Changes in Composition of Peptidoglycan during Maturation of the Cell Wall in Pneumococci

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An experimental system which allows the selective reisolation and structural analysis of a newly made (nascent) segment of pneumococcal peptidoglycan at various times after its incorporation into the preexisting old cell wall was developed. Age-related changes were observed in each one of the major nine wall peptide components resolvable by a high-performance liquid chromatography method. The nascent wall segment (made in 1.7% of a generation time) contained 60% of its peptides as the alanine-isoglutamyl-lysine tripeptide monomer, 12% as the directly cross-linked peptide dimer (tri-tetra peptide), and a total of 2% as the two major peptide trimers. In the mature wall segment reisolated 1 h later (1 generation time), the proportion of the tripeptide monomer dropped to 40%, while the major dimer and trimers increased to 23% and 8%, respectively. The age-related structural changes were completely inhibited by cefotaxime. The observations indicate that covalent bonds in the structure of pneumococcal peptidoglycan undergo substantial secondary rearrangements after incorporation into the preexisting wall. These changes are likely to be related to the movement of the conserved cell wall segments within the cell surface during cell division.

Streptococcus pneumoniae is a gram-positive coccus with a single wall growth zone located at the cellular equator, where newly made wall units of peptidoglycan and teichoic acid are added to the preexisting wall (2). The wall material becomes part of a conserved segregational unit, which then moves in a symmetrical fashion to the "left" and "right" of the cellular equator during the enlargement of the bacterial cell (25). Virtually nothing is known about the possible structural changes that may accompany these cell division-related movements of the cell wall units on the pneumococcal cell surface.

Previous studies with *Escherichia coli* (4), *Neisseria gonorrhoeae* (6, 16, 17), *Staphylococcus aureus* (21), and *Streptococcus faecalis* (5) resulted in data indicating that newly made peptidoglycan in each of these bacteria had a relative abundance of un-cross-linked stem peptides, some of which then became cross-linked in a maturation process that followed the incorporation of wall precursors into the cell wall. All these experiments were done by using radioactively labeled compounds in pulse-chase experiments. The results have unambiguously shown that the peptidoglycans of both gram-positive and gram-negative bacteria undergo secondary structural changes after their covalent linkage to the preexisting wall. Nevertheless, the existence of pools of unknown size of the radioactive compounds used and the phenomenon of cell wall turnover (which exists in most of the systems used) introduce elements of uncertainty into the qualitative interpretation of the pulse-chase experiments. Recent studies of *E. coli* indicate that peptidoglycan material released during turnover can be selectively reutilized for wall synthesis (15) and that these recycled compounds can enter the biosynthetic pathway closer to the fully formed precursors than the radioactive amino acids or hexosamines used in the pulse-chase experiments can.

Some unique features of the pneumococcal physiology allow an experimental system that can bypass these problems of interpretation to be defined. The cell wall of this

bacterium does not seem to undergo turnover (27), and in the case of pneumococci it is possible to design a method that allows a physical separation of newly made from mature cell wall segments which can then be compared for compositional differences by direct chemical analysis.

The method is based on the high degree of specificity of the pneumococcal autolysin (*N*-acetylmuramoyl-L-alanine amidase) in dissecting out wall segments with choline-containing teichoic acid chains from amid wall segments that contain ethanolamine (EA) teichoic acids and are resistant to the hydrolytic action of the amidase (19). In the basic experimental design, we grew pneumococci in EA medium. At a defined cell concentration these bacteria were exposed for a short (1-min) period to a pulse of choline added to the medium. Pneumococci growing in EA medium and manufacturing EA-containing (lysis-resistant) wall polymers are known to shift instantly to the synthesis of choline-containing teichoic acids upon the addition of choline to the medium (24). It is also known that the choline-containing teichoic acid units synthesized link up with peptidoglycan precursors synthesized at the same time and that the copolymers then incorporate into the cell surface to form a narrow segment of autolysin-sensitive wall located at the cell equator and surrounded on both sides by the old, EA-containing (autolysin-resistant) hemispheres of the cell wall (23, 25). One can isolate and purify these hybrid cell walls by the usual techniques (19). Upon incubation with the pneumococcal amidase, the newly made (choline-containing) wall material is selectively and quantitatively released, leaving the bulk of the cell wall preparation (composed of the old, EA-containing segments) intact. The family of stem peptides released is then put through a newly developed high-performance liquid chromatography (HPLC) analytical system that allows a high degree of resolution of individual peptides (9). A pictorial presentation of this method is shown in Fig. 1.

We used this technique to compare the compositions of newly made and mature peptidoglycan in pneumococci. Our results confirm the existence of a biphasic wall-synthetic process in the pneumococcus; the nascent peptidoglycan of pneumococci is rich in monomeric peptides, and the deficit

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FIG. 1. Design of the medium-shift (EA to choline) experiments. Pneumococcal cells grown in EA-containing medium were pulsed with L-[4,5- ^3H (N)]lysine and choline. The hybrid walls, composed of newly made (black area) and old (stippled area) segments, were digested with the amidase. Peptides were released only from the newly made portion of the wall. Undigested (old) walls were removed by centrifugation, and the peptides were released into the supernatant and analyzed by HPLC.

in cross-linked material is compensated for by a maturation process that follows the incorporation of subunits into the cell wall. The results suggest that the peptide dimers are formed in a heterogeneous-phase maturation reaction between monomeric tripeptide acceptors already polymerized into the cell wall and pentapeptide-containing cell wall precursors that act as donors.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The unencapsulated *S. pneumoniae* strain R6, a derivative of strain R36A, was used in all experiments (1). Bacteria were grown at 37°C in a chemically defined medium, Cden (A. Tomasz, Bacteriol. Proc., abstr. no. G87, p. 29, 1964), modified by reducing the concentration of lysine to 10 $\mu\text{g}/\text{ml}$ when radioactive label was used. Choline (5 $\mu\text{g}/\text{ml}$) was replaced in some experiments by EA (40 $\mu\text{g}/\text{ml}$).

Isolation and digestion of cell walls. Pneumococcal cell walls which were composed of hybrid wall segments with both choline- and EA-containing teichoic acids (see introduction; Fig. 1) (2 to 25 mg) were purified as described elsewhere (11) and were digested at 37°C for 24 h with pneumococcal autolysin (amidase) produced in *E. coli* CM21 (8). The amidase-catalyzed solubilization of choline-contain-

ing portions of the cell wall (Fig. 1) was measured by release of radioactive lysine. After enzyme digestion, 80 to 97% of the radioactive label became nonsedimentable, indicating almost complete solubilization of the peptidoglycan that was synthesized by the bacteria growing in choline-containing medium. The absolute number of counts per minute was never lower in hydrolysates of mature cells than in newly made cells, indicating a permanent association of teichoic acid and peptidoglycan which did not change during cell maturation.

Analytical HPLC. The chromatographic system consisted of two pumps, a 721 system controller, a U6K injector, and a 730 data module (Waters Associates, Inc., Milford, Mass.), and a Spectroflow 757 detector (Applied Biosystems, Inc., Ramsey, N.J.). The analytical column used was a Vydac 218TP54 (250 by 4.6 mm) (The Separation Group, Hesperia, Calif.) provided with a Newguard RP-18 precolumn (15 by 3.2 mm; Pierce Chemical Co., Rockford, Ill.). The chromatographic conditions were described earlier (9, 10).

Design of radioactive lysine pulse-chase experiment. Pneumococci were grown in choline-containing Cden medium with a low concentration (10 $\mu\text{g}/\text{ml}$) of lysine. At an optical density at 620 nm (OD_{620}) of 0.3, the culture received 0.5 μCi (1 $\mu\text{g}/\text{ml}$) of [^3H]lysine (L-[4,5- ^3H (N)]lysine; Du Pont, NEN Research Products, Boston, Mass.). The culture was rapidly chilled and washed, and half of the pelleted cells were used to prepare nascent cell walls. The other half of the pelleted cells was suspended in isotope-free medium, allowed to grow for one doubling time, and used for the preparation of mature walls. After separation of peptides by HPLC, the radioactivity of each collected peak was determined by scintillation counting.

Design of medium-shift experiments. Pneumococcal strain R6 was grown at 37°C in EA (40 $\mu\text{g}/\text{ml}$)-containing chemically defined medium (Cden). In order to introduce radioactive label into the cell walls, radioactive lysine (L-[4,5- ^3H (N)]lysine (0.5- μCi ; 1- $\mu\text{g}/\text{ml}$ final concentration) and choline (5 $\mu\text{g}/\text{ml}$) were added to the growth medium when the culture reached an OD of 0.3 (corresponding to about 3×10^8 CFU/ml). The culture was further incubated at 37°C for either 1 or 4 min (pulse). The temperature was thereafter rapidly (in 30 s) lowered below 5°C by pouring the culture into a container filled with crushed ice. The container was immersed in an ice-ethanol bath. The cells were harvested by centrifugation at 4°C. One-half of the pelleted cells (to be used for the analysis of newly made cells) were processed for cell wall preparation. The other half of the pellet was washed twice with 100 ml of ice-cold growth medium containing no amino alcohol component but a normal concentration of lysine (225 $\mu\text{g}/\text{ml}$). The cells were resuspended in the same medium, which was then supplemented either with choline (5 $\mu\text{g}/\text{ml}$) (chase condition A) or with EA (40 $\mu\text{g}/\text{ml}$) (chase condition B) as the amino alcohol component and grown at 37°C for an additional doubling time. In chase condition A, the cells continue to synthesize autolysin-susceptible wall, while in chase condition B the bacteria shift back to the production of autolysin-resistant (EA-containing) wall segments. After the chase period, the cultures were chilled and harvested, and mature cell walls were prepared as described above.

In some experiments, cefotaxime (0.1 $\mu\text{g}/\text{ml}$ [10 times the MIC]) was added to the cultures 5 min before the addition of choline and radioactive lysine. After the addition of choline and lysine, the culture was further incubated in the presence of the antibiotic for another 10 min before the bacteria were harvested for cell wall preparation. In order to prevent

FIG. 2. Localization of the newly made (nascent) wall segments at the cellular equator. Choline-pulsed, EA-grown pneumococci were treated with amidase as described in Materials and Methods. Arrows indicate the location of newly made (amidase-sensitive) wall segments that were removed by the enzyme.

possible loss of cell wall peptides during the antibiotic treatment, an isogenic autolysin-defective derivative of R6 (R6 lyt 4-4) was used in these experiments.

Topography of amidase digestion in pneumococci containing autolysis-susceptible nascent cell wall and autolysis-resistant mature cell wall. EA-grown pneumococci pulsed with choline were treated with amidase in buffer. After fixation and sectioning, the cell surface was examined by electron microscopy for sites of autolysin action (25). The site of wall hydrolysis was restricted to the equatorial zone of the bacteria (Fig. 2). These areas represent segments of the wall envelope where choline (and [^3H]Lys) incorporation occurred, leaving the EA-containing hemispheres of wall intact.

RESULTS

Pulse-chase experiments with radioactive lysine. In this study, traditional radioactive pulse-chase experiments without a shift in the amino alcohol content (EA to choline) of the medium were performed.

A culture of exponentially growing pneumococci in choline medium received [^3H]lysine in the medium. After 1

min of incubation the culture was rapidly chilled, and half of the cell pellet was immediately processed to isolate cell walls (preparation A). The other half of the cell pellet was suspended in fresh, nonradioactive medium and was grown for an additional mass-doubling period (chase), after which cell walls were isolated (preparation B). Both preparations A (representing radioactive label in the newly made [nascent] cell wall) and B (with radioactive label chased into the mature cell wall) were purified and hydrolyzed quantitatively with amidase, and the peptides were separated by HPLC. The distribution of radioactive lysine in the nine major peptide components was then quantitated, and the data are shown in Table 1. Striking compositional differences between the nascent and matured peptidoglycan are apparent. In the nascent wall (preparation A), most of the radioactive lysine (44%) was present in the monomeric peptide (peptide 1, a tripeptide composed of alanyl-isoglutamyl-lysine). The proportion of this peptide dropped to 28% during the chase period. In parallel, 16% of the radioactive lysine was present in the tri-tetra peptide dimer of the nascent wall, and this value rose to 29% by the end of the chase period. The percent distribution of radioactivity at the end of the chase

TABLE 1. Distribution of [³H]lysine in various cell wall stem peptides in pneumococcal R6 cells grown in choline-containing Cden

Cell wall preparation	Amt of lysine (% of total) in indicated peptide ^a											
	Monomers				Dimers					Trimers		
	1	2	3	Total	4	5	6	7	Total	8	9	Total
Nascent	44	6	3	53	16	9	6	9	40	4	3	7
Chased	28	3	3	34	29	12	9	6	56	6	4	10
Average ^b	26	2	5	33	24	13	12	6	54	7	5	12

^a Data are calculated from collected fractions (measured in counts per minute) corresponding to peaks 1 to 9 in a chromatogram (9).

^b Cells continuously grown in [³H]lysine-containing medium.

period closely resembled that of an average cell wall preparation in which bacteria were grown in the continued presence of [³H]lysine (Table 1).

Medium shift from EA to choline. The availability of the unique medium shift system for pneumococci has allowed us to test and possibly confirm the results of the radioactive pulse-chase experiment. Pneumococci were grown in EA-containing medium. At a culture of OD₆₂₀ of 0.3 (corresponding to 3×10^8 viable units per ml), choline was added to the growth medium. In some experiments, radioactive lysine was added along with choline. After exactly 1 min (or, in some experiments, 4 min) of incubation at 37°C, the temperature of the culture was rapidly lowered to below 5°C, and the cells were harvested by centrifugation at 4°C. Half of the pelleted culture was used for preparation of nascent walls; the other half was washed twice, resuspended in choline medium, and grown at 37°C for an additional doubling time before preparation of mature cell walls. Both newly made and mature cell walls were treated with amidase, and the peptides released were analyzed by high-resolution liquid chromatography. Five to twenty-five nanomoles of total peptide material was loaded on an HPLC column from each one of the following wall preparations: nascent walls synthesized during either 1 or 4 min of choline pulse, walls allowed to mature in choline medium for one mass-doubling time following a 4-min pulse of choline, and walls prepared from an exponential phase culture grown without pulse or chase in choline medium (average wall). The corresponding data are shown in Table 2.

TABLE 2. Relative amounts of structurally different stem peptides in the newly made and mature cell walls^a

Cell wall preparation	Amt of indicated peptide (% of total of peptides 1 to 9) ^b											
	Monomers				Dimers					Trimers		
	1	2	3	Total	4	5	6	7	Total	8	9	Total
1 min (choline)	60	4	2	66	12	12	5	3	32	1	1	2
4 min (choline)	47	6	5	58	14	13	8	3	38	2	2	4
Mature (4 min [choline])	40	5	2	47	23	12	9	3	45	4	2	8
Average ^c	32	2	7	41	22	14	10	5	51	4	4	8

^a Cell walls were radiolabeled with [³H]Lys or labeled and chased for various time periods (Fig. 1). Walls were prepared and analyzed as described in Materials and Methods.

^b UV absorbance data are expressed as percentages of the sum of the content of peptides 1 to 9. All of the identified peptides make up about 80% of the total peptide components of the peptidoglycan.

^c Cells grown in normal choline-containing medium.

MONOMERS

DIMERS

TRIMERS

FIG. 3. Structures of main wall peptides of pneumococcal strain R6 (9).

The results shown in Table 2 fully confirm the data obtained in the radioactive pulse-chase experiment of Table 1.

Double medium shift: EA to choline and back to EA. Examination of the data in Tables 1 and 2 poses a puzzle concerning the source of the peptide material in the dimeric stem peptides, particularly the tri-tetra peptide dimer (peptide 4), which virtually doubled in amount during the chase period. The tripeptide component of peptide 4 could have arisen from peptide 1 (the monomeric tripeptide), which accumulated in large excess in the nascent wall and then decreased rapidly during maturation. However, none of the monomeric peptides incorporated into the nascent wall has the appropriate chemical structure to provide a source for the dimeric compounds by simply cross-linking monomeric units that have already become part of the polymerized cell wall. The structures of the peptide components are shown in Fig. 3. Therefore, one must assume that the increase in dimer 4 (peptide 4) during maturation resulted from a biosynthetic cross-linking reaction between the monomeric tripeptide (already incorporated into the cell wall), acting as an acceptor, and some pentapeptide-containing wall precursor (which continued to arrive at the cell surface during the process of maturation), acting as a donor. If this were true, then one would expect continued incorporation of radioactive lysine into the nascent wall segments during the maturation process. A modification of the medium shift experi-

TABLE 3. Percent distribution of monomers, dimers, and trimers in medium shift experiments with an isogenic autolysin-defective derivative of *S. pneumoniae* R6

Cell wall preparation ^a	Amt of indicated peptides (% of total) ^b		
	Monomers	Dimers	Trimers
R6 lyt 4-4 (1-min pulse)	61	36	3
R6 lyt 4-4 (chased)	49	44	7
R6 lyt 4-4 (cefotaxime)	74	22	4
R6 lyt 4-6 (average) ^c	48	45	7

^a See Materials and Methods for details.

^b Data are calculated from HPLC chromatograms (9). Monomers, peptides 1 to 3; dimers, peptides 4 to 7; trimers, peptides 8 and 9.

^c Pneumococcal cells continuously grown in normal choline medium.

mental design visualized in Fig. 1 allows this hypothesis to be tested.

Pneumococcal cells were grown in EA-containing medium. At an OD₆₂₀ of 0.3, the culture was pulsed for exactly 1 min by adding choline to the medium. After 1 min the temperature was rapidly lowered to below 5°C; the cells were harvested at 4°C, washed twice, resuspended in EA medium, and grown for an additional generation time (60 min). Fifteen minutes before the end of this chase period (i.e., 45 min after the shift of the culture back to the EA medium), radioactive lysine was also added to the culture, which was incubated for the rest of the chase period (i.e., for a further 15 min); at the end of the chase period, the culture was harvested and cell walls were prepared. It was found that 336 cpm/nmol of glutamic acid residue (about 19% of the total radioactive material incorporated into the whole cell wall preparation) was released as amidase-sensitive wall material, indicating that this fraction of the radioactive lysine had incorporated into the choline-containing segment of the cell wall during the maturation process.

Inhibition of the maturation process by cefotaxime. A culture of the autolysin-defective pneumococcal strain R6 lyt 4-4 was grown exactly as described for the EA-to-choline medium shift experiment. Bacteria were grown in EA medium, and shortly before the culture would have reached an OD₂₆₀ of 0.3, cefotaxime was added (0.1 µg/ml [10 times the MIC]) to one half of the culture (the other half served as a control). Five minutes later, both the control and drug-treated cultures received choline, and incubation was continued. A portion of the control culture was chilled 1 min after the addition of choline (to prepare nascent cell wall); the other half of the control was allowed to grow for another doubling time before cell wall preparation, as described in Materials and Methods. The cefotaxime-treated culture was allowed to incubate for 10 min with the antibiotic and choline before the cells were harvested and the cell walls were prepared. The amount of peptide material incorporated into the cell walls during the choline pulse in control cultures and in cultures incubated with cefotaxime was estimated from the total amount of peptides released by amidase treatment. As much as 75% of the amount of peptides incorporated into the cell wall of the control culture (1 min) was found to have incorporated during the 10-min treatment with cefotaxime. Thus, wall synthesis seems to have continued to a substantial degree in the presence of the antibiotic. On the other hand, HPLC analysis showed that the chemical composition of this material was still that of a typical nascent wall, in spite of the prolonged (10-min) time of incubation (Table 3).

DISCUSSION

The results of our studies indicate that in pneumococci, as in other bacteria, there exists a cell wall maturation process that follows the polymerization of cell wall building blocks into the texture of the preexisting wall. The maturation process involves an increase in the cross-linking of the newly incorporated stem peptides. This type of general structural change, from a monomer-rich nascent wall with a relatively low level of cross-linking towards a more cross-linked mature wall, has already been described for the gram-positive *S. faecalis* (5, 20) and *Staphylococcus aureus* (21, 22) and the gram-negative *E. coli* (3, 4), *Proteus mirabilis* (13, 14), and *N. gonorrhoeae* (6, 16, 17). In *Staphylococcus aureus* walls, the compositional shift from monomer rich to oligomer rich was reported as complete in 3 min (21). In pneumococci the process appears to be slower. The proportions of monomeric, dimeric, and trimeric peptides were 66, 32, 2%, respectively, in the newly made (1-min pulse) pneumococcal wall. When the pulse period was extended to 4 min, a change in newly made material was observed. The monomer fraction decreased to 58%, the dimers increased to 38%, and the two trimers increased to a total of 4%. When the same 4-min wall segment was further aged for one generation and analyzed by HPLC, the monomeric fraction was further diminished to 47%, the dimers were increased to 45%, and the trimers were up to 8%. This distribution is close to the typical composition of the average cell walls of the strain tested (monomers, 41%; dimers, 51%; and trimers, 8%). Thus, about half of the compositional change (decline in monomeric peptides and increase in cross-linked peptides) was complete in 4 min, with the rest of the change having taken place during one doubling time (Table 2).

In staphylococci, the major peptide species participating in the age-related structural changes were peptide monomers (abundant in the nascent wall and decreasing during maturation) and the oligomeric peptides (increasing in abundance with time). The proportion of peptide dimers and trimers was reported to be unchanged (21). In pneumococci, the nascent wall was also high in peptide monomer, but the increase during maturation involved primarily peptide dimers and trimers.

Recently, high-resolution analytical techniques were introduced to the analysis of wall composition in some bacteria (7, 9, 12, 26). By using such techniques, it was shown that in *E. coli* nascent peptidoglycan is rich in pentapeptides and tetrapeptides (B. Glauner, Ph.D. thesis, University of Tübingen, Tübingen, Federal Republic of Germany, 1986). The chemical nature of the monomeric peptide(s) accumulating in newly made staphylococcal walls is not known, but presumably it includes tetrapeptides (22). In pneumococci, the proportion of individual stem peptides in the nascent and mature cell walls was estimated by integrating the areas under the peaks from HPLC chromatograms (9). In the newly made cell wall of strain R6 (after both 1- and 4-min pulses) the linear tripeptide (peptide 1 [alanyl-isoglutamyl-lysine]) made up approximately one-half of the total stem peptides. At the early stages of wall synthesis, two dimeric peptides were predominant, existing in equal amounts: the tri-tetra peptide with the serine-alanine interbridge (peptide 5) followed by the tri-tetra peptide (peptide 4) (Fig. 3).

In the mature wall, the percent distribution of stem peptides changed. The major monomeric tripeptide accounted for only 32 to 40% of the total stem peptides. Quantitatively, the highest amounts of the dipeptides were observed in the following fractions in decreasing order: the

tri-tetra peptide (peptide 4), the tri-tetra peptide with the serine-alanine interbridge (peptide 5), and peptide 6, which consisted of two tri-tetrameric peptides (one with a serine-alanine side chain and the other with an alanine-alanine interbridge). In the mature wall, the trimeric peptides (peptides 8 and 9) were also doubled in amounts compared with the newly made cell wall.

The substantial increase in the amount of the tri-tetra dipeptide during maturation raises the question of the material source of the tetrapeptide portion of these compounds. Both the radioactive pulse-chase experiments and the medium shift studies clearly showed the rapid movement of material from the monomeric tripeptide (which was the single most abundant component of newly made walls) to the tri-tetra peptide during the process of maturation. The decline in the quantity of tripeptide was roughly proportional to the increase in the quantity of peptide dimer. Thus, the most likely source of the tripeptide part of the dimer is the monomeric peptide 1. However, there were no peptides in the nascent wall that could account for the increase in the tetrapeptide component of dimer 4 by any of the known transpeptidation processes. The monomeric pentapeptide (peptide 2) was not present in sufficient quantities in the nascent cell wall. Furthermore, the concentration of this compound underwent, if anything, a minor increase (rather than the expected decrease) in concentration during the maturation process. Clearly, the source of the tetrapeptide component must be some peptide capable of acting as a donor in the transpeptidation reaction. Our data do not exclude the possibility that this donor is peptide 2 (the linear pentapeptide), which may have a high turnover rate in the nascent cell wall. Alternatively, a bactoprenol-linked disaccharide-pentapeptide precursor may serve as a direct reaction partner with the already incorporated (wall structure-bound) tripeptides acting as acceptors. The continued incorporation of radioactive lysine into already matured wall segments (see Results) is compatible with this suggestion.

We suggest that in pneumococci newly made peptidoglycan units incorporate into the cell wall initially through a relatively fast transglycosylase reaction, either as tripeptide monomers (i.e., converted from the pentapeptide to the tripeptide already on the precursor level) or as pentapeptides, which are then rapidly trimmed to tripeptides, through the action of D,D- and L,D-carboxypeptidases. Such a process of trimming should prime the newly made wall for equatorial growth, i.e., it should enrich the newly made peptidoglycan in peptides that can only serve as acceptors, while most of the peptide partners needed for cross-linking would arrive in a slower maturation process that may involve a heterogeneous-phase transpeptidation process between tripeptides (already incorporated into the cell wall) acting as preferred acceptors and wall precursor pentapeptides acting as donors. Such a mechanism should suit bacteria such as pneumococci in which wall enlargement occurs predominantly through zonal (equatorial) growth. This type of model has already been proposed to explain the basic mechanistic difference between septal and longitudinal wall synthesis in bacilli (18).

The biphasic nature of cell wall synthesis in pneumococci implies the sequential activity of at least two sets of enzymes, at least some of which may be penicillin-binding proteins (PBPs). The virtually complete inhibition of the maturation process by cefotaxime, a relatively poor inhibitor of PBP 2b, without a comparably severe inhibition of the formation of the nascent wall, suggests that PBP 2b may perform an important role in this process (i.e., the initial

incorporation of precursors into the wall). Such a role has already been suggested for PBP 2b on the basis of earlier studies (27).

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