Coordinated Incorporation of Nascent Peptidoglycan and Teichoic Acid into Pneumococcal Cell Walls and Conservation of Peptidoglycan during Growth*

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SUMMARY

Choline-containing pneumococcal cell walls are sensitive to autolysin, whereas ethanolamine-containing walls are not. Bacteria were labeled with radioactive peptidoglycan precursors while growing either in choline or in ethanolamine-containing media. Subsequently, the labeled cells were allowed to grow for four to five generations in nonradioactive medium supplemented with the alternative amino alcohol source (i.e., cells labeled in choline medium → ethanolamine; cells labeled in ethanolamine medium → choline). The autolysin sensitivity of the isotope label in cell walls prepared from such bacteria indicates that nascent peptidoglycan and teichoic acid units that are synthesized at the same time are attached to one another, incorporated into the cell surface at the cellular equator, and remain conserved during growth and division of the bacteria.

The cell wall of gram-positive bacteria is most frequently made up of two types of macromolecular components, a phosphorus-containing polysaccharide (teichoic acid) and the peptidoglycan (1). In pneumococci the corresponding macromolecules are covalently linked. One is a complex teichoic acid containing choline (in addition to glucose, ribitol, phosphorus, 2,4,6-tri-O-acetylated galactosamine) (2, 3). The other is a lysine-containing peptidoglycan (3).

The mechanism of enlargement of the cell envelope and its mode of inheritance during cellular growth and division are fundamental problems of cell biology. In previous work with the pneumococcal system it has been shown that choline-containing teichoic acids are incorporated into the cell surface at a limited number of sites that are located at the cellular equator (4). During growth, the choline-containing macromolecules move symmetrically to the "left" and to the "right" of the growth zone and are passed on to daughter cells intact (at least within the resolution of the autoradiographic method) (4).

In the present investigation, we addressed ourselves to two further questions. (a) Are nascent teichoic acid and peptidoglycan units incorporated into the cell wall in a coordinated fashion? (b) Is the peptidoglycan portion of the cell wall also conserved during growth?

In these experiments we exploited two features of the pneumococcal system. First, these bacteria have a nutritional requirement for choline which is located exclusively in the teichoic acids (5). Second, pneumococci growing on choline analogs (ethanolamine) synthesize cell walls that are resistant to the action of the pneumococcal autolytic enzyme, an N-acetylmuramyl-L-alanine amidase (3).

MATERIALS AND METHODS

The R36A strain of Diplococcus pneumoniae was used in all experiments. The composition of the chemically defined growth medium (Ca+) was described in an earlier publication (6). Growth of cultures was monitored by a Coleman nephelometer calibrated to allow estimation of the viable titer of pneumococci. Since ethanolamine-grown pneumococci form chains it was necessary to disperse such samples in order to obtain a more realistic estimate of the number of bacterial viable units. This was achieved by vigorous blowing of a 0.1 ml sample into the dilution medium (10 ml) followed by three stirrings on a Vortex mixer. Methods used for the preparation of pneumococcal cell walls, autolysin, and the assay of autolysin activity are available in the literature (5). Biosynthetic labeling of cell walls with various isotopes was carried out by growing the bacteria in the Ca+ medium supplemented with the appropriate radioactive compound(s). Detailed methods are described in the legends to the illustrations of the corresponding experiments. The following radioactive isotopes were purchased from New England Nuclear: L-[U-3H]lysine (NEC290), L-[6-3H]lysine (NET247), L-[3-3H]alanine (NET244), L-[U-3C]glutamic acid (NEC290), D-[U-3H]glucose (NEC582), [1,2-3H]ethanolamine (NEC068), and [methyl-3H]choline (NEC109). Radioactivity in cell wall and soluble fractions was determined by pipetting 100-μl portions onto GF/A (Whatman glass fiber paper) 2.4-cm filter discs; after drying (100°, 10 min), the discs were counted in toluene base scintillator liquid with the use of a Nuclear-Chicago Mark II spectrometer.

The composition of SFSH solution is 0.15 M NaCl, 0.01 M K2HPO4, and 3 mM 2-mercaptoethanol. The cytological procedure used for the preparation of thin sections was described earlier (7); the only modification was the introduction of an extra fixation step (2.5% glutaraldehyde in 0.1 M potassium phosphate buffer for 30 min at room temperature) prior to the osmium fixation. A Hitachi Perkin-Elmer type HU-11-C-1 electron microscope was used at 75 kv. Amino acids and amino sugars were determined with a Beckman model 190B automatic amino acid analyzer after acid hydrolysis (3). We are grateful to Dr. J. Manning of this university for these analyses.

Isotope Labeling of Pneumococci Immediately after Shifting Bacteria from Ethanolamine- to Choline-containing Medium—A 400-ml culture of pneumococcal precultured to the ethanolamine-
containing medium (by over 100 generations of growth in that medium) was used in this experiment. The growth of the culture (at 37°) was monitored with a nephelometer calibrated to allow the estimation of the viable titer of the bacteria. When the cell concentration had reached 9 x 10^9 bacteria per ml, the cells were removed from the ethanolamine medium by centrifugation (10 min at 10,000 X g) at room temperature and were resuspended in 400 ml of prewarmed (37°) Cd_4 medium lacking four normal constituents of this medium: glucose, alanine, lysine, and choline. The suspension was next divided into four portions (A, B, C, D), 100 ml each, and these were used for the biochemical labeling of pneumococci with the following isotopes: [U-14C]glucose (5 μCi and 100 μg per ml) in A, L-3-[H]alanine (2.5 μCi and 5 μg per ml) in B, [U-3H]lysine (2 μCi and 2 μg per ml) in C, and [methyL-3H]-choline (1 μCi and 0.14 μg per ml) in D. At the same time all media were supplemented with the normal concentrations of the lacking nonradioactive media components. Five minutes after resuspension in the choline- (and isotope-) containing media, 1-ml samples were removed and tested for deoxycholate-1 drop of 5% solution added) induced lysis; as expected, all four cultures showed complete clearing of the cell suspensions. Incubation of the cultures continued for 30 min at 37° during which time the viable titer of the four cultures increased to 1.4 x 10^10 cells per ml. At this time, the cultures were chilled on crushed ice; pH of the suspensions was lowered to 4 by the addition of 0.1 ml of 1 N HCl to each 100 ml of chilled culture) in order to suppress action of endogenous autolysin during harvesting of the cells. The cells were harvested by centrifugation in the cold at 9,000 X g for 10 min. After resuspension in 3 ml of 0.15 NaCl solution containing 0.05 M K_HPO_4, the cells were heated at 100° for 10 min and used for cell wall preparation.

RESULTS

Differential Autolysin Sensitivity in "Hybrid" (Choline- and Ethanolamine-containing) Cell Walls

The autolysin resistance of ethanolamine-containing pneumococcal cell walls (and the role of choline residues in lysis sensitivity) has been described earlier (3). For the purposes of the experiments to be described in this paper it was necessary to establish that the control of autolysin sensitivity by the amino acid residues was valid even in "hybrid" cell walls, i.e., in walls prepared from bacteria in which the choline- and ethanolamine-containing teichoic acids share the surface of the same individual cells. Table I shows that autolysin treatment of such hybrid walls results in the selective removal of choline label without virtually any loss of ethanolamine label.

Coordinated Incorporation of Peptidoglycan and Teichoic Acid into Cell Wall

Experiment 1—Pneumococci were grown in [14C]ethanolamine-containing medium. At an appropriate cell concentration the culture was divided in two parts; to one of the cultures [3H]choline was added (Culture B), while the other culture was undisturbed (Culture A). With the relative concentrations of ethanolamine and choline used, ethanolamine utilization is stopped and choline incorporation commences without detectable delay.

After 5 min of exposure to choline, Culture B regained complete sensitivity to detergent-induced lysis, while Culture A showed resistance to lysis, characteristic of ethanolamine cells (8). The larger portion of both cultures was used for the preparation of cell walls; smaller portions were treated with purified pneumococcal autolysin (containing about 2 x 10^8 cell equivalents of bacterial lysate in 1 ml). After determination of the amounts of [14C]ethanolamine and [3H]choline released by the autolysin treatment, the cells were fixed with glutaraldehyde and processed for serial sectioning and electron microscopic observation. The results of this experiment showed that autolysin treatment of choline-pulsed ethanolamine cells caused release of over 80% of the incorporated choline (11,000 cpm released out of a maximum of 13,000). In addition, these cells showed autolytic cell wall damage localized to the cellular equator (Fig. 1). There was no evidence for cell wall damage elsewhere on the cell surface. Neither [14C]ethanolamine release nor cytological wall damage was observable in the control (Culture A) cells. Since autolysin only attacks choline-containing portions of the cell walls (see Table I), the location of wall damage must represent sites of incorporation of new, choline-containing teichoic acid. It should be noted that in Fig. 1, the entire width of anatomically identifiable cell wall was removed by the autolytic "microsurgery" treatment, indicating that we are observing localized release of both teichoic acids and peptidoglycan from the cellular equator through the normal action of autolysin. After centrifugation, the supernatant solution (containing autolytic products of the choline-containing "nascent" cell wall) and the residue (containing the autolysin resistant, ethanolamine-containing cell wall) were analyzed (after hydrolysis) for amino sugar and amino acid composition. The results in Table II indicate that along with the choline the autolysin has released peptidoglycan also. No significant differences in the average composition of "nascent" and "old" cell walls are evident from the data.

Experiment 2—The design of this experiment was similar to the one in Experiment 1, with the exception that along with the choline, ethanolamine cells in Culture B also received radioactive amino acids (lysine and glutamic acid) capable of labeling the peptide portion of newly made cell wall. After 30 min of exposure to these amino acid tracers in choline-containing medium, the bacteria were harvested, cell walls prepared, and the purified cell walls were exposed to autolysin. Table III shows that each marker isotope was nearly quantitatively removed from the cell wall.
The details of this experiment were as follows. Glaser have designed an isotope-labeling strategy which allowed Pneumococci were labeled with [14C]lysine (A) or [14C]glutamic acid (B) for several generations. At a cell concentration of 5.5 × 10^7 cells/ml, radioactive choline ([methyl-3H]choline, 5 µg and 1 µCi per ml) was added to culture B, and incubation was continued for 5 min (representing about 8% of the bacterial generation time). Both cultures were chilled on ice, centrifuged (10,000 × g, 5 min), resuspended in 5 ml of SPSH, and the suspensions heated at 70° for 10 min. After 3 washings (with 10 ml of cold SPSH each time), the cells were resuspended in 5 ml of SPSH. Portions (100 µl) were used to determine incorporated radioactivity; 0.9 ml of each cell suspension was incubated with 0.1 ml of autolysin at 37° for 60 min. Half-milliliter aliquots of the incubation mixture were centrifuged (10,000 × g, 5 min), and the supernatants were used to determine released radioactivity. The rest of the autolysin-treated cell suspensions were fixed with glutaraldehyde and processed for electron microscopy.

Most of the heat-inactivated cell suspensions (4 ml) of cultures A and B were used for cell wall preparations (see Table II). Magnification: × 121,000.

walls by the enzyme treatment. This finding indicates that newly synthesized teichoic acids are attached exclusively to peptidoglycan units synthesized at the same time. Practically no nascent lysine or glutamic acid label gets associated with "old," ethanolamine-containing (and thus autolysin-resistant) portions of the cell wall.

Segregation of Peptidoglycan during Cell Division—Lysis-resistant (ethanolamine-containing) pneumococci were labeled in the peptidoglycan with amino acids, and retention of the label in the ethanolamine-containing portion of cell wall was tested after growth of the prelabeled cells in choline containing medium for four generations. The details of this experiment were as follows. Pneumococci were labeled with [14C]lysine (A) or [14C]glutamic acid (B) for several generations. At a cell concentration of 5.5 × 10^7 viable units per ml the culture was divided; most of the cells were used for cell wall preparation (A1 and B1); a smaller portion was washed free of ethanolamine and radioactive amino acids and was diluted 20-fold into choline containing growth medium free of isotopes. This culture was then allowed to grow in the choline medium for four generations after which cell walls were prepared (A2 and B2). Cell walls were compared for the amounts of incorporated amino acids and for the solubilization of these labels during treatment with autolysin. Table IV shows that there was no loss in the absolute amounts of radioactive glutamic acid or lysine during growth in the choline medium. The observed increase in the absolute amounts of radioactivity in preparations A2 and B2 is probably due to the continued incorporation of isotopes from cellular pools during transfer of ethanolamine cells to the choline medium (see experimental procedure in legend to Table IV). Table IV further shows the complete resistance of peptidoglycan label to autolysin even after four generations of growth of the cells in choline-containing medium.

These findings indicate that teichoic acid and peptidoglycan units incorporating into the wall at the same biosynthetic time become permanently linked with one another and are physically conserved during subsequent growth and division.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid and amino sugar composition of &quot;nascent&quot; and &quot;old&quot; portions of pneumococc cell walls</th>
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</thead>
<tbody>
<tr>
<td>Molar ratio</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Muramic acid</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Glucosamine</td>
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<tr>
<td>Galactosamine</td>
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</tbody>
</table>

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**DISCUSSION**

In an elegant series of experiments Glaser et al. examined the mode of cell wall assembly in *Bacillus subtilis* (9, 10). Exploiting the observation of Ellwood and Temppest (11) that at low phosphate concentration *B. subtilis* stops synthesizing teichoic acids and shifts to the production of teichuronic acid, Mauck and Glaser have designed an isotope-labeling strategy which allowed them to examine the addition of nascent teichoic acid and peptidoglycan units to the cell wall. Two important conclusions...
Autolysin sensitivity of peptidoglycan synthesized immediately after shifting ethanolamine cells to choline-containing growth medium

Total isotope incorporated into the cell walls (prepared as described in Experiment 2) was determined by pipetting 100-μl portions of the cell wall suspensions (in distilled water) onto paper filter disks (Whatman No. 3MM) and burning in the Oxymat isotope combustion apparatus (Intertechnique). The 3H2O and 14C from the combusted tritium- or carbon-14- (or both) containing cell walls was assayed by liquid scintillation counting with a Nuclear-Chicago Mark II spectrometer. 3H2O was counted in a mixture of 700 ml of dioxane, 300 ml of toluene, 20 g of naphthalene, and 7 g of butyl-2-(4-biphenyl-5-phenyl-1,3,4-oxadiazole). 14CO2 was trapped in a solution of 330 ml of phenylethylamine, 220 ml of methanol, 400 ml of toluene, and 50 ml of H2O containing 7 g of butyl-2-(4-biphenyl-5-phenyl-1,3,4-oxadiazole).

Other portions of the cell wall suspensions were treated with crude pneumococcal autolysin (0.1 ml of autolysin to 0.9 ml of cell wall suspension in saline, pH 6.5 to 7.0) for 3 hours at 37°. After centrifugation at 10,000 × g for 15 min, radioactivity in the supernatants was determined in 100-μl portions after combustion in the Oxymat (see above). Autolysin treatment (with fresh 100-μl portions of the enzyme) was repeated four consecutive times. There was no further isotope release into nonsedimentable form upon still further incubations with autolysin (up to eight consecutive autolysin treatments).

### Table III

<table>
<thead>
<tr>
<th>Compound used for cell wall labeling</th>
<th>Radioactivity in cell wall preparation</th>
<th>Radioactivity released by autolysin treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before autolysin treatments</td>
<td>After autolysin treatments</td>
</tr>
<tr>
<td></td>
<td>cpm (cpm)</td>
<td>cpm (cpm)</td>
</tr>
<tr>
<td>[U-14C]Glucose.</td>
<td>35,180 (100%)</td>
<td>3,040 (8.6%)</td>
</tr>
<tr>
<td>L-[3-H]Alanine.</td>
<td>3,480 (100%)</td>
<td>20 (0.6%)</td>
</tr>
<tr>
<td>L-[U-14C]Lysine.</td>
<td>215,400 (100%)</td>
<td>1,770 (0.9%)</td>
</tr>
<tr>
<td>[methyl-3H]Choline.</td>
<td>4,786,920 (100%)</td>
<td>186,960 (3.9%)</td>
</tr>
</tbody>
</table>

### Table IV

Conservation of peptidoglycan during growth of pneumococci

Two pneumococcal cultures (200 ml each) were grown in ethanolamine-containing C6H10O6 medium, supplemented with radioactive isotopes. Both cultures A and B had 40 μg per ml of ethanolamine; culture A was supplemented with 1 μCi and 2.5 μg per ml of L-[U-14C]glutamic acid, while culture B received 1 μCi and 2.5 μg of L-[U-14C]lysine. After five generations of growth in these media (from an initial cell concentration of 2.5 × 10⁶ cells per ml to a final concentration of 7.5 × 10⁸ cells per ml), each culture was divided in two parts: 100 ml were used for cell wall preparation (A1 and B1); another 100 ml were washed free of extracellular radioactivity, resuspended each in 2 liters of amino alcohol free growth medium, and incubated for 10 min at 37°. The media were then supplemented with choline (5 μg per ml), and the two cultures (A2 and B2) were allowed to grow for four generations in the choline-containing media, after which cell walls were prepared from each culture (see under “Materials and Methods”).

The four cell wall preparations (A1, A2, B1, and B2) were resuspended each in 2 ml of SPSH solution; after determination of the total incorporated 3H and 14C radioactivity (in 100-μl samples), the rest of the wall suspensions were incubated with 200-μl portions of standardized autolysin at 37° for 16 hours. After centrifugation (15,000 × g, 20 min), the amount of radioactivity released in sedimentable form was determined.

<table>
<thead>
<tr>
<th>Source of cell walls</th>
<th>Radioactivity released by autolysin treatments</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In cell wall before autolysin treatment</td>
</tr>
<tr>
<td></td>
<td>cpm per 100 μl</td>
</tr>
<tr>
<td>Cells labeled in ethanolamine medium with [14C]glutamic acid (A1)</td>
<td>3009</td>
</tr>
<tr>
<td>Cells labeled in ethanolamine medium with [14C]glutamic acid and grown for 4 generations in choline medium (A2)</td>
<td>3752</td>
</tr>
<tr>
<td>Cells labeled in ethanolamine medium with [3H]lysine (B1).</td>
<td>4702</td>
</tr>
<tr>
<td>Cells labeled in ethanolamine medium with [3H]lysine and grown for 4 generations in choline medium (B2).</td>
<td>4797</td>
</tr>
</tbody>
</table>

have emerged from these studies. First, newly made teichoic acid chains were attached to glycopeptide units made at the same time. Second, these authors have observed extensive randomization and turnover of cell wall components during growth of the bacilli.

In the experiments described in this paper, we have examined the same problems in a coccus-shaped bacterium, *D. pneumoniae*. In the design of our experiments we exploited two physiological properties unique to pneumococci, their nutritional requirement for a specific structural component of teichoic acid (choline) (5) and the fact that replacement of choline with certain choline analogs (ethanolamine) causes the production of autolysin-resistant cell walls (8). One of the conclusions of our studies is identical with the first conclusion in Mauck and Glaser’s study; in pneumococci too, teichoic acid and peptidoglycan units made at the same time become associated with one another. The second conclusion of our studies, however, shows a striking difference between the pneumococcal and *B. subtilis* systems. In contrast to the high turnover rates of bacillary walls, the cell walls of pneumococci show remarkably little, if any, turnover (see Table IV). Furthermore, both peptidoglycan and teichoic acid enter the cell surface in a growth zone located at the cellular equator. Once physically linked in the growth zone, the teichoic acid-peptidoglycan complex of pneumococci seems to form a conservatively segregating unit which is passed on to daughter cells intact. One should mention that a higher turnover in the peptide portion of cell walls would not be a priori incompatible with the autoradiographic evidence of conservation of pneumococcal teichoic acids. One could imagine extensive breakdown and resynthesis of the peptidoglycan portion of cell walls without necessarily affecting the glycan-teichoic acid part of the cell wall. Rearrangement of “new” peptidoglycan units with “older” portions of the cell wall could occur, for instance, by an amidase-catalyzed mechanism, as suggested by Ghuyen (12). Our data indicate that such rearrangement occurs rarely if at all in pneumococci.

It should be noted that the existence of peptidoglycan turnover is a somewhat controversial issue at the present time; positive findings have been reported in the cases of *Bacillus megaterium* (13), *Lactobacillus acidophilus* (14), and the strain W23 of *D. subtilis* (10), while no evidence for turnover was found in a mu-
tant strain of *B. megatherium* (15) and in *Streptococcus faecalis* (14).

It is tempting to conclude that the differences in the mode of segregation of cell walls of pneumococci and *B. subtilis* reflect a principal difference in the mechanism of surface extension in coccus-shaped versus rod-shaped bacteria. Indeed, it has been suggested that cocci might have only one mechanism for surface enlargement (through synthesis at the growth zone), while bacilli may have an additional mechanism for longitudinal surface growth (16). Equatorially located growth zone (16, 17), symmetrical and conserved segregation (18, 19), and lack of turnover (14) of cell walls have been shown to characterize *S. faecalis*. In this connection it may be interesting to note the striking difference in the physiological response of pneumococci and bacilli to agents that can inhibit cell division (ultraviolet radiation, low doses of mitomycin or penicillin, fluorodeoxyuridine, and thymidine starvation). Unlike bacilli, pneumococci show no filament formation by these agents.

The mechanism that controls the closely coordinated incorporation of teichoic acid and peptidoglycan units into the cell wall growth zone is unknown at the present time.

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