JOURNAL OF BACTERIOLOGY, Nov. 1990, p. 6573–6575
0021-9193/90/116573-03$02.00/0

Vol. 172, No. 11

Biosynthesis of a Membrane Adhesion Zone Fraction throughout the Cell Cycle of *Escherichia coli*

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Received 12 February 1990/Accepted 30 July 1990

Synchronized cells of *Escherichia coli* were pulse-labeled with [^{3}H]leucine and subjected to membrane fractionation to determine whether a fraction that is enriched for membrane-murein adhesion zones (fraction OM_L) was preferentially generated at specific times during the cell cycle, as previously suggested from studies of *lkyD* and *cha* mutants. Contrary to this prediction, the experiments showed that OM_L was formed continuously during the division cycle.

Escherichia coli and other gram-negative bacteria are surrounded by a cell envelope that includes two membranes, the cytoplasmic (inner) membrane and the outer membrane. The region between the two membranes (the periplasmic space) contains the rigid murein sacculus that determines cell shape. It was shown by Bayer (1) that the inner membrane is closely associated with the murein-outer membrane layer at numerous sites within the cell envelope, forming zones of adhesion that can be seen in thin-section electron micrographs. The adhesion zones have been implicated in several cellular processes, including the export of proteins and lipopolysaccharides from inner membrane to outer membrane (reviewed in reference 2).

Membrane-murein attachments also appear to play a role in the cell division process, as shown by the observation that the division site is flanked by two circumferential zones of adhesion, the periseptal annuli, that extend completely around the cell cylinder (7, 10). The periseptal annuli appear at the division site before the onset of septal invagination (6). Their formation represents the earliest detectable morphological event in the differentiation of these sites.

Membrane isolation procedures have been described that permit the isolation of a fraction (OM_L [8]) that appears to be enriched in membrane-murein adhesion zones (3, 8). In vivo and in vitro studies have indicated that the OM_L fraction participates in lipopolysaccharide export to the outer membrane and in the incorporation of new murein subunits into the sacculus (8), supporting its assignment as an adhesion zone fraction. It is possible that this fraction includes the periseptal annuli, but there is no direct evidence for this.

It was previously shown that the OM_L fraction accumulates to high levels in certain cell division mutants of *E. coli* and *Salmonella typhimurium* (*cha* and *lkyD*, respectively) that are blocked at a late stage of the division process (5). This finding suggested a relationship between OM_L and cell division. DNA synthesis was required for the accumulation of OM_L in the mutant strains, further linking OM_L to the division cycle. In one suggested model to explain these observations, the membrane junctions contained in OM_L are assembled at a specific stage of the cell cycle (5). In this model, the *lkyD*$^+$ and *cha*$^+$ gene products are required for progression to the next stage of the cycle, which is normally associated with a transient cessation in the formation of OM_L. This would explain the accumulation of OM_L in Cha$^-$ and LkyD$^-$ cells. This model predicts a discontinuous mode of synthesis of OM_L during the cell cycle in wild-type cells.

On the other hand, studies of the development of periseptal annuli have shown that new annuli are generated early in the division cycle (6). The annuli appear to continue their growth around the cylinder during much of the remainder of the cycle, during and after displacement to their final locations within the cell (6, 7). If the OM_L fraction includes the membrane-murein junctions that comprise the periseptal annuli, this process would be consistent with a continuous mode of synthesis of OM_L during most of the division cycle.

To determine whether OM_L was synthesized continuously or discontinuously during the cell cycle, we have studied the incorporation of [^{3}H]leucine into membrane fractions in synchronized cells of *E. coli*.

E. coli AB1157 was synchronized by the method of Kepes and Kepes (9) by subjecting the culture to 15 cycles of phosphate depletion and refeeding. The culture was then diluted into the same glucose-minimal medium (9) containing a nonlimiting concentration of phosphate (1 mM) and grown at 37°C with vigorous aeration. Cell number (by Coulter Counter analysis) and optical density at 600 nm were determined at intervals. This procedure resulted in a synchronous doubling of the cell number every 65 min during the duration of the experiment (Fig. 1a); the cell mass increased exponentially during this time. The labeling experiments described below were performed during the second and third division cycles after the culture was diluted into nonlimiting medium.

Samples of the synchronized culture were pulse-labeled with [^{3}H]leucine at 10-min intervals during two successive division cycles. The pulse-labeled samples were then mixed with samples of a nonsynchronized culture that was labeled with [^{14}C]leucine for several generations to provide an internal control for experimental variations during the remainder of the membrane isolation procedure.

A 120-ml portion of the culture was used for steady-state labeling with [^{14}C]leucine by adding 1.8 ml of L-[U-^{14}C] leucine (0.25 mCi/ml; 300 mCi/mmol) at the time of dilution into nonlimiting medium and growing the cells for 3 h. From a parallel culture, duplicate 6-ml samples were removed at 10-min intervals during the second and third division cycles, and 0.4 ml of L-[3,4,5-^{3}H]leucine (1 mCi/ml; 1 Ci/mmol) was added to each sample. After 8 min of incubation, growth was stopped by addition of ice plus a mixture of chilled nonra-

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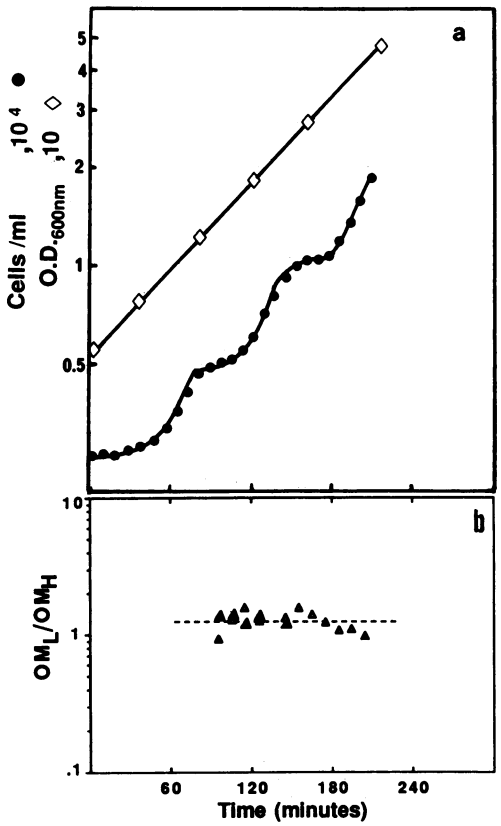

FIG. 1. Rate of OM_L synthesis in synchronized cells. (a) Growth and division pattern of a synchronized culture; (b) incorporation of [^{3}H]leucine into OM_L. Membrane fractions were prepared as described in the legend to Fig. 2. For each SG2 gradient, the $^3H/^{14}C$ ratio of the pooled OM_L fraction was divided by the $^3H/^{14}C$ ratio of the OM_H fraction to express the incorporation of [^{3}H]leucine into OM_L relative to incorporation into outer membrane (OM_L/OM_H; vertical axis). ---, Average of all values in all samples. The coefficient of variation of the experimental points was 14%. For the last six time points, only one of the duplicate samples was carried through the SG2 fractionation. Open and closed symbols represent the results for the duplicate samples that were carried through the membrane fractionation procedures. $O.D._{600nm}$, Optical density at 600 nm.

dioactive leucine (0.6 ml; 10 mg/ml) and carbonyl cyanide *m*-chlorophenylhydrazone (0.04 ml; 1 mM). A sufficient amount of the [^{14}C]leucine-labeled culture was added to each sample to obtain a $^3H/^{14}C$ ratio of approximately 3:1. The samples were then used for membrane fractionation as described in the legend to Fig. 2. The $^3H/^{14}C$ ratios were used to compare rates of synthesis of specific membrane fractions at different stages of the cell cycle.

Purification of the OM_L fraction was carried out by the combined use of sedimentation and flotation gradient centrifugation as described by Ishidate et al. (8). The mixtures of [^{3}H]leucine- and [^{14}C]leucine-labeled cells were disrupted in a French pressure cell, and crude cell envelope was collected, applied to the top of a sucrose density gradient, and subjected to overnight centrifugation (8). Each fraction was analyzed for refractive index (as an indication of sucrose concentration) and for radioactivity from ^{3}H and ^{14}C. Buoyant density was calculated from sucrose concentration.

After the initial sedimentation gradient (SG1; Fig. 2a), fractions from the OM_L region were placed at the bottom of a second gradient and subjected to a round of flotation centrifugation for 72 h (SG2; Fig. 2b). This procedure further resolved OM_L from residual outer membrane material (OM_H).

Comparison of the $^3H/^{14}C$ ratios of the purified fractions showed that the rate of synthesis of OM_L was relatively constant throughout the two division cycles whether expressed relative to synthesis of outer membrane (Fig. 1b) or relative to total cell envelope synthesis (data not shown).

Analysis of the incorporation of labeled leucine into OM_H

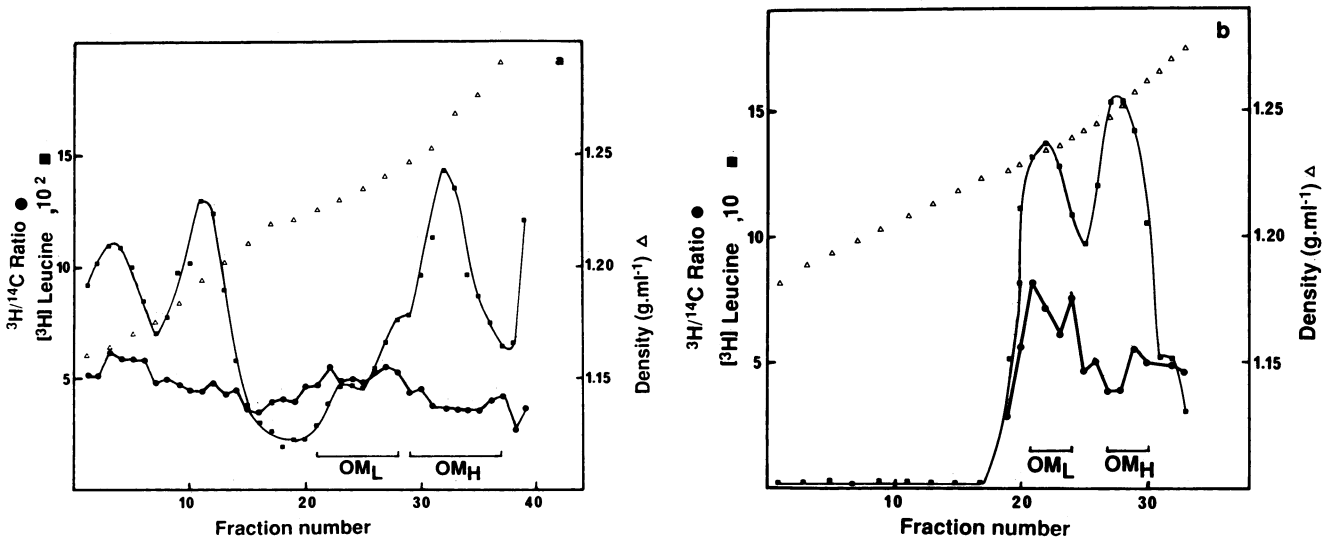

FIG. 2. Membrane fractionation. (a) SG1. Shown is a representative gradient. The fractions indicated by the brackets were pooled to give the OM_L and OM_H fractions, based on the appearance of the gradients and the densities of the fractions as described by Ishidate et al. (8). (b) SG2. A portion of the OM_L fraction from SG1 (see above) was subjected to flotation gradient centrifugation (8). The fractions indicated by the brackets were pooled to give the OM_L and OM_H fractions.

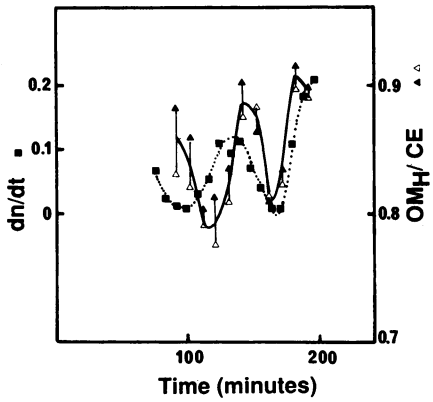

FIG. 3. Rate of outer membrane synthesis in synchronized cells. For each SG1 gradient (Fig. 2a), the $^3H/^{14}C$ ratio of the pooled OM_H fraction was divided by the $^3H/^{14}C$ ratio of the entire gradient, thereby expressing the incorporation of [^{3}H]leucine into OM_H relative to the incorporation into total cell envelope material (OM_H/CE; ——). △ and ▲, Results from the duplicate SG1 gradients for each time point; – – –, rate of change in cell number (*dn/dt*) in each time interval, calculated from the data shown in Fig. 1a, where *n* is cell number and *t* is time.

in the same experiment showed that the ratio of the rate of OM_H synthesis to the rate of total cell envelope synthesis varied cyclically during the cell cycle, with the peaks of the oscillations being separated by approximately one generation time (Fig. 3). These results are consistent with the previous suggestion of Boyd and Holland (4), based on differences in the patterns of incorporation into Sarkosyl-soluble and Sarkosyl-insoluble cell envelope material, that the outer membrane shows a bilinear mode of synthesis during the division cycle. We note that the observed cyclical change in the ratio of the rate of synthesis of OM_H to the rate of synthesis of total cell envelope in our study would be equally consistent with inner membrane being synthesized bilinearly while outer membrane was synthesized exponentially during the cycle.

These results suggest that the bulk of OM_L is synthesized continuously during the division cycle and do not support the hypothesis that the membrane adhesion zones that are thought to comprise the bulk of the OM_L fraction are formed at a specific time in the cell cycle. If this fraction includes the periseptal annular adhesion zones, the results would be consistent with the morphological evidence that these structures continue to develop during most of the cycle. It remains possible that either the rate of degradation of the OM_L fraction or the rate of formation of a small subset of this fraction, such as the periseptal annuli, varies during the cell cycle. A test of this possibility must await the availability of a specific molecular marker for the annuli.

This study was supported by grants from the National Institutes of Health, the National Science Foundation, and the Centre Nationale de Recherche Scientifique.

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