall ghosts. Growth of the organism in the presence of para-fluorophenylalanine prevents the antibiotic-induced autolysis, which, in a control culture, can be induced by penicillin G or polymyxin B. Autolysis of whole cells and walls of cells grown in the presence of para-fluorophenylalanine is significantly inhibited. On the basis of these results, and others, it is an obvious conclusion that growth in the presence of para-fluorophenylalanine leads to an inhibition of the autolytic system of Bacillus sp. #4.

Growth of the organism in the presence of ortho- or meta-fluorophenylalanine, which do not inhibit cell division, prevents autolysis of whole cells and cell walls in a manner similar to that induced by growth in the presence of para-fluorophenylalanine. Thus, it was a simple task to demonstrate that there is no correlation of the inhibitions of cell division and autolysis induced by growth in the presence of para-fluorophenylalanine. This conclusion is indeed disappointing. Data, obtained by Schwarz et al. (1969), have implicated the involvement of autolytic enzymes in the cell division process. However, in

the case of inhibition of cell division by para-fluorophenylalanine, the results indicate that the inhibitor acts by some mechanism other than the inhibition of autolytic activity, since inhibition of cell division and of autolytic activity appear to be separable effects of para-fluorophenylalanine. A more subtle mechanism of action of para-fluorophenylalanine, such as a specific inhibition of the portion of the autolytic system involved in cell division (which does not occur in the case of ortho- or meta-flourophenylalanine), cannot be ruled out, but this seems unlikely.

Growth of Bacillus sp. #4 in the presence of para-fluorophenylalanine has no significant effect on the enzyme aspartase. Further, it has been shown that growth in the presence of para-fluorophenylalanine does not alter the rates of synthesis of DNA, RNA, or protein. It can be concluded that cells grown in the presence of the analog are in a balanced state of growth with respect to synthesis of those macromolecules. Therefore, it can be inferred that the inhibitions caused by growth in the presence of para-fluorophenylalanine are not the result of an unbalanced state of growth.

The results which have been discussed thus far are merely descriptive, they describe the effects of para-fluorophenylalanine on cell division, growth, and some other cellular activities and define some of the parameters by which these effects can be controlled, but do not provide

data relevant to the mechanism by which the inhibitor acts. The mechanism by which para-fluorophenylalanine inhibits cell division remains obscure, at best. Clearly, research in this area must continue in the future, in the event that these investigations might provide a more lucid understanding of how filamentation is induced, and more importantly, the mechanism by which bacteria divide.

The remainder of this study has been concerned with elucidation of the mechanism by which para-fluorophenylalanine (and the other isomers of fluorophenylalanine) inhibits autolytic activity of Bacillus sp. #4. Several experiments have demonstrated that growth in the presence of any one of the fluorophenylalanines results in inhibition of autolytic activity. Cells grown in the presence of one of the fluorophenylalanines are resistant to antibiotic-induced cell lysis. Also, cells grown in the presence of one of the fluorophenylalanines are resistant to autolysis, as demonstrated by their greatly lowered ability to autolyze under conditions in which mid-log phase control whole cells will autolyze. Autolytic enzyme assays, using isolated cell wall preparations, have shown that walls prepared from cells grown in the presence of one of the fluorophenylalanines are resistant to degradation by native autolysins, in situ. Data confirming these findings have been obtained, using isolated enzyme assays. Crude autolytic enzyme extracts, prepared from walls of cells grown in the presence of one of the fluorophenylalanines, have greatly lowered ability to

degrade substrate cell walls.

Whole cell autolysis assays have shown that addition of one of the fluorophenylalanines to an early-log phase culture results in inhibition of autolysis within 1.75 hours after the addition. Addition of para-chlorophenylalanine to an early-log phase culture does not inhibit autolysis, indicating that only those phenylalanine analogs which slow growth of the organism are able to inhibit autolysis. It is significant, then, that a natural slowing of growth rate, as in the case of a control culture entering stationary phase, is accompanied by a loss of autolytic activity; control cultures retain autolytic activity throughout logarithmic growth, but abruptly lose activity upon entering stationary phase. The inability of stationary phase cells to autolyze is a common phenomenon, and has been demonstrated in Streptococcus pneumoniae, Streptococcus faecalis, and Bacillus subtilis (Mosser and Tomasz, 1970; Cornett et al., 1978; deBoer et al., 1982).

One way in which growth in the presence of para-fluorophenylalanine could act to inhibit autolysis would be to induce alteration(s) in the mucopeptide such that it can no longer serve as a substrate for the native autolysins. In fact, one difference between mid-log phase control cells and cells grown in the presence of para-fluorophenylalanine has been found. Growth in the presence of the analog results in an increase in the amount of cell wall per unit of cell dry weight, and electron microscopy

shows that these cells have thicker walls. However, other data indicate that walls prepared from cells grown in the presence of para-fluorophenylalanine, stationary phase control cells, and mid-log phase control cells are equally susceptible to degradation by the autolytic enzyme system extracted from mid-log control Bacillus sp. #4 cells and are also equally susceptible to degradation by lysozyme. Other experiments indicate that walls prepared from cells grown in the presence of para-fluorophenylalanine, mid-log control cells, and stationary phase control cells are identical in the basic composition of their cell wall mucopeptide. Thus, it has been concluded that the effect of para-fluorophenylalanine on autolysis is not the result of an alteration of the cell wall mucopeptide, either in its basic composition or in its ability to serve as an autolytic enzyme substrate.

Another way in which the fluorophenylalanines could act to inhibit autolytic activity would be through the specific loss of either of the autolytic enzymes which are present in the control cell autolytic system. However, the data presented herein indicate that both amidase and glycosidase activities are present in cells grown in the presence of para-fluorophenylalanine. Another experiment has demonstrated that the effects of various pH values on the rate of autolysis of mid-log phase control cells and of cells grown in the presence of para-fluorophenylalanine are remarkably similar. The data indicate that growth in the presence of para-fluorophenylalanine does not result in

specific loss of either autolytic activity. Rather, the data indicate an overall loss of autolytic activity, presumably the result of a lesser rate of both types of activity, or the result of lesser amounts of both amidase and glycosidase present in the cell walls.

Studies on the protein and autolysin content of cell walls indicates that cells grown in the presence of any one of the isomers of fluorophenylalanine and stationary phase control cells all contain less total protein in their cell walls (by comparison with walls of mid-log phase control cells). The data also indicate that walls of these autolytic-deficient cells contain far less extractable autolytic activity. In addition, the apparent specific activities of the autolytic enzyme systems are lower than the reduction expected as a result of the lesser amount of extractable protein present. Again, it is significant that cultures grown in the presence of para-fluorophenylalanine and stationary phase control cultures give remarkably similar results. Data similar to those for Bacillus sp. #4 have been obtained using Bacillus subtilis and Streptococcus pneumoniae (Williamson and Ward, 1981).

Incorporation of para-fluorophenylalanine into the autolytic enzymes could result in a lower rate of autolytic activity, rather than a complete inhibition of activity. However, the lack of a high rate of autolysis of stationary phase control cells indicates that an alteration of protein structure, caused by incorporation of para-fluorophenyl-

alanine, is not the only way in which autolytic activity can be lowered. The inhibited autolytic activity of cells entering stationary phase demonstrates that this loss of autolytic activity is, in all probability, a natural phenomenon which accompanies the slowing of growth rate.

Lowered autolytic activity could be the result of an inability to transport autolysins to the cell walls. However, all cell walls prepared from cells grown in the presence of one of the isomers of fluorophenylalanine have contained at least some autolytic activity. Also, filamentous cells, growing on a solid medium which contains starch and para-fluorophenylalanine, are able to hydrolyze the starch, indicating that the filamentous cells are able to produce extracellular amylase. Although the data are not conclusive, it is believed that inhibited autolysis is not the result of an inability to transport the autolytic enzymes across the cell membrane to the cell wall.

Lipoteichoic acids have been shown to inhibit autolytic activity, both in vivo and in vitro, in a number of genera and species of bacteria (Shockman et al., 1974; Holtje and Tomasz, 1975; Cleveland et al., 1975). It is now generally believed that lipoteichoic acids play a major role in control of autolytic activity (deBoer et al., 1982). Shungu et al. (1979) have shown that autolytic-deficient mutants of Streptococcus faecium have increased levels of both cellular lipids and lipoteichoic acids. These workers believe that the increased levels of lipoteichoic acids present in these

mutants is the primary factor resulting in inhibited autolytic activity of these cultures.

Experiments with Bacillus sp. #4 show that cells grown in the presence of para-fluorophenylalanine contain higher levels of cellular lipids (by comparison with mid-log phase control cells). However, cells grown in the presence of para-fluorophenylalanine and stationary phase control cells contain less total lipoteichoic acids than do mid-log control cells. The difference between the autolytic-deficient and autolytic-competent cultures lies in the ratios of extracellular to intracellular lipoteichoic acids. The autolytic-deficient cultures (para-fluorophenylalanine grown and stationary phase cells) have a higher ratio of extracellular to intracellular lipoteichoic acids. Although it is speculation, it is possible that release of intracellular lipoteichoic acids to the culture medium (now extracellular) by cells grown in the presence of para-fluorophenylalanine and by stationary phase control cells is the reason for the inhibition of autolytic activity of these cultures. Again, it appears significant that cells grown in the presence of para-fluorophenylalanine give results quite similar to those for stationary phase control cells.

After considering the data collected to date, it appears that the effect of growth in the presence of one of the isomers of fluorophenylalanine on autolysis is secondary in nature, that it is not the direct result of incorporation of fluorophenylalanine into the autolytic enzymes themselves

or into the enzymes involved in determination of cell wall structure. Rather, it is believed that the initial slowing of growth rate caused by addition of fluorophenylalanine to a culture mimics the inhibition of autolytic activity which occurs at the end of logarithmic growth (entry into stationary phase) in a control culture. This inhibition of autolysis in stationary phase cells is, presumably, the result of a normal mechanism of control of autolytic activity and lipoteichoic acids appear to play a major role in this phenomenon.

LITERATURE CITED

Brown, W. C. 1973. Rapid methods for extracting autolysins from Bacillus subtilis. Appl. Microbiol. 25: 295-300.

Buchanan, R. E. and N. E. Gibbons (ed.). 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Carpenter, C. and S. B. Binkley. 1968. Effect of p-fluorophenylalanine on chromosome replication in Escherichia coli. J. Bacteriol. 96: 939-949.

Champlin, F. R. and E. A. Grula. 1979. Noninvolvement of beauvericin in the entomopathology of Beauveria bassiana. Appl. Environ. Microbiol. 37: 1122-1125.

Chung, K. L. 1971. Thickened cell walls of Bacillus cereus grown in the presence of chloramphenicol: their fate during growth. Can. J. Microbiol. 17: 1561-156

Cleveland, R. F., J. V. Holtje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman. 1975. Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. 67: 1128-1135.

Cornett, J. B., B. E. Redman, and G. D. Shockman. 1978. Autolytic defective mutant of Streptococcus faecalis. J. Bacteriol. 133: 631-640.

Coyette, J. and G.D. Shockman. 1973. Some properties of the autolytic N-acetylmuramidase of Lactobacillus acidophilus. J. Bacteriol. 114: 34-41.

deBoer, W. R., F. J. Kruyssen, and J. T. M. Wouters. 1981. Cell wall metabolism in Bacillus subtilis subsp. niger: accumulation of wall polymers in the supernatant of chemostat cultures. J. Bacteriol. 146: 877-884.

deBoer, W. R., D. Meyer, C. G. Jordens, F. J. Kruyssen, and J. T. M. Wouters. 1982. Cell wall turnover in growing and non-growing cultures of Bacillus subtilis. J. Bacteriol. 149: 977-984.

Fan, D. P. 1970. Autolysin(s) of Bacillus subtilis as dechaining enzyme. J. Bacteriol. 103: 494-499.

Fan, D. P. and M. M. Beckman. 1971. Mutant of Bacillus subtilis demonstrating the requirement of lysis for growth. J. Bacteriol. 105: 629-636.

Fan, D. P. and M. M. Beckman. 1972. New centrifugation technique for isolating enzymes from large cell structures: isolation and characterization of two Bacillus subtilis autolysins. J. Bacteriol. 109: 1258-1265.

Fan, D. P. and M. M. Beckman. 1972. Ultrastructural studies on a mutant of Bacillus subtilis whose growth is inhibited due to insufficient autolysin production. J. Bacteriol. 109: 1247-1258.

Fein, J. E. and H. J. Rogers. 1976. Autolytic enzyme-deficient mutants of Bacillus subtilis 168. J. Bacteriol. 127: 1427-1442.

Fiedler, F. and L. Glaser. 1973. Assembly of bacterial cell walls. Biochim. Biophys. Acta. 300: 467-485.

Forsberg, C. W. and H. J. Rogers. 1974. Characterization of Bacillus lichenformis 6346 mutants which have altered lytic enzyme activities. J. Bacteriol. 118: 358-368.

Gerhardt, P. 1981. Diluents and biomass measurement, p. 504-507. In: P. Gerhardt, R. G. E. Murphy, R. N. Costilow, E. W. Nester, W. A. Woods, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D. C.

Glaser, L. and B. Lindsay. 1977. Relation between cell wall turnover and cell growth in Bacillus subtilis. J. Bacteriol. 130: 610-619.

Grula, E. A. 1960a. Cell division in a species of Erwinia. II. Initial observations relating to nutritional dependency. J. Bacteriol. 80: 369-374.

Grula, E. A. 1960b. Cell division in a species of Erwinia. II. Inhibition of division by D-amino acids. J. Bacteriol. 80: 375-385.

Grula, E. A. and M. M. Grula. 1962a. Cell division in a species of Erwinia. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. J. Bacteriol. 83: 981-988.

Grula, E. A. and M. M. Grula. 1962b. Cell division in a species of Erwinia. IV. Metabolic blocks in pantothenate biosynthesis and their relationship to inhibition of cell division. J. Bacteriol. 83: 989-997.

Grula, E. A. and M. M. Grula. 1964. Cell division in a species of Erwinia. VII. Amino sugar content of dividing and non-dividing cells. Biochem. Biophys. Res. Commun. 44: 341-346.

Grula, E. A. and R. L. Hopfer. 1972. Cell division in a species of Erwinia. XIII. Leakage from the peripheral area of filamentous cells. Biochim. Biophys. Acta. 255: 822-832.

Grula, E. A. and R. D. King. 1970. Inhibition of cell division in Micrococcus lysodeikticus dis-II. Can. J. Microbiol. 16: 317-324.

Grula, E. A. and R. D. King. 1971. Changes in cell membranes of dividing and non-dividing cells of Micrococcus lysodeikticus dis-IIp$^+$. Biochem. Biophys. Res. Commun. 44: 1356-1363.

Grula, E. A. and G. L. Smith. 1965. Cell division in a species of Erwinia. IX. Electron microscopy of normally dividing cells. J. Bacteriol. 90: 1054-1058.

Grula, E. A., G. L. Smith, and M. M. Grula. 1965. Cell division in a species of Erwinia. VIII. Amino acid composition of the mucopeptide in dividing and non-dividing cells. Can. J. Microbiol. 11: 605-610.

Grula, M. M. and E. A. Grula. 1962. Reversal of mitomycin c-induced growth and division inhibition in a species of Erwinia. Nature (London). 195: 1126-1127.

Grula, M. M. and E. A. Grula. 1976. Requirement for pantothenate for filament formation by Erwinia carotovora. J. Bacteriol. 125: 968-974.

Grula, M. M., R. W. Smith, C. F. Parham, and E. A. Grula. 1968. Cell division in a species of Erwinia. XII. A study of nutritional influences in D-serine inhibition of growth and division of Erwinia sp., and of certain specific sites of D-serine action. Can J. Microbiol. 14: 1225-1238.

Hanson, R. S. and J. A. Phillips. 1981. Chemical composition, p. 328-364. In: P. Gerhardt, R. E. G. Murphy, E. W. Nester, W. A. Woods, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D. C.

Hardy, C. and S. B. Binkley. 1967. The effect of p-fluorophenylalanine on nucleic acid biosynthesis and cell division in Escherichia coli. Biochem. 6: 1892-1898.

Heathcote, J. D., and K. Jones. 1965. The rapid resolution of complex mixtures of the naturally occurring amino acids by two-dimensional thin-layer chromatography on cellulose powder. Biochem. J. 97: 15.

Helmstetter, C. E. 1974. Initiation of chromosome replication in Escherichia coli. II. analysis of the control mechanism. 84: 21-36.

Herbold, D. R. and L. Glaser. 1975. Interaction of N-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. 250: 7231-7238.

Higgins, M. L. and G. D. Shockman. 1971. Procaryotic cell division with respect to walls and membranes. Crit. Rev. Microbiol. 1: 29-72.

Holtje, J. V., D. Mirelman, N. Sharon, and U. Schwarz. 1975. Novel type of murein transglycosylase in Escherichia coli. J. Bacteriol. 124: 1067-1076.

Holtje, J. V. and A. Tomasz. 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Nat. Acad. Sci. 72: 1690-1694.

Holtje, J. V. and A. Tomasz. 1976. Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical heterogeneity. J. Biol. Chem. 251: 4199-4207.

Horne, D., R. Hackenback, and A. Tomasz. 1977. Secretion of lipids induced by inhibition of peptidoglycan synthesis in streptococci. J. Bacteriol. 132: 704-717.

Hughes, R. C., P. J. Tanner, and E. Stokes. Cell Wall thickening in Bacillus subtilis. Biochem. J. 120: 159-170.

Inouye, M. and A. B. Pardee. 1970. Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of Escherichia coli. J. Biol. Chem. 245: 5813-5819.

Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28: 329-348.

Johnson, J. H.and E. A. Grula. 1980. Cell membrane phospholipids and their constituent fatty acids in dividing and non-dividing cells of Micrococcus lysodeikticus. Can. J. Microbiol. 26: 658-665.

Kang, S. and A. Markovitz. 1967. Derepression of alkaline phopphatase in E. coli by p-flourophenylalanine. J. Bacteriol. 94: 87-91.

Kessler, R. E. and G. D. Shockman. 1979. Precursor-product relationship of intracellular and extracellular lipoteichoic acids of Streptococcus faecium. J. Bacteriol. 137: 869-877.

King, R. D. and E. A. Grula. 1972. Condition of cell wall mucopeptide in dividing and non-dividing cells of Micrococcus lysodeikticus dis-IIp$^+$. Can. J. Microbiol. 18: 519-529.

Leduc, M., R. Kasra, and J. van Heijenoort. 1982. Induction and control of the autolytic system of Escherichia coli. J. Bacteriol. 152: 26-34.

Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mauck, J. and L. Glaser. 1970. Turnover of the cell wall of B. subtilis W-23 during logarithmic growth. Biochem. Biophys. Res. Commun. 39: 699-706.

Mauck, J. and L. Glaser. 1971. Turnover of the cell wall of Gram-positive bacteria. J. Biol. Chem. 246: 1820-1827.

Morse, D. and B. L. Horecker. 1966. Thin layer chromatographic separation of DNS-amino acids. Anal. Biochem. 14: 429-433.

Mosser, J. L. and A. Tomasz. 1970. Choline-containing teichoic acid as a structural component of preumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. 245: 287-298.

Newton, B. A. 1956. The properties and mode of action of the polymyxins. Bacteriol. Rev. 20: 14-27.

Park, J. T. and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of Staphylococcus aureus. J. Gen. Microbiol. 22: 249-258.

Pooley, H. M. 1976a. Turnover and spreading of old wall during surface growth of Bacillus subtilis. J. Bacteriol. 125: 1127-1148.

Pooley, H. M. 1976b. Layered distribution, according to age, within the cell wall of Bacillus subtilis. J. Bacteriol. 125: 1139-1147.

Previc, E. P. and S. B. Binkley. 1964. Slow exponential growth of Escherichia coli in the presence of p-fluorophenylalanine. Effect of the analog on aromatic biosynthesis. Biochim. Biophys. Acta. 87: 277-290.

Rana, R. S. and H. O. Halvorson. 1972. Method of restricting incorporation of radioactivity from ^{3}H-thymidine into deoxyribonucleic acid only during outgrowth of spores of Bacillus cereus T. J. Bacteriol. 109: 599-605.

Rhuland, L. E., E. Work, R. F. Denman, and D. S. Hoare. 1955. The behavior of the isomers of α, ε diaminopimelic acid on paper chromatography. J. Amer. Chem. Soc. 70: 4844-4846.

Richmond, M. H. 1963. Random replacement of phenylalanine by p-fluorophenylalanine in alkaline phosphatase(s) formed during biosynthesis by E. coli. J. Mol. Biol. 6: 284-294.

Rogers, H. J. 1970. Bacterial growth and the cell envelope. Microbiol. Rev. 34: 194-214.

Sandler, N. and A. Keynan. 1981. Cell wall synthesis and initiation of deoxyribonucleic acid replication in Bacillus subtilis. J. Bacteriol. 148: 443-449.

Schwarz, U., A. Asmus, and H. Frank. 1969. Autolytic enzymes and cell division in Escherichia coli. J. Mol. Biol. 41: 419-429.

Shapiro, B. M., A. G. Siccardi, Y. Hirota, and F. Jacob. 1970. On the process of cellular division in Escherichia coli. II. Membrane protein alterations associated with mutations affecting the initiation of DNA synthesis. J. Mol. Biol. 52: 75-89.

Sharon, N. and S. Seifter. 1964. A transglycosylation reaction catalyzed by lysozyme. J. Biol. Chem. 239: 2398-2399.

Shockman, G. D., L. Daneo-Moore, and M. L. Higgins. 1974. Problems of cell wall and membrane growth, enlargement, and division. Ann. N. Y. Acad. Sci. 235: 161-197.

Shungu, D. L., J. B. Cornett, and G. D. Shockman. 1979. Morphological and physiological study of autolytic-defective Streptococcus faecium strains. J. Bacteriol. 138: 598-608.

Steinberg, W. and H. O. Halvorson. 1968a. Timing of enzyme synthesis during outgrowth of spores of Bacillus cereus. I. Ordered enzyme synthesis. J. Bacteriol. 95: 469-478.

Steinberg, W. and H. O. Halvorson. 1968b. Timing of enzyme synthesis during outgrowth of spores of Bacillus cereus. II. Relationship between ordered enzyme synthesis and deoxyribonucleic acid replication. J. Bacteriol. 95: 479-489.

Strominger, J. L. and J. M. Ghuysen. 1967. Mechanisms of enzymatic bacteriolysis. Science. 156: 213-221.

Tomasz, A. 1979. The mechanism of the irreversible antimicrobial effects of penicillins: How the beta-lactam antibiotics kill and lyse bacteria. Ann. Rev. Microbiol. 33: 113-137.

Williamson, R. and J. B. Ward. 1981. Deficiency of autolytic activity in Bacillus subtilis and Streptococcus pneumoniae is associated with a decreased permeability of the wall. J. Gen. Microbiol. 125: 325-334.

Yoshida, A. 1960. Studies on the mechanism of protein synthesis: Incorporation of p-fluorophenylalanine into α-amylase of Bacillus subtilis. Biochim. Biophys. Acta. 41: 98-103.

Young, F. E. 1966. autolytic enzyme associated with cell walls of Bacillus subtilis. J. Biol. Chem. 241: 3426-3467.

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