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Turnover of Cell Walls in Microorganisms

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INTRODUCTION

The purpose of this article is to review cell wall turnover (see below for a definition) in various microorganisms. Emphasis is placed on the role of turnover in surface extension and cell wall assembly. In addition, characteristic features of turnover in different kinds of organisms are discussed. When possible, an analysis of the methods used in some turnover experiments is presented. Extensive reviews on bacterial cell walls have been published by Rogers et al. (129), Shockman and Barrett (138), and Park (117). Reviews on the surfaces of members of the genus *Bacillus* have been provided by Munson and Glaser (116) and Doyle and Koch (48). Additional reviews on surface stress theory and turnover (97–100) and bacterial surface extension (39, 134) are available. Mathematical analyses of various models for wall turnover have been published by Archibald (3), de Boer et al. (42–44), and Koch and Doyle (99). It is not our purpose to create redundant literature, but to provide, for the first time, a review devoted solely to cell wall turnover.

DEFINITION OF CELL WALL TURNOVER

Cell wall turnover is defined as any enzymatic process that leads to the excision of fragments from preexisting insoluble wall material. Necessary corollaries to the definition are that the enzymes must be produced by the microorganisms that are undergoing turnover or the enzymes must be produced by other organisms in coculture and that the microorganisms undergoing turnover must be viable. It is not necessary that the organism be growing. In contrast to the traditional view, it is not essential that wall turnover products be liberated into the surrounding medium. It is required, of course, that any wall materials released into the medium be replaced by metabolic processes.

Other words have been used to describe wall turnover processes. These include wall excision, shedding, and wall catabolism. Because the term *turnover* has been used so widely and for so long, it seems appropriate that it be retained to fit the definition described above.

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GENERAL CHARACTERISTICS OF AND ASSAY FOR CELL WALL TURNOVER

It has now been over a quarter of a century since cell wall turnover was first described in *Bacillus megaterium* (25, 26). The physiologic bases for the need for wall turnover are just now becoming understood. Wall turnover has been reported for gram-positive bacilli, gram-positive cocci, gram-negative rods, gram-negative cocci, blue-green algae (cyanobacteria), and yeasts. It appears that surface turnover is a much more common process than previously believed. Table 1 lists the genus and species for some microorganisms reported to exhibit wall turnover. The most extensively studied organisms are members of the genus *Bacillus*. Recent studies with *Escherichia coli*, especially by Goodell and colleagues, have served to define turnover more clearly in gram-negative rods.

Turnover may require only one enzyme (autolysin). It is thought that an *N*-acetyl-muramoyl-L-alanine amidase (amidase) is responsible for the turnover in *Bacillus subtilis* (111, 115). Structural determinations of the turnover products can be used to identify the autolysin causing turnover. For example, an amidase should give a product characterized by terminal L-alanine residues. An *N*-acetylmuramidase would give rise to turnover products containing reducing *N*-acetylmuramic acid.

Goodell and Schwarz (71) have established the following criteria (with our modifications) to show that cell wall turnover occurs in exponential cultures of *E. coli*. (i) Murein must be initially labeled with the appropriate probe. There must be no label in internal or external pools. Only then can intracellular and extracellular products of murein degradation be detected. (ii) The turnover products must be liberated at a constant and predictable rate during growth. (iii) The kinetics of appearance of turnover products in the culture medium should be distinct from the kinetics of uptake and secretion through the cytoplasmic membrane of cell wall metabolites. (iv) Any reincorporation of turnover products must be blocked or independently assessed. These criteria can be safely used for any bacterium and, with appropriate consideration of the structures of their respective cell walls, for algae and fungi as well.

Turnover is usually measured by the use of radioactive cell wall precursors. The precursor *N*-acetyl-D-glucosamine

TABLE 1. Some microorganisms reported to exhibit cell wall turnover[a]

Microorganism	Reference(s)
Acinetobacter sp.	148
Agmenellum quadruplicatum	47
Bacillus anthracis[b]	25, 26
Bacillus cereus	35
Bacillus megaterium	
Bacillus subtilis	112
Chlamydomonas reinhardii	160
Chlorella ellipsoidea	146
Escherichia coli	29
Lactobacillus acidophilus (plantarum)	14
Listeria monocytogenes	50
Myxococcus xanthus	41
Neisseria gonorrhoeae	77, 78
Neisseria subflava	107
Nocardia restricta	109
Proteus mirabilis	65, 66
Salmonella typhimurium	70
Saccharomyces cerevisiae	105
Staphylococcus aureus	128
Streptococcus mutans	107

[a] Only the original reports are cited in this table.
[b] Unpublished results of S. Ekwunife, J. W. Ezzell, and R. J. Doyle.

(GlcNAc) or *meso*-DL-diaminopimelic acid (DAP) is frequently used. After a suitable period of time, the culture may be rapidly filtered, washed, and suspended in fresh growth medium. Turnover products may then appear in the growth medium or the amount of label remaining cell associated may be determined. An alternative method followed by many laboratories is simply to chase the radioactive pulse with nonradioactive precursor and study the amount of label remaining in the cells as a function of time. It must be pointed out that, when pulse-chase experiments are undertaken, the chase must be at a concentration of at least 100 K_ms for the incorporation of the precursor. In many cases, this precaution has not been followed. Instead, the investigators add a high concentration of nonradioactive precursor and assume that an adequate chase has been made. Another factor involved in the choice of a precursor is ability to assess how effectively the microorganism can specifically insert the precursor into cell wall. For example, work in Chaloupka's laboratory (20, 22, 23, 28, 30) has shown that, although DAP is readily incorporated into the peptidoglycan of *B. megaterium*, a portion of it is decarboxylated to lysine, which is then incorporated into protein. In *B. megaterium*, as well as in some other bacilli, there may be significant protein turnover. The addition of lysine to the culture may repress or inhibit the DAP-decarboxylase (133). Figure 1 provides a summary of results describing the use of DAP as a precursor for bacteria which contain the diamino acid in their peptidoglycans. Finally, as regards the use of GlcNAc as a precursor for peptidoglycan, there are several precautions that must be considered. In *B. subtilis*, at least 95% of the GlcNAc pulse is incorporated into peptidoglycan and about 5% is incorporated into protein or other stable components. For short-term turnover experiments of 1 to 3 generations, the GlcNAc makes an excellent precursor. For longer experiments, such as those describing the turnover of cell poles, much of the label will be lost into the culture medium, but the label remaining cell associated can be mostly attributed to protein (115, 145). This is because there is very little protein turnover in *B. subtilis* (115; G. Kirchner, M. A. Kemper, A. L. Koch, and R. J. Doyle, Ann. Microbiol. [Paris], in press). It is therefore necessary to distinguish label found in peptidoglycan from that found in protein to accurately assess wall turnover during growth (Kirchner et al., in press). Doyle and Koch (48) have recently provided a brief discussion of the metabolism of GlcNAc in *B. subtilis* in a general review about autolysins and growth of the organism.

In certain streptococci or staphylococci, the peptidoglycan may be labeled with a radioactive amino acid such as L-lysine or glycine, respectively (128, 139). When these amino acids are used, it is necessary to fractionate the cell walls (or turnover products) from protein so that turnover can be quantitated.

In general, it is best to use only short pulse times (<0.1 generation) so that the protein pool is not enriched. Kinetics of incorporation of label and its subsequent turnover are also more easily interpreted by use of short pulses. The growth of

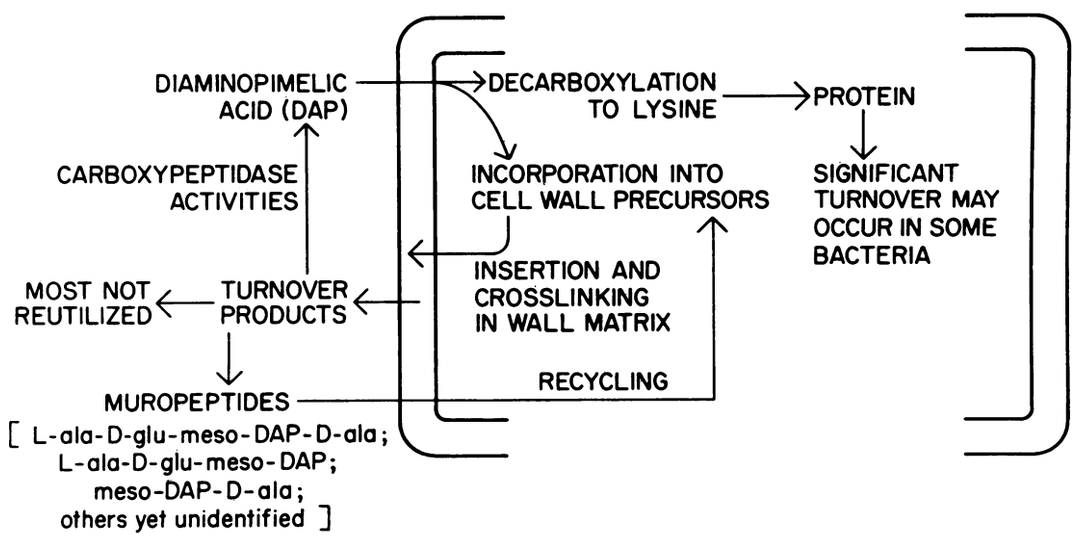

FIG. 1. Use of DAP as a precursor for cell wall in bacteria. Adapted from Chaloupka and Křečková (23), Goodell (67, 68), Goodell and Schwarz (71), and Goodell and Higgins (70).

B. subtilis, for example, in the presence of [^{3}H]- or [^{14}C]GlcNAc for five to six generations may provide a wall that is nearly uniformly labeled, but it also will provide a cell population which has a very high content of labeled protein.

In *B. subtilis*, it is unknown how radioactive GlcNAc could label proteins. Glycoproteins in the bacterium are unknown. Contaminants in the labeled GlcNAc may be one means of providing radioactivity to non-wall components. In addition, the deacetylation and subsequent deamination of GlcNAc may ultimately lead to fructose-6-phosphate, a readily utilizable sugar phosphate (7, 8, 31). Fructose-6-phosphate may yield storage carbohydrates, nucleic acids, and other cellular constituents.

Table 2 summarizes some of the characteristics of cell wall turnover in selected microorganisms. More extensive discussion of turnover of various cell types is provided in the following sections.

TURNOVER OF CELL WALL IN GRAM-POSITIVE BACILLI

In members of the genus *Bacillus*, growth processes seem to be directly coupled with cell wall turnover. Length extension in the bacteria is probably by an inside-to-outside growth mechanism (48, 99, 115, 123, 124). This mechanism allows only for growth at many sites in the cell side walls and rules out growth from a single zone as is found in streptococci (11, 79–81, 101, 102, 137). The surface stress theory (97, 98, 101) has provided one explanation for the inside-to-outside growth of the gram-positive bacilli which is compatible with experimental results. The theory states that new cell wall material is inserted at many sites in side walls. When the newly inserted wall material is cross-linked, it then assumes tension and can stretch. The addition of more underlying wall, and the subsequent stretching of the older wall, will result in elongation of the cylinder if the addition of new wall material is at enough sites. The outermost (oldest) wall is the most highly stressed, and it is this wall that is turned over. Turnover eases the tension in the outermost cross-linked peptidoglycan, thereby making it possible for new wall material to be "pushed" to the surface without breaking en route. It is important to consider that turnover may not be an absolute requirement for surface extension. The foregoing explanation of the coupling of turnover to growth requires that autolysin(s) clip two bonds which hold a segment of peptidoglycan into the wall. The clipping of a single bond reduces the surface tension on the outermost peptidoglycan, and the clipping of the second bond actually causes the turnover event. An analogy is the breaking of a string between two opposing forces. Once broken, the string is no longer under tension, but an additional cut in the string is required to free it from its anchor.

Cells deficient in autolysin turn their walls over slowly (54, 115, 156, 157) and also exhibit unusually rough or serrated surfaces (53, 54). Enough autolysin may be present in these mutants to cleave only the most stressed, and therefore highly sensitive, bonds. To date, mutants completely lacking in autolysin have not been described. It should also be pointed out in this regard that wild-type *B. subtilis* cells have outer cell wall surfaces that are rougher in appearance than the inner wall faces (142, 143). This could be due to breakage by turgor or the actions of autolysins on the most peripheral wall or both.

When radioactive GlcNAc is pulsed and chased in *B. subtilis*, its fate can be followed in at least three phases. The first phase is defined as the time required for the label to be cross-linked and exteriorized into the wall. This lag phase requires about a single generation and is characterized by metabolic stability. The second phase consists of a rapidly turning over cylinder compartment in which approximately 50% of the label is turned over per generation. The final phase consists of a slowly turning over compartment, probably the cell pole. Actual turnover experiments yield results that must be corrected for the label in the various compartments. Koch and Doyle (99) have provided equations that can be used in computer programs to estimate the contributions of each compartment to turnover. In these calculations, it is essential to know the length of the pulse, the generation time of the culture, and the extent of loss of radioactivity from the cells during growth.

Two papers by Howard Pooley (123, 124) were essential to the present understanding of cell wall turnover and wall assembly in *B. subtilis*. Pooley found that, regardless of pulse length, turnover rates were nearly constant (at least for the rapidly turning over compartment). Pooley furthermore found that only the wall near one generation of age was turned over by endogenous autolysin or removed by excess exogenously added autolysin. This led to the concept of "layering," in which very new wall is the innermost layer and wall of about one generation or older is the outermost, turnover-susceptible layer. An equally important view developed by Pooley was that wall material "spread" as it aged. This explained why turnover of wall pulsed for a very short time was equal in rate to turnover of wall pulsed for a relatively long time. Surface stress theory provides one explanation for the "spreading" phenomenon (99).

The view that cell cylinders turn over at a relatively rapid rate, compared with cell poles, is now widely accepted. A series of clever experiments from Archibald's laboratory has been critical in establishing the pattern of wall turnover in *B. subtilis* (1–4, 32, 145). It is known that some bacteriophages of *B. subtilis* bind to only teichoic acids and not to teichuronic acids. When cultures of the organism are limited in phosphate, teichuronic acid synthesis occurs. Therefore, periods of phosphate sufficiency followed by phosphate deficiency can result in cells initially able to adsorb phage particles but later unable to bind the viruses. Archibald and Coapes (4) applied continuous-culture techniques to study the distribution of bacteriophage SP50 binding sites on the surface of *B. subtilis* W23. Phosphate-limited bacteria did not adsorb phage SP50. When the cells were switched to phosphate sufficiency and limiting potassium, teichoic acid synthesis quickly ensued. When the teichoic acid-containing wall had become about one generation old, bacteriophages were seen to adsorb to many sites on the cell cylinder, but not to old poles. In contrast, when cells were switched to potassium sufficiency and limiting phosphate, SP50 binding sites survived until the teichoic acid content became very low. The insight of Archibald and Coapes led to the following statements: "This newly incorporated material may be located initially at a level underlying the surface of the cell and may become exposed at the surface only during subsequent growth. Incorporation of new material may also proceed rapidly into the developing septa, but new wall material is incorporated into existing polar caps more slowly, or perhaps not at all." We now recognize the foregoing statements as essential for understanding the inside-to-outside growth concept (48, 94, 115). Archibald (2) and Anderson et al. (1) later used pulse-chase experiments in the chemostat to study the appearance or loss of bacteriophage binding sites in *B. subtilis*, but the same general conclusions outlined above were always obtained. In none of the work from

TABLE 2. Summary of results from turnover studies on various microorganisms

Organism	Results	Reference(s)
Agmenellum quadruplicatum	This blue-green bacterium turns over its cell wall at a rate comparable to that of members of the genus *Bacillus*. Turnover does not appear to be correlated with septation or with cell separation.	47
Bacillus megaterium	Turnover of cell walls proceeds proportionally to the growth rate. About 30–50% of prelabeled cell wall is released to the medium during one cell cycle. A portion of degradation products is trichloroacetic acid precipitable and is composed of peptidoglycan and teichoic acid. The other part is the end product of a degradation pathway, e.g., DAP. Inhibitors of protein and peptidoglycan synthesis slow down the cell wall degradation. During the stationary phase and during most of the sporulation phases, cell wall turnover is negligible. Filamentous growth caused by a supraoptimal temperature results in a decreased peptidoglycan degradation. The lack of cell wall turnover in one strain has been reported.	
Bacillus subtilis	Newly inserted side wall requires about 1 generation before turnover commences. Turnover on poles is very slow, proceeding from the side wall-pole junction to the tip of the pole. Proteases reduce rate of turnover. Rate of turnover of wild-type cells is proportional to growth rate. During exponential turnover, approximately 50% of the side walls are shed each generation. Turnover is not markedly changed by exogenous lipoteichoic acid or phospholipids. Division-arrested or stationary-phase cells turn over walls very slowly. Turnover products are those expected by the actions of *N*-acetylmuramoyl-L-alanine amidases. Some turnover appears to occur in Lyt^- strains.	1–2, 4, 32, 34, 35, 42–44, 50–52, 64, 87–89, 94, 106, 110, 111, 113, 114, 123–125, 135, 136, 145, 153, 156–159, 169
Chlamydomonas reinhardii	This green unicellular alga possesses a cell wall rich in hydroxyproline-containing glycoproteins. As the cell enlarges, some of the glycoprotein is shed into the medium. The liberation of zoospores from the sporangia is accompanied by a second period of cell wall degradation and appearance of soluble hydroxyproline-containing glycoproteins.	160, 161
Chlorella ellipsoidea	Rigid and nonrigid components of the cell wall turn over during the cell cycle. Turnover is delayed by at least one cell cycle after incorporation of ^{14}C label in the cell wall.	146
Escherichia coli	Up to 8 % of the peptides of peptidoglycan may be lost into the medium per generation. These peptides (Fig. 1) may be reutilized to form new peptidoglycan precursors. Mutants unable to transport the peptides have been isolated. The rate of peptidoglycan turnover is up to 50% per generation, similar to *B. subtilis*, but most of the turnover products are reincorporated.	29, 68, 70–72
Lactobacillus plantarum (*acidophilus*)	In exponential cells, there is a loss of up to 30% of wall per generation. In pulse-labeled cells (<0.2 generation), there is a lag of about 2 generations before turnover commences. A fraction of wall (about 15%) does not turn over at all, even after several generations. Very short pulses (<0.08 generation) of wall precursor do not turn over. When amino acid-starved cells (thickened walls) are placed in amino acid-sufficient media, all of the wall becomes susceptible to turnover.	14, 38
Myxococcus xanthus	In exponential cultures of the organism, there is low turnover of cell wall. When myxospore formation is induced by glycerol, cell wall turnover exhibits an increased rate. Chloramphenicol prevents myxospore formation but only slowly reduces the rate of expected cell wall turnover.	41
Neisseria gonorrhoeae	Exponential cells turn over their cell walls at about 50% per generation. Turnover rates of 7–20% per generation have also been reported. An accessory polymer, similar to Braun's lipoprotein, may limit extent of turnover. Whether a true lag period is required before the initiation of turnover is unknown.	69, 72, 76–78, 132, 141
Saccharomyces cerevisiae	Cell wall-bound mannoproteins have been reported to appear in the culture fluids during growth. Only a small amount of cell wall mannoprotein appears to become susceptible to turnover, and then only shortly after incorporation into the insoluble wall. Periplasmic mannoproteins, which possess high turnover rates, may be precursors for wall mannoproteins.	105, 119

Continued on following page

TABLE 2—*Continued*

Organism	Results	Reference(s)
Staphylococcus aureus	For wild-type cells the extent of turnover is about 25% per generation. Newly added wall and aged wall were claimed to turn over at the same rates, although Blümel et al. (12) stated that there was about a 0.5-generation delay after a [^{3}H]GlcNAc pulse before release of label began. Turnover occurs in both the exponential and stationary phases of growth. Protein synthesis inhibitors reduce turnover. Turnover products are highly immunodominant. Upon thickening of wall induced by chloramphenicol and removal of the antibiotic, old wall is sloughed off first. Sodium polyanethol sulfonate "liquoid" greatly reduces wall turnover, but does not inhibit growth. The liquoid presumably binds strongly to cell wall teichoic acids, thereby reducing the activity of autolysins on the wall substrate.	11–13, 128, 167, 168
Streptococcus mutans	Turnover does not occur in regularly growing cells. Fluoride anion, at noncidal concentrations, induces turnover of exponentially growing cells.	107

Archibald's laboratory were there indications of zonal growth or wall assembly. The results showed the tight coupling between wall growth and wall turnover, a relationship which took some time to understand. Now other independent methods have been employed, using both Lyt$^-$ and Lyt$^+$ strains of *B. subtilis*, to describe not only side wall turnover, but also cell pole turnover (115; Kirchner et al., in press).

Recently, Kirchner et al. (in press) observed in a temperature-sensitive phosphoglucomutase mutant of *B. subtilis* that concanavalin A-reactive sites (glucosylated teichoic acids) disappeared very slowly from cell poles during growth at the nonpermissive temperature. The pattern of loss of concanavalin A receptors revealed that wall material very near the side wall-pole junction disappeared first and then proceeded slowly to the very tip of the pole. Some cells were observed with poles still having the ability to bind the lectin after at least 15 generations. Similarly, when the cells were shifted from a phosphate sufficiency to phosphate limitation, loss of the capacity to bind bacteriophage was very slow on the cell poles but quite rapid, as expected, on side walls. A composite view of the results for turnover of the cell wall of *B. subtilis* is shown in Fig. 2. What is not completely clear is how poles can remain so resistant to turnover. Fein and Rogers (54), Rogers et al. (130), and Vitkovic (157) have shown that autolysins may be passed from cell to cell and that low amounts of the enzymes may be found in the growth media. According to Fréhel and Ryter (59), the released autolysins of *B. megaterium* are responsible for splitting large trichloroacetic acid-insoluble cell wall fragments containing peptidoglycan as well as teichoic acid. It would be expected that the autolysins could bind to cell poles and thereby initiate turnover. Koch and Burdett (98) have suggested that cell poles are not under as much tension as side walls and are therefore more resistant to turnover. Doyle and Koch (48) and Kirchner et al. (in press) have speculated that poles may have much less autolysin than side wall, because the side walls are assembled by an inside-to-outside and diffuse mechanism, whereas the pole is assembled solely from a completed septum (the thickness of the completed septum is more than twice as thick as the thickness of a side wall or end wall [9, 10, 16]). This requires a type of compartmentation for the secretion of autolysins which is not presently supported experimentally. Work is now in progress to assay the concentrations of autolysins over various parts of the cell surface of *B. subtilis*.

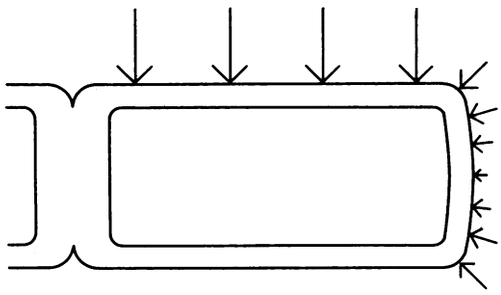

FIG. 2. Sites of turnover of cell wall in *B. subtilis*. The short arrows at the cell pole tip reflect a low rate of turnover, whereas the longer arrows reflect higher rates of wall turnover.

Any kind of enzymatic process (or processes) responsible for excising wall materials and either reutilizing or replacing the turned-over product must be finely regulated. Various bacteria seem to have various methods for the regulation of autolysins (39, 48, 129). In *B. subtilis*, the proton motive force appears to be one component of the regulatory mechanism for autolysins. Conditions which deplete the proton motive force (anaerobiosis, starvation for carbon source, or addition of metabolic poisons) result in lysis of the organism (88). Stimulation of the proton motive force (by oxygenation, addition of carbon source, or removal of poison) results in cessation of the uncontrolled autolysis (88). Exactly how proton motive force can regulate autolysin(s) is uncertain. It may be that, as cells with energized membrane secrete protons, the protons become bound to the cell wall. The wall matrix would then assume a pH dictated by the pKs of the groups binding the protons. These groups would likely be carboxylate and phosphate. The pH of the wall matrix would then be low enough to reduce the activity of the autolysins. Kemper, Koch, and Doyle (submitted for publication) have recently found that levansucrase of *B. subtilis* does not become active until about one generation (the time for inside-to-outside wall growth) after induction by sucrose. It may be that the levansucrase is also held in the low-pH wall matrix until it can be pushed to the surface by normal wall growth. Compounds, such as phospholipids and lipoteichoic acids, do not seem to markedly alter the rate of wall turnover in *B. subtilis* (115; R. J. Doyle and L. K. Jolliffe, unpublished data). When autolysin is pushed to the cell periphery by inside-to-outside wall growth, it may be sufficiently shielded from the influence of the energized membrane that it becomes active. This argument has been used to explain how gram-positive rods maintain constant thicknesses of cell walls, how the bacteria can split their septa right down the

middle, and the origin of wall bands (96) in splitting septa (96, 103). Other explanations of the above are possible and even likely, but at present none are more attractive.

In *B. subtilis*, regulation of turnover at an additional level has been recognized. Mutants unable to secrete extracellular protease demonstrate higher turnover rates than wild-type protease-sufficient cells (87). Protease-hyperproducing strains, in contrast, exhibit a reduced rate of wall turnover compared with the wild-type cells. Furthermore, when a protease inhibitor such as phenylmethylsulfonyl fluoride is added to the medium of wild-type cells, turnover rates of cell wall increase. Further experiments suggested that autolysin is partially protected from proteolysis when bound to the cell wall, but soluble autolysin is highly susceptible to inactivation by proteases. The results suggest that any autolysin released from growing cells may be rapidly inactivated. Protease-digested autolysin may then serve as a source of amino acids for the cell (48). Vitkovic (157, 158) has suggested that secreted autolysin may function to kill other, possibly unrelated bacteria. Because members of the genus *Bacillus* are generally soil bacteria, it would be of interest to determine the effects of the autolysin(s) of *B. subtilis* on other bacilli. However, it seems that the inactivation of autolysins by extracellular proteolytic enzymes may not represent a general mechanism of their control. *B. megaterium*, in which cell wall turnover proceeds similarly to that in *B. subtilis* (22–24, 26), does not produce an extracellular serine protease, a probable candidate for the role of an inactivating enzyme. Moreover, the metalloprotease, which is the only exocellular protease synthesized by this organism, is excreted in substantial quantity during growth in a mineral salts-plus-glucose medium, in which the cell wall turnover proceeds at a rate of 30 to 50% per cell cycle. The repression of the protease formation by amino acids (27) has no effect on the extent of cell wall turnover.

Turnover experiments with other gram-positive rods have given results similar to those with *B. subtilis*. In *Listeria monocytogenes*, [1-^{3}H]GlcNAc was found to be a good label for peptidoglycan (50). The labeled wall could not be solubilized by hen egg white lysozyme, but was readily solubilized by mutanolysin. It was shown that about 25% of the label was lost into the medium per generation following a 0.5-generation pulse with the radioactive GlcNAc. In addition, there was nearly a one-generation lag prior to the initiation of turnover and turnover markedly slowed after three additional generations. The results suggested that about 27 to 30% of the label was "immune" to turnover. Earlier, Boothby et al. (14) and Daneo-Moore et al. (38) observed that in *Lactobacillus acidophilus* (*plantarum*) a delay of over 1.5 generations was needed for turnover to begin after a pulse of about 0.2 generation. The results with both the *Listeria* and *Lactobacillus* turnover experiments are compatible with the inside-to-outside growth model (48, 97). Furthermore, it is likely that the fractions resistant to rapid turnover are cell poles. Surface stress theory offers an explanation for turnover, but there is no a priori requirement that a certain amount of wall be lost for each generation. Rather, the amount of wall excised each generation may depend on internal turgor, the amount of available autolysin(s), "accessory" polymers associated with the peptidoglycan, and the means of regulation of autolysin(s).

GRAM-NEGATIVE RODS

In 1972, Chaloupka and Strnadová (29) observed that in a DAP-requiring mutant of *E. coli* up to 15% of [^{14}C]DAP-labeled wall was lost into the growth medium over three to four generations. Control experiments established that some lysis occurred as the labeled cells were filtered, washed, and suspended in a fresh medium. The amount of radioactivity lost by lysis was much less than that observed to occur during growth. The experiment clearly demonstrated that DAP-containing materials were lost into the growth medium (turned over) during cell division processes. Since that time, there have been additional experiments documenting cell wall turnover in *E. coli* (68, 70–72). The work of Greenway and Perkins (72) made it clear that the glycan portion of the peptidoglycan also turns over, probably at a rate comparable to that of the muropeptides. These researchers labeled *E. coli* for 2 to 2.5 generations, centrifuged the cells, and then suspended the cells in fresh medium. Loss of label occurred from the cells at a rate of about 8% per generation. Control experiments established that the peptidoglycan was specifically labeled by D-[1-^{14}C]glucosamine.

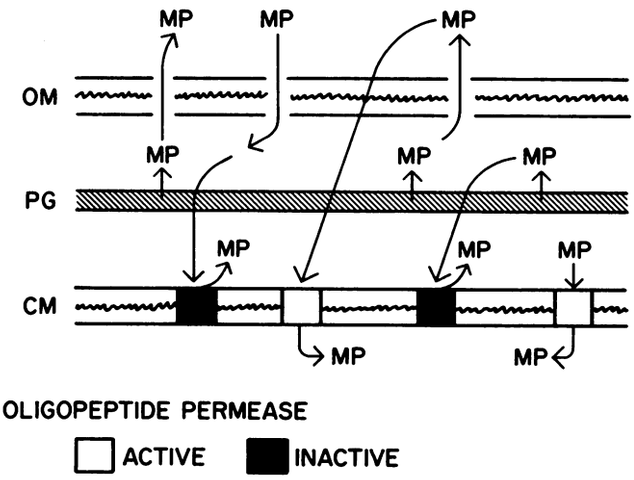

FIG. 3. Turnover of cell wall of *E. coli*. Adapted from Goodell (68), Goodell and Schwarz (71), and Goodell and Higgins (70). MP, Muropeptide; CM, cytoplasmic membrane; PG, peptidoglycan; OM, outer membrane.

The most extensive work on cell wall turnover in *E. coli* has come from Goodell et al. (67, 68, 70, 71). Goodell and Schwarz (71) found that at least three muropeptides (peptides from peptidoglycan) were released into the culture medium during growth: tetrapeptide (L-Ala-D-Glu-*meso*-DAP-D-Ala), tripeptide (L-Ala-D-Glu-*meso*-DAP), and a dipeptide (*meso*-DAP-D-Ala). Greenway and Perkins (72) were unable to show that the peptide components of the peptidoglycan turned over. Goodell and Schwarz (71) established that some of the muropeptides, once produced from peptidoglycan, penetrate the outer membrane and may or may not be reutilized. In absolute terms, about 50% of the peptides of peptidoglycan are excised per generation, but only approximately 8% of the excised muropeptides are found in culture supernatants. To produce the muropeptides from insoluble cell wall, several enzymes (periplasmic space location) are probably involved: the amidase (liberating a terminal L-Ala-containing peptide), endopeptidases, and a D-Ala carboxypeptidase. In addition, a DD-carboxypeptidase may be involved as well. It thus seems that several enzymes are involved in cell wall turnover in *E. coli*. Leutgeb and Schwarz (108) have previously suggested that *E. coli* possesses endopeptidases which act specifically on peptidoglycan.

A composite view of the turnover of cell wall in *E. coli* is shown in Fig. 3. During growth, muropeptides are excised from the peptidoglycan by specific enzymes probably located in the periplasmic space. The muropeptides may be modified further by additional enzymes (the tetrapeptide may be converted to the tripeptide, for example). Some of the muropeptides escape into the growth medium, but most are transported back into the cell via oligopeptide permeases. The cell is efficient in reutilizing excised muropeptides, as most of the peptides never escape through the outer membrane. We believe that turnover occurs as a result of the need for the cell to expand its surface. In *E. coli*, most of the turnover products are reutilized, but it is impossible to say whether or not turnover is creating new acceptor sites for growing peptidoglycan. This would require knowledge of the structure of any turned-over glycan components. What are the sizes of the glycan components that may be in the culture medium or in the periplasmic space? What are the reducing groups? Are anhydro-containing carbohydrate residues generated? The surface stress theory would welcome cell wall turnover in any bacterium, but it would also applaud the bacterium clever enough to reutilize turnover products.

Goodell and Higgins (70) used a peptide permease mutant of *E. coli* to demonstrate that turnover products may be reutilized. In the mutant, muropeptides are incapable of being transported into the cytoplasm and are therefore lost into the culture medium. Once in the cytoplasm, muropeptides may be attached to uridine diphosphate-muramic acid forming part of the precursors for new wall. The oligopeptide permease for the uptake of the muropeptides is not specific for cell wall peptides, but can transport a variety of peptides. Many transport systems in bacteria must be induced. The oligopeptide permease of *E. coli*, however, is constitutive. This would be expected if there was a constant need for the system during growth.

Results obtained by Goodell and Higgins for *E. coli* seemed to fit nicely with results from *Salmonella typhimurium*, suggesting that both bacteria exhibit cell wall turnover. Cooper (37), however, recently could find no evidence for wall turnover in *S. typhimurium*. Cooper found that the specific activity of radioactive labels for both protein and peptidoglycan decreased by the same rates during balanced growth. If wall turnover had been occurring, it would have been expected that the ratio of protein label to peptidoglycan label would increase with time. Cooper may have used a strain of *Salmonella* sp. which prevented the loss of turnover products through the outer membrane, resulting in the total reincorporation of turned-over wall components. The observations of Goodell and Higgins now mandate that, when wall turnover is studied in the gram-negative rod, consideration must be given to the effects of the outer membrane in the trapping of turnover products. We have become accustomed to thinking of the outer membrane as a structure which prohibits the entry of deleterious agents to the plasma membrane and cytoplasm. We must now consider that the outer membrane also keeps turned-over, but utilizable, molecules accessible to the cell and helps to conserve energy sources. This seems to be a heretofore unrecognized function of the outer membrane.

Growth of the cell cylinders of *E. coli* is by a diffuse process (17–19, 166) by which cell wall is added at many sites. The addition of wall in the cylinder requires that sites be available for the covalent attachment of new peptidoglycan and for cross-linkings. To our knowledge, a systematic study of turnover following pulse-labeling of cell walls in *E. coli* has not been done. It would be helpful to know whether recently inserted wall is as susceptible to turnover as wall a generation or more old. Goodell (personal communication) has shown that, in *Salmonella* spp., turnover commences within a few minutes of a pulse of labeled DAP. Furthermore, does glycan turn over at the same age as the peptides? Are poles resistant to turnover, as they are in *B. subtilis*? What are the characteristics of turnover-defective mutants? How are autolytic enzymes regulated in vivo in *E. coli* (there are several papers describing the activation of autolysins, but none bear directly on the turnover of wall components)? The studies of Goodell and colleagues have now made it possible to address the physiological role of wall turnover during growth of *E. coli* and *Salmonella* spp. A thesis in this review is that turnover occurs only on stressed (cross-linked) wall. In gram-negative rods, there is no possibility of an extensive inside-to-outside growth mechanism. The newly inserted and cross-linked wall segment may have the same probability for being turned over as very old wall, as all portions of the wall would be expected to be under the same degree of tension or stretch. The breaking of stressed peptidoglycan by autolysins, its solubilization by another autolysin, the insertion of new wall segments, and the subsequent reincorporation of excised wall may constitute a type of steady state required for orderly division processes in the gram-negative rod. In this sense, surface expansion of the gram-negative rod is akin to that of the gram-positive rod in which stretched peptidoglycan (by autolysins or by partial physical breakage as in the case of the Lyt^- phenotype) must be cleaved before growth can occur.

TURNOVER OF CELL WALL IN BACTERIAL COCCI

For bacterial cocci, two organisms, *Staphylococcus aureus* and *Neisseria gonorrhoeae*, have received considerable attention in studies relating to cell wall turnover. It appears that many of the factors governing turnover in *S. aureus* and *N. gonorrhoeae* may be similar to those for *B. subtilis*, although the bacteria have different modes of cell division. Unlike *Streptococcus faecalis* and other streptococci, *S. aureus* is more nearly a true coccus. *N. gonorrhoeae* has been described as coccobacillary (164). There is no compelling evidence (61, 151, 152) that either staphylococci or gonococci enlarge their surfaces by discrete growth zones. Both organisms exhibit a high rate of cell wall turnover.

Rogers (128) labeled the pentaglycine bridge in the peptidoglycan of *S. aureus* with [1-^{14}C]glycine and found that the label could be chased away by nonradioactive glycine. As far as is known, this is the first report on cell wall turnover in *S. aureus*. Later, Hisatsune et al. (82) were able to fractionate soluble peptidoglycan-teichoic acid complexes from culture supernatants of *S. aureus*. Interestingly, a turnover product, Glu-Lys-Ala-Gly-NH_2 (1:1:2:5:1), was found to be highly immunodominant in the cell wall. It is surprising that there is a paucity of literature on the role of turnover products in pathogenesis of microbial diseases. The release of muramyl peptides into tissues may provoke various immune reactions (5, 6, 13, 162).

In *S. aureus*, Wong et al. (167, 168) found that the wild-type bacteria turned over wall in both the exponential and stationary phases of growth. Therefore, unlike *B. subtilis*, the high rates of turnover are not correlated with surface expansion, but may be correlated with peptidoglycan synthesis. Wong et al. also found that both old and new wall turned over at the same rate. Loss of label followed pseudo-first-order kinetics until at least 90% of the label had been turned over. Furthermore, the rate of turnover of peptido-

glycan was identical to that of teichoic acid (63), a result that could have been predicted if the teichoic acid is covalently bound to the peptidoglycan and if the teichoic acid did not influence the autolysin. Wong et al. (167, 168) failed to use very short pulses (<0.1 generation) for their labeling procedures. In fact, in many of the turnover experiments reported by Wong et al. labeling times of up to several generations were used. The results from the turnover experiments contrast with those obtained from permitting the cells to autolyze in buffers (loss of carbon source may result in loss of protonic potential). Old wall is solubilized first in autolyzing cells and then is followed by new wall.

Gilpin et al. (62) found that when *S. aureus* was double labeled with [^{3}H]GlcNAc (steady state) and [^{14}C]GlcNAc (pulse) there was a higher rate of release of steady-state labeled wall, regardless of whether whole cells or cell walls were used. The foregoing results of Wong et al. (167, 168) and Gilpin et al. (62) contrast with those of Blümel et al. (12), who found that there was at least a 0.5-generation delay before turnover began after a pulse of [^{3}H]GlcNAc. The work of Giesbrecht and colleagues (61) has provided considerable insight into the mechanism of surface enlargement in *S. aureus*. A thick annular ring is laid down all the way through the cell. This ring or septum will split when the two daughter cells have achieved a critical size or volume. Discounting contributions by the developing septum, surface expansion in staphylococci may require diffuse addition of wall. Thus, the inside-to-outside growth of the wall would give rise to peptidoglycans most stressed on the cell periphery, where turnover would transiently alleviate the stress. The inside-to-outside growth would result in a lag before turnover began, consistent with the results of Blümel et al. (12). Blümel et al. (12) stated that their results indicated that " . . . newly synthesized wall material was placed layer by layer beneath the inner surface of the old wall." We believe that these results are consistent with turnover of exteriorized wall. There is a tacit assumption that autolysins are somehow regulated until they reach the surface by being pushed along with wall materials. In staphylococci, lipoteichoic acids may have a role in regulating the autolysins (55, 75), although the lysis of carbon-starved *S. aureus* suggests that proton motive force may also play a role in cellular autolysis (62) (see also reference 163). In addition, a prediction based on surface stress theory is that the outer wall would be much rougher than the inner wall face (97, 100, 102). In this regard, Umeda et al. (152) recently observed that the outer part of the cell wall of *S. aureus* possessed a "fuzzy coat." We believe that additional studies on the kinetics of wall turnover in *S. aureus* are warranted. The possibility, as suggested by Giesbrecht et al. (60) and Reinicke et al. (126), that autolysins are packaged into special compartments termed murosomes is interesting. This may mean that turnover is restricted to a few localized areas on the *S. aureus* cell surface, a view at odds with surface stress theory.

In *N. gonorrhoeae*, several reports show that the turnover of peptidoglycan occurs. Hebeler and Young (77, 78), Goodell et al. (69), Rosenthal (131), Sinha and Rosenthal (141), and Greenway and Perkins (72) have observed a time-dependent loss of labeled cell wall in various strains of *N. gonorrhoeae*. The work of Hebeler and Young suggests that the loss of wall material into the growth medium is of the order of 50% per generation. Goodell et al. (69) reported turnover rates of 7 to 29% per generation. Rosenthal (131) reported a turnover rate of 31 to 40% per generation. Greenway and Perkins (72) reported turnover rates to vary between 9 and 16% per generation, depending on the strain.

The work of Greenway and Perkins (72) seems to verify that only aged wall turns over in *N. gonorrhoeae*. They found that there was a delay of about 1 generation before pulsed radioactivity began to be lost into the growth medium. Furthermore, they observed that both O-acetylated and non-O-acetylated forms of peptidoglycan were susceptible to turnover. Because [^{3}H]glucosamine was used as a label for cell wall, the results showed that the glycan turned over.

N. gonorrhoeae has been described as a short rod, but never undergoes a rod-sphere-rod transition (164). The bacterium forms a complete septum and has a wall thickness typical of gram-negative bacteria (164) (see also reference 84). The evidence for a single growth zone, analogous to that of a gram-positive streptococcus, is not strong. Surface expansion may be by septum splitting and by diffuse addition of wall material at nonseptal sites. Surface stress theory (97, 102) would predict that newly cross-linked cell wall would be under tension and more readily cleaved by autolysins. It is interesting that there is a delay before turnover begins after the incorporation of wall label (131). The inside-to-outside migration of wall in *N. gonorrhoeae* would not be expected to require 1 generation. It may be possible that newly inserted wall does not become cross-linked until it has aged. Furthermore, cell wall of *N. gonorrhoeae* would probably not be under as much tension as wall in a gram-positive species, possibly reducing a role of turgor in promoting turnover.

Work in Rosenthal's laboratory (131, 139, 141) has helped to define the nature of the turnover products in *N. gonorrhoeae*. Several autolysins may be required to generate soluble turnover products. Both *N*-acetylglucosaminidase and *N*-acetyl-muramoyl-L-alanine amidase activities have been implicated. The major turnover fraction consisted of *N*-acetylglucosaminyl-β-1,4-1,6-anhydro-*N*-acetylmuramyl-L-Ala-D-Glu-*meso*-DAP, whereas most of the remainder was composed of the corresponding disaccharide tetrapeptide containing a terminal D-alanine residue. The presence of anhydro-muramyl (nonreducing end) reflected the activity of a transglycosylase (hexosaminidase). As far as is known, there is no release of murein peptides, similar to those observed in *E. coli*. The biological role for the transglycosylase activity, which also occurs in some other gram-negative bacteria, remains obscure. It is unlikely that the anhydro-containing residues could be reutilized once they are shed into the culture medium. Anhydro-muramyl-peptide transport systems also have not been described.

We suggest that turnover is required for normal surface expansion, much in the same way that turnover accompanies cell growth in gram-positive bacilli. It would be helpful if a better understanding of the regulation of autolysins in gonococci were available. As far as we know, there are no mechanisms proposed for amidase or transglycosylase regulation in *N. gonorrhoeae*.

CELL WALL TURNOVER IN ORAL BACTERIA

Lesher et al. (107) reported that the addition of fluoride to growth media resulted in the loss of [^{14}C]GlcNAc or [^{3}H]lysine from cell walls of several oral bacteria. Labeled cells were washed and suspended in fresh media, and growth was monitored by measuring turbidities of the suspensions. Following suitable periods of growth, the cells were washed and fractionated by the Park-Hancock scheme (118). When noncidal concentrations (usually 5 mM) of fluoride were used, there was a loss of radioactivity from the cells.

Neisseria subflava, for example, exhibited a loss of up to 36% per doubling, whereas *Streptococcus mutans* exhibited losses of 16 to 56%, depending on the strain. Furthermore, when fluoride-grown cells were washed and suspended in dilute buffers, spontaneous lysis occurred. Lysis was observed for *Streptococcus sanguis*, *Bacteroides matruchotii*, *E. coli*, *Actinomyces viscosus*, *Streptococcus faecalis*, *Lactobacillus plantarum*, *Neisseria flavescens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. The authors did not report cell wall turnover for some of these bacteria. For *Streptococcus mutans*, in the absence of fluoride, there was no loss of wall during exponential growth (107).

Lesher et al. (107) showed that fluoride did not inhibit the synthesis of peptidoglycan. How fluoride could activate or enhance autolysins from such a wide variety of bacteria is unclear. Surely, there must be a common means for regulation of the autolysins (140). It is unlikely that salts activate the autolysins, because cell walls of many of the bacteria, when suspended in various buffer-salt solutions, do not autolyze. An increased cellular turgor pressure could play a role in the activities of autolysins (97), but there is no reason to suggest that fluoride would elevate the turgor of a cell. In fact, it seems more likely that the anion would decrease turgor. At present, there is no satisfactory explanation for the results reported by Lesher et al. (107) on fluoride-enhanced autolysis or cell wall turnover. For many of the bacteria, however, the loss of proton motive force due to fluoride (93) may explain enhanced cell wall turnover or cellular autolysis.

It is known that dental plaque may accumulate fluoride (40) and that plaque harbors a large number of different kinds of microorganisms. We suggest that, although fluoride may be effective in reducing dental caries, the anion may actually enhance inflammation of soft oral tissues. This speculation is based on the assumption that cell wall turnover products play a role in the development of periodontal disease (6). It would also be interesting to determine whether the bacillus-like morphology exhibited by *Streptococcus mutans* in low bicarbonate-high potassium media (147) has a requirement for cell wall turnover as predicted by surface stress theory.

CELL WALL TURNOVER DURING CELL DIFFERENTIATION IN PROCARYOTES

Sporulation of bacilli and the formation of myxospores in myxococci represent the typical examples of differentiation in procaryotic cells (57, 165). The development of the spore of a bacillus, its germination, and its postgerminative development are accompanied by distinct changes in the peptidoglycan surface layers, including their synthesis and degradation. Similar phenomena have been found in the myxospores.

In bacilli, the onset of stationary phase is characterized by a substantial decrease in the synthesis of cell wall material up to the first step of sporogenesis: formation of the asymmetric prespore septum (120–122). Simultaneously, the synthesis of the cell wall teichoic acid also decreases (88). However, a basal synthesis of the peptidoglycan is necessary in this stage, because penicillin and some other antibiotics interfering with the formation of the cell wall also inhibit forespore septation (83). The first increase in the rate of peptidoglycan synthesis occurs during the formation of the future germ cell wall (stage III of sporogenesis); the second indicates the synthesis of the cortical endogenous cell wall (stage IV) (154; Fig. 4A). This second "wave" of synthetic activity is lacking in mutants with a defect in

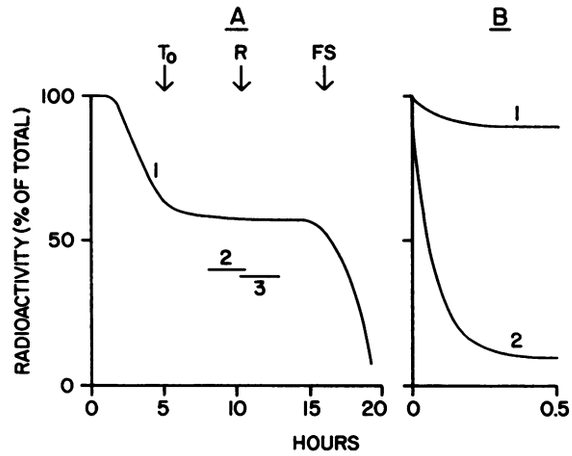

FIG. 4. Synthesis and degradation of peptidoglycan components during differentiation of bacilli. (A) Sporulation: 1, loss of radioactivity from the prelabeled cell during growth and sporulation; 2, period of synthesis of the germ cell wall; 3, period of cortex synthesis; T_0, end of growth phase; R, appearance of a refractile prespore in the sporangium; FS, release of mature spores. (B) Germination: 1, loss of radioactivity from the prelabeled germ cell wall and, 2, from the cortex. Adapted from Vinter (154, 155), Pearce and Fitz-James (120), and Chaloupka and Křećková (22).

cortex formation (120). A group of enzymes involved in the synthesis of the spore peptidoglycans, especially of the cortex, was found to be distinct from those participating in the formation of the cell wall of proliferating vegetative cells (144, 149, 150). Several enzymes presumably engaged in the synthesis of the germ cell wall or cortex, whose activity may be increased during sporogenesis, e.g., *N*-acetylmuramidase, D-glutamyl-*meso*-diaminopimelyl endopeptidase, and *N*-acetyl-muramoyl-L-alanine amidase, can be, however, involved in peptidoglycan hydrolysis as well, and their synthesis or activation or both may be related to the programmed autolysis of the sporangial compartment at the end of sporulation (73, 74, 95). This holds mostly for the amidase, which seems to be the dominant enzyme participating in cell wall degradation during growth as well as in lysis of the sporangium (33, 95).

The cell wall of the previously proliferating vegetative cell, converting into the sporangial cell wall during sporogenesis, either does not undergo turnover or undergoes it to a negligible extent only. Its components are not utilized for the synthesis of either spore germ cell wall or the cortical layer (30, 121, 154). The addition of decoyinine, an antibiotic inducing sporogenesis in the presence of nutrients, suppresses peptidoglycan turnover as well as the autolytic activity of the population (153).

The sporangial cell wall is degraded at the end of the maturation stage of sporogenesis only when the free spores are gradually released from sporangia. The autolytic enzyme split products of the sporangial wall are smaller than those found in the medium during growth of the culture (23). Unlike cell wall teichoic acid and peptidoglycan, the membrane-bound teichoic acid exhibits turnover during sporulation (86). It is not clear whether the two peptidoglycan moieties of the spore germ cell wall or cortex may undergo turnover during their synthesis, because the difficulties with equilibration between the external amino acids with their pool in the spore compartment make this question impossible to assess experimentally. The association of several

hydrolytic enzymes with the developing sporangium and spore indicates that some kind of turnover of spore envelopes might exist (74).

The germination of the bacterial spore is accompanied by a rapid degradation of spore cortex to relatively large fragments (85, 121, 155). Several enzymes participate in its hydrolysis. Some of them, e.g., amidase, are typical cell wall lytic enzymes involved also in peptidoglycan turnover during growth. Some of the enzymes which change the refractility of the permeabilized spore seem to be germination specific (56). The triggering of the cortex lysis may be a complex phenomenon involving also the action of a proteolytic enzyme (15). Unlike the cortex, the germ cell wall is degraded to a limited extent only, if at all, during germination and outgrowth of the spore (Fig. 4B). The slightly decreased radioactivity in the germ cell wall fraction, labeled during forespore development, can be due to the simultaneous degradation of a portion of cortex labeled concomitantly. Moreover, the degradation ceases before the switching on of the synthesis of new cell wall during the development of the germinated spore into a vegetative cell (121, 154). However, the occurrence of some turnover of the cell wall during the postgerminative development is supported by the finding that the loss of radioactivity in this fraction continues in the presence of penicillin, while in its absence the radioactivity in the cell wall slightly increases (155).

Unlike bacilli, myxococci represent the gram-negative type of procaryote capable of a complex cell differentiation (cytodifferentiation). The differentiation of the vegetative cell resulting in the formation of a myxospore can proceed via two pathways. One resembles the development of *Dictyostelium* sp. It is triggered by starvation and includes the aggregation of the cell population and the formation of a fruiting body. The other pathway proceeds at the "one cell" level and can be induced by glycerol (166). The latter is obviously more suitable for biochemical analyses, because aggregation of the cells occurring in the first pathway is accompanied by a massive lysis of the population.

Similar to *E. coli* and in contrast to bacilli, *Myxococcus xanthus* exerts only a low level of turnover of peptidoglycan during growth. The induction of the cell to differentiate into myxospores was accompanied by a substantial increase of cell wall turnover. This turnover was measured as the incorporation and release of labeled DAP to and from the peptidoglycan fraction (41). The autolytic activity of the cell population increased at the same rate (104). An increased peptidoglycan turnover and simultaneous sensitivity of the cells to autolysis was also characteristic for germination of the myxospores (91, 92).

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