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Mode of Peptidoglycan Synthesis in *Salmonella typhimurium*: Single-Strand Insertion

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The synthesis of peptidoglycan by *Salmonella typhimurium* at the molecular level has been analyzed by studying the pattern of insertion of newly synthesized strands into the preexisting cell wall. We have measured the acceptor-donor radioactivity ratio during short labeling periods, and we found values between 0 and 0.2. This is less than the ratio observed by Burman and Park (Proc. Natl. Acad. Sci. USA, 81:1844–1848) for peptidoglycan synthesis in *Escherichia coli*. We propose that insertion of new strands occurs as single strands.

The murein sacculus or peptidoglycan coat of a gram-negative bacterium is a macromolecule that surrounds the cytoplasmic membrane of the cell. The peptidoglycan appears to be a single layer of strands arranged perpendicular to the axis of the rod-shaped cells (8, 11). Burman and Park (1) have proposed a fecund and heuristically explicit model for the mode of synthesis of the peptidoglycan of *Escherichia coli*. They proposed that bonds between adjacent peptidoglycan strands are broken and that between these separated chains newer chains are inserted in pairs. They further proposed that the chains proceed helically around the cell and are inserted between the initially inserted material and the older peptidoglycan. This model is based on the acceptor-donor radioactivity ratios (ADRRs; described in detail below) of cells after short- and long-term labeling.

The peptidoglycan is a unique polysaccharide made up of repeating subunits composed of alternating N-acetylglucosamine and N-acetylmuramic acid moieties. Attached to each muramic acid is a short peptide composed of four amino acids: L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (DAP), and D-alanine. This rigid "exoskeleton" is formed by a unique biosynthetic pathway. Chains of peptidoglycan are extended by the addition of the disaccharide subunits with an attached pentapeptide. Soon after chain extension, a cross-link between subunits is formed when the penultimate D-alanine forms a peptide link between its carboxyl group and the free amino group of a neighboring DAP. During this cross-linking reaction, the final D-alanine (the fifth amino acid) is lost. It is assumed that the carboxyterminal D-Ala–D-Ala link provides the energy for the crosslinking reaction (8, 10). Even if no cross-linking reaction occurs, a carboxypeptidase activity removes the last D-alanine from the inserted pentapeptides that are not involved in cross-linking reactions. Thus, all of the peptide chains soon have only four amino acids (1, 3, 8). The removal of the D-alanine residues occurs within a few minutes of insertion. Therefore, cross-linking occurs either immediately or not at all, and for these reasons it is assumed that only the newly inserted peptide chain can act as a donor. (For further analysis of this assumption, see Discussion.)

The ADRR is determined on dimers released by muramidase digestion of radioactively labeled peptidoglycan. The ADRR is the ratio in a peptidoglycan fragment of radioactive DAP that is found with no free amino group (an acceptor muropeptide) to that found with a free amino group (a donor muropeptide). In dimers, the acceptor DAP has no free amino group because one amino group of the DAP is blocked in a peptide bond during the formation of the original pentapeptide, and the other amino group is blocked by the formation of a peptide bond during cross-linking. The DAP adjacent to the donor D-alanine (a donor DAP) still retains its free amino group. The different DAP types can be distinguished because the donor DAP with a free amino group can be converted to hydroxyaminopimelic acid (HAP) by treatment with an oxidizing agent. The acceptor DAP is resistant to oxidation. Subsequent hydrolysis and thin-layer chromatography allow separation and determination of the radioactivity in the donor or acceptor peptide.

When the ADRR was measured by Burman and Park (1), they obtained initial values of approximately 0.25. Burman and Park reasoned that if a single strand was being inserted between separated strands of unlabeled peptidoglycan, the expected ADRR would be 0 (i.e., no acceptor label), as only newly inserted strands can form cross-links and are donors to adjacent acceptor DAP types. To account for this nonzero ADRR, Burman and Park (1) proposed that new strands are inserted in pairs. Since both strands could act as donors, they would form cross-links with the newly inserted adjacent radioactive strand, as well as with the adjacent nonradioactive peptidoglycan, thus producing some labeled DAP in the acceptor form.

The initial ADRR remained constant for approximately 8 min before rising to approximately 1.25 (1). Burman and Park explained this initial plateau by proposing that the inserted strands proceed helically around the circumference of the cell and, after one turn, are inserted adjacent to the material initially inserted. Since insertion of the strands proceeds by the breakage of existing interstrand cross-links and the formation of new cross-links between the newly inserted strand(s) and the adjacent peptidoglycan, the new cross-links convert some of the original donor subunits into acceptor subunits.

We have analyzed the ADRR after pulse-labeling *Salmonella typhimurium* and have found significantly lower ADRRs. We can even obtain ADRR values of 0. This indicates a model of peptidoglycan growth in which single strands are inserted into the preexisting peptidoglycan.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* strains were obtained from Kenneth E. Sanderson of the University of Calgary, Calgary, Alberta, Canada. Strain 2616 (LT2 *lys*) was the

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most efficient strain with regard to the incorporation of radioactive DAP into the peptidoglycan, but the efficiency rates of the other strains were not less than 50%. In all cases the incorporation of DAP into *S. typhimurium* was over 25 times more efficient than that in any *E. coli* strain studied (2).

Growth and labeling of bacteria. Bacteria were grown in medium C (7), which contains 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 2 g of NH_4Cl, 3 g of NaCl, and 0.25 g of $MgSO_4$ per liter. The medium was supplemented, when required, with 0.2% glucose or glycerol and 40 μg of lysine per ml. When faster growth rates were required, 19 amino acids (no cysteine) were added at a concentration of 80 μg/ml. Bacteria were grown at 37°C with rotary shaking.

Labeling was started by adding *meso*-[^{3}H]DAP (1 μCi/ml, 36.5 Ci/mM; Research Products International, Mount Prospect, Ill.) and was stopped either by boiling or by rapid cooling with ice in the presence of trichloroacetic acid.

ADRR determination. The ADRRs of labeled samples were determined by deamination by a procedure described by J. T.Park (5, 6; personal communication). Isolated sacculi are treated with nitrous acid to convert DAP residues with free amino groups to HAP residues. Acid hydrolysis of isolated dimers and subsequent separation of the radioactive material gives the ADRR.

Samples of cells were harvested by centrifugation (3,000 × *g*, 5 min), transferred to a Microfuge tube, and suspended in 4% sodium dodecyl sulfate. The cells were boiled for 30 min, concentrated by centrifugation (15,000 × *g*, 12 min), and treated for another 15 min (100°C) with 4% sodium dodecyl sulfate. The sacculi were then washed twice with distilled water. The final pellets were suspended in 0.8 ml of 0.5 M $NaNO_2$. Glacial acetic acid (130 μl) was added, and the tubes were vortexed and incubated at 0°C for 2 h.

We did not digest the sacculi to remove lipoprotein. The ADRR determinations were therefore carried out on that 80% of the peptidoglycan that was not covalently associated with lipopolysaccharide.

The deaminated sacculi were washed twice with 0.02 M sodium acetate and digested overnight with lysozyme (0.2 ml, 300 μg/ml in distilled water; other experiments in which sodium acetate buffer was used [pH 4.8] gave comparable results) at 37°C. After treatment at 100°C for 10 min, the undigested material was removed by centrifugation (15,000 × *g*, 5 to 12 min), and the supernatant was lyophilized. The resulting residue was applied to a silica gel plate (LK5D; Whatman, Inc., Clifton, N.J.) and dried. The plate was developed twice with isobutyric acid-water-triethylamine (500:270:20). After the plates were sprayed with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), they were autoradiographed with Kodak X-Omat film.

The monomer and dimer fractions identified by autoradiography were cut out of the plate, eluted overnight at 37°C with distilled water, placed in a glass tube, and lyophilized. The dried residue was hydrolyzed overnight with 6 N HCl at 105°C. The hydrolysate was lyophilized, taken up in 10 μl of 1% triethylamine, and spotted on a cellulose thin-layer chromatographic plate (Uniplate, Cellulose MN300; Analtech). The plate was developed once with methanol–water–pyridine–6 N HCl (80:16:40:4), dried, treated with En^{3}Hance, and autoradiographed. The DAP (R_f = 0.2) and HAP (R_f = 0.4) were quantitatively scraped off the plate and placed in scintillation vials with 1 ml of water and 4 ml of Safety-Solve (Research Products International).

Calculation of the ADRR. If the deamination of the sacculus was complete and the separation of HAP from the DAP was perfect, one would expect an ADRR for monomers of 0.0. In practice it is not 0, indicating that either or both of the assumptions are not valid. In any case, this would lead to elevated ADRR values for dimers. Therefore, we correct each of the dimer values for the possible incomplete deamination or separation by using the values for the monomer to correct the results obtained for the dimer. For example, if a monomer was found to have 20% of its counts in DAP (2,000 cpm in DAP and 8,000 cpm in HAP), we would correct the measured values found in the dimer from the same sample (for example, 2,800 cpm in DAP and 7,200 cpm in HAP; an initial ADRR of 0.39) by removing 25% (calculated as 2,000/8,000, the monomer results) of the HAP counts from the DAP counts and adding those counts back to the HAP counts to get the appropriate ADRR (1,000 cpm in DAP and 9,000 cpm in HAP; an ADRR of 0.1). A similar correction for the monomer would lead to an ADRR value of 0.0.

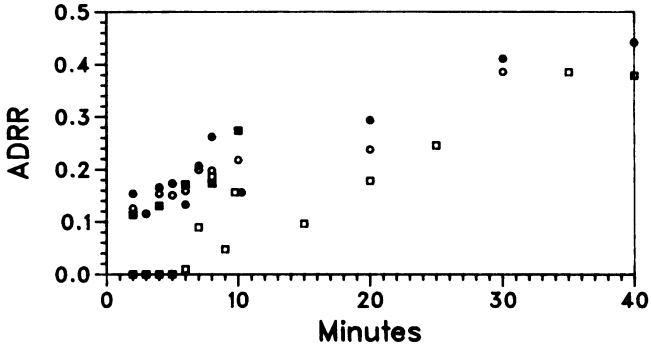

FIG. 1. ADRR determinations for *S. typhimurium* growing on glycerol minimal medium. The ADRRs were measured and calculated as described in Methods and Materials. Cells were labeled with [^{3}H]DAP (●) or [^{14}C]DAP (○) for the times indicated, and the incorporation was stopped by adding the cells to an equal volume of boiling water. Also shown are results from experiments in which the cells were stopped with ice (■) or ice and 5% trichloroacetic acid (□).

RESULTS

Search for ADRRs of 0. Because one would expect an ADRR value of 0 for dimers that were made with single-strand insertion, we studied very short labeling times at lowered temperatures to see whether we could find an ADRR of 0. Because extremely short labeling times lead to extremely low incorporation, we used large volumes of cells. For example, we labeled 600 ml of *S. typhimurium* growing at 21°C for 1 min (incorporation stopped by boiling) and obtained an ADRR of 0.004. In this culture the doubling time was 210 min. The equivalent labeling time at 37°C would be approximately 15 s. In another experiment run at 25°C, we found an ADRR of 0.0 at 1 min of labeling.

Kinetics of the ADRR. We have also determined the ADRR values for cells growing and labeled in glycerol minimal medium (doubling time of 60 min) (Fig. 1). Note that at labeling times between 2 and 10 min, values of the ADRR are less than 0.2 and extrapolation of the points to time zero would give a value of approximately 0.08. Note that when incorporation was stopped with ice and trichloroacetic acid, the initial ADRR values were 0.0 for up to 4 min.

Low ADRR values (less than 0.2) have also been observed for cells growing at different rates (45-min doubling in glucose plus 19 amino acids and 50-min doubling in glucose minimal medium). Any evidence for a plateau in the first 8 to 10 min is ambiguous. The data do not allow us to decide whether any initial plateau does or does not exist.

Methodological considerations. We have investigated some of the technical aspects of the methods used to determine the ADRR in the hope of improving the determination and obtaining a value that reflects the actual condition of the inserted peptidoglycan. Our initial method of stopping the incorporation of DAP was to add the cells to an equal volume of boiling water. It is possible that there may be some residual incorporation when the cells experience the increased temperature. This could lead to an effective increase in the labeling time. In order to overcome this problem, we tried other methods such as adding cells to ice or adding cells to ice with final concentrations of 5 and 10% trichloroacetic acid. Although there is some indication that stopping growth and incorporation with ice and trichloroacetic acid may lead to lower ADRRs—for example, we have consistently obtained values of 0.1 or less for the short labeling times (Fig. 1)—we feel that the results and model presented in this paper are independent of the particular method used to end the labeling period.

We have also analyzed the deamination procedure. The ADRR is not affected by additional deaminations, and additional deaminations do not lower the monomer correction values. This result also suggests that the deamination of the DAP types is essentially complete after one deamination. In any event, it should be noted that the correction factor, approximately 15 to 20% in our experiments (although occasionally lower), may give the low ADRR values. We note this caveat while suggesting that the consistency of the results, along with various internal controls (e.g., a dimer consisting of two incompletely digested monomers gives an ADRR of 0 as theoretically expected), argues that the results we have obtained are not due to an artifact associated with the correction factor.

DISCUSSION

ADRR and single-strand insertion. The ADRR values presented in this paper support a model of cell wall synthesis in *S. typhimurium* in which single strands of peptidoglycan are inserted into the growing sacculus. Cross-links are formed between the newly inserted material, which acts as a donor during cross-link formation, and the resident peptidoglycan. The evidence supporting this model are low ADRRs (0.0 to 0.1) for cells labeled for short times.

How does one account for the observation of nonzero ADRRs during short labeling periods, as observed by Burman and Park (1)? While it is possible that there are differences between their experimental object (*E. coli*) and ours (*S. typhimurium*), we feel that this is probably less important than other considerations. One possibility is to question the assumption (1, 3) that there is a complete removal of the final D-alanine from monomers in the peptidoglycan. If this removal was not complete, then there would remain some ability of the resident cell wall material to act as a donor in a cross-linking reaction. We note that the time scale of this decarboxypeptidation reaction is in the order of minutes, while our experiments are over much shorter time scales. If in a localized region of the cell wall there is a rapid succession of single-chain insertions (hot spots), one would find the new chains to be the objects of donation by adjacent pentapeptides in the resident cell wall. This would give nonzero acceptor values short labeling times.

Furthermore, the recent finding of a new type of cross-linkage, a DAP-DAP link between monomers (4), opens the possibility that a tetrapeptide can form links between newly inserted strands. The chemistry of the DAP types in these relatively rare (approximately 20% of dimers) linkages would lead to the same identification of acceptor and donor moieties. Thus, the absence of pentapeptides is no bar to the formation of interstrand cross-links.

Model of Burman and Park. Burman and Park (1) found initial ADRR values of not less than 0.2 and proposed that this nonzero result indicated that peptidoglycan strands were inserted in pairs. Our results of ADRRs of 0, or at the very least values less than 0.1, suggest that at least in *S. typhimurium*, peptidoglycan insertion is by single strands. The "constant" periods (approximately 8 min) reported by Burman and Park (1) before the rise in the acceptor-donor ratio are not confirmed by our experiments.

Park (10) has recently proposed that the two-strand mode of peptidoglycan insertion can be explained by the structure of peptidoglycan chains (9). If the successive monomers in a peptidoglycan chain are at 90° angles, then one would expect that only every other peptide would be in the plane of the sacculus. Park (10) proposed that only two-strand insertion can have the newly inserted material in perfect register with the resident acceptor peptidoglycan. Our calculations for such a model predict that the ADRR at initial labeling times should be 0.33. Our experimental determinations, as well as those of Burman and Park (1), are significantly below this value, and we suggest that the two-stranded model based on structural considerations is not supported by the available data.

One additional possibility to explain the differences between the results reported here and those of Burman and Park (1) should be noted. Because Burman and Park used a DAP-requiring cell, they had to remove the DAP for up to 25 min prior to labeling. This period of starvation or depletion may have caused disturbances or changes in the pattern of cell wall synthesis. Because labeling of cell wall with DAP is rapid and efficient in *S. typhimurium*, even in a cell that does not require DAP (2), no starvation for DAP was required in our experiments.

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