MEMBRANE ASSEMBLY IN *ESCHERICHIA COLI* II. SEGREGATION OF PREFORMED AND NEWLY FORMED MEMBRANE PROTEINS INTO CELLS AND MINICELLS

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SUMMARY. Cells of a minicell producing strain of *Escherichia coli* were freed of minicells and innoculated into fresh medium for further growth. The protein composition of the minicell membranes formed during periods of further growth reflected that of the total cellular membrane proteins, rather than being representative of the membrane proteins formed during the period of production of the minicells. These data, when viewed together with published observations on minicell morphogenesis, do not support models of membrane assembly where the membrane protein matrix grows preferentially either at the poles or at the equitorial perimeter of this organism with conservation of structure of preformed and newly formed membrane.

In the accompanying paper we tested models for the assembly of the *E. coli* membrane using a lipid density label. In this report, we describe studies on the incorporation of preformed and newly synthesized proteins into the membranes of minicells and their parent cells. Minicells are small enucleate cells which are approximately one-tenth the size of, and thus easily separable from the minicell producing parent bacteria (1). Since minicells are formed by fission near one or both poles of the parent cells (1-2), it is possible to test directly a model for minicell morphogenesis where proteins (but not necessarily lipids) are preferentially assembled into the minicell membranes at a fixed site(s) near the polar regions or equitorial perimeter of the parent cell with concomitant conservation of structure of the protein matrices of the preformed and newly synthesized membranes.

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METHODS. *Bacterial strains and media.* The minicell producing strain of *E. coli* K 12, strain P678-54 was obtained from Dr. H. Adler. A β-glucoside fermenting variant (*bgl*$^+$)[1] was isolated as described previously (3), and the β-glucoside fermenting strain was converted to *lac*$^+$ by conjugation with an Hfr Cavalli lactose prototroph provided by Dr. W. Epstein. The product of these selection procedures, strain P678-54 *bgl*$^+$ *lac*$^+$ was used in the experiments described here. For studies where the β-glucoside and β-galactoside fermentation systems were induced, the medium for growth of the 500 ml cultures consisted of medium A (4), 2 µg per ml of thiamine·HCl, 1% Difco casamino acids, and inducer (0.1 mM phenyl-1-thio-β-D-glucopyranoside for induction of the β-glucoside system and 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for induction of the β-galactoside system). Where cellular protein was labeled, the 500 ml cultures contained medium A, 0.4% glycerol, 2 µg per ml of thiamine·HCl, and 50 µg per ml each of L-threonine and L-leucine. For isotopic labeling, 5 µCi of ^{3}H-isoleucine, or 10 µCi of ^{14}C-isoleucine was included where indicated. In both cases, cellular growth was at 37° C with rotary shaking of the 2 liter flasks.

Separation of minicells from parent cells. The method was essentially that of Dvorak, Wetzel and Heppel (5). Cells from a 500 ml culture were harvested by centrifugation and suspended in 100 ml of 0.15 M NaCl. This suspension was centrifuged for 3 minutes at 2,000 x g and 25° C in the Sorvall SS34 rotor to sediment cells, and the supernatant fraction which contained most of the minicells was centrifuged for an additional 10 minutes under the same conditions to remove large cells. This supernatant solution was then centrifuged for 15 minutes at 14,500 x g to pellet the minicells. The minicell pellet was suspended in 0.5 ml of 0.15 M NaCl and applied to a 10 ml 20% to 5% linear sucrose gradient containing 50 mM potassium phosphate buffer of pH 7.0 in a 15 ml Corex tube, for a 10 minute centrifugation at 2,500 rpm

[1] Symbols used are: *bgl*$^+$, β-glucoside fermentation; *lac*$^+$, lactose fermentation.

and 25° C in the Sorvall HB-4 rotor. The minicells remained near the top of the gradient as a sharp band and were collected and washed free of sucrose. Minicells purified by this method contained less than 10 colony forming cells per 10^4 minicells, based on the estimate that an absorbance of 0.3 at 650 nm represents 10^9 minicells per ml (6). The original 2,000 x g pellet, which contained mostly large cells, was suspended in 2 ml of 0.15 M NaCl and applied to two 20 ml 20% to 5% linear sucrose gradients in 25 ml Corex tubes for an 8 minute centrifugation at 2,500 rpm and 25° C using the Sorvall HB-4 rotor. The broad band of large cells in the lower two-thirds of the gradient was collected, and the cells were washed free of sucrose.

RESULTS. Since minicells divide from their parent cells by fission only near the polar regions (1-2), a minicell producing strain can be used to test for models of membrane growth where proteins are inserted into the membrane at the equitorial perimeter or at the polar region(s). To test these possibilities, the distribution of previously synthesized and newly synthesized membrane proteins between cells and newly formed minicells has been studied in two experiments. In the first experiment (Table I), the proteins of a minicell producing strain were labeled during 3 generations of growth in a medium labeled with a ^{3}H-amino acid. The cells were then separated from the minicells in the population, and suspended in a medium supplemented with a ^{14}C-amino acid. If minicell membranes are formed by a linear extension of the membrane protein matrix at the polar region, with conservation of previously formed and newly formed protein matrices, then the membrane proteins of the newly formed minicells should have a higher $^{14}C/^3H$ ratio than the membrane proteins of the cells from which the minicells were derived. The membrane proteins of minicells formed at the later time periods (for example between 1.0 and 1.6 generations of growth after shifting from ^{14}C- to ^{3}H-medium) might possibly exhibit a $^{14}C/^3H$ ratio approaching infinity. If proteins are inserted into membrane at the equitorial perimeter, on the other hand, the proteins of the membranes of minicells formed initially should have a $^{14}C/^3H$ ratio of zero. The $^{14}C/^3H$ ratio

TABLE I. Distribution of prelabeled and newly synthesized proteins between the cell envelopes of cells and newly formed minicells.

Sample	Generations of growth after shifting from ^{3}H- to ^{14}C-medium	Cell envelope ^{3}H cpm/μg	Cell envelope ^{14}C cpm/μg
cells	0	11.0	---
minicells		10.8	---
cells	0.3	8.0	0.9
minicells		7.8	0.7
cells	1.0	6.1	2.1
minicells		5.8	1.6
cells	1.6	3.2	2.7
minicells		3.5	2.7

Cells of the minicell producing strain, grown as described in Methods for 3 generations in ^{3}H-amino acid labeled medium (^{3}H-medium), were processed sterilely to separate cells and minicells. After removing a sample of the cells for assay of cell envelope protein and radioactivity, the bulk portion of the cells was transferred to ^{14}C-amino acid labeled medium (^{14}C-medium) and grown for 0.3 generations of growth as determined by the increase of turbidity measured with a Klett colorimeter. The cells grown for 0.3 generations in ^{14}C-medium were processed to separate cells and newly formed minicells, the bulk of the cells again being returned to ^{14}C-medium for an additional 0.7 generations (1.0 generations of growth in ^{14}C-medium). This procedure was repeated once more to generate the samples for 1.6 generations of growth in ^{14}C-medium. The cell envelope preparations were obtained by sonication of the cell suspensions followed by a 60 minute centrifugation at 4° C and 100,000 x g. The protein contents of the cell envelope preparations were estimated by the method of Lowry, *et al.* (7), and radioactivity, which is expressed as counts per minute per μg of cell envelope protein, was determined by scintillation counting in the solution described by Patterson and Green (8) containing 3:1 of toluene and Triton X-100 (Rohm and Haas).

of proteins in the cell envelopes of newly formed minicells, however, does not differ from that of proteins in the envelopes of the cells from which the minicells were derived.

A second experiment (Table II) to test the stated models for membrane growth employs cells which have been induced for β-glucoside transport. After removal of the minicells, the cells are induced for β-galactoside transport, and the distribution of the transport systems between cells and minicells is determined. Since the glycosidases corresponding to the transport systems are

TABLE II. Distribution of components of the β-glucoside and β-galactoside fermentation systems between cells and newly formed minicells after shifting from conditions of induction for the β-glucoside system to conditions of induction for the β-galactoside system.

Sample	Generations of growth after shifting to induction for the β-galactoside system	RATIO β-galactoside transport / β-galactosidase	RATIO β-glucoside transport / phospho-β-glucosidase
cells	0	-----	0.14
minicells		-----	0.12
cells	0.3	0.055	0.13
minicells		0.060	0.12
cells	0.7	0.060	0.11
minicells		0.060	0.14
cells	1.5	0.065	0.12
minicells		0.055	0.12
cells	2.5	0.060	0.15
minicells		0.050	0.13

The minicell producing strain was induced for the β-glucoside system during 3 generations of growth, and the cells and minicells were separated as described in Methods. A sample of cells was removed for the assay of β-glucoside transport and phospho-β-glucosidase, and the bulk portion of the cells was suspended in fresh medium containing an inducer of the β-galactoside system, but no inducer for the β-glucoside system. From this point, the course of growth and separation of cells and minicells was essentially that as described for Table I. In all cases the medium in which the cells separated from minicells were suspended contained an inducer for the β-galactoside system, but no inducer for the β-glucoside system. The activities of the soluble enzymes β-galactosidase and phospho-β-glucosidase were determined by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-nitrophenyl-β-D-glucopyranoside-6-phosphate respectively in sonic extracts (3,9). Since these glycosidases are present in considerable excess intracellularly, the rate determining step in the hydrolysis of ONPG and *p*-nitrophenyl-β-D-glucopyranoside by intact cells is their transport which was assayed by published procedures (3,9), except that sodium azide was omitted in the assay for β-galactoside transport.

coordinately induced with the transport systems, their distribution will be representative of the soluble protein distribution between cells and minicells. We have therefore normalized the transport activities to the activities of the corresponding glycosidases. The ratios of transport to glycosidase activity remain constant for cells and minicells for each sample tested, a result similar to that obtained for the radioactive labeling experiment.

DISCUSSION. The data presented here are open to the following alternative interpretations. 1) Proteins are inserted into the membrane matrix at many points. 2) Proteins are first inserted at one or a few points as described in the models in the accompanying paper, and then diffuse throughout the membrane. Though no direct data on the diffusion of a chemically defined protein or proteins in membranes is available, diffusion of membrane antigens has been observed by immunological techniques following cell-cell fusion (10). 3) In cells giving rise to minicells, proteins are inserted along a distinct perimeter displaced from the equitorial perimeter with growth occurring towards both poles at rates directly proportional to the distances from the displaced perimeter to the given poles. We favor the first alternative.

We have demonstrated that certain newly synthesized proteins are incorporated into membrane together with lipids also made *de novo* (9,11-13). The formation of certainly some and perhaps many membrane sites is thus not a purely random process. Though the data presented in this and the accompanying paper do not support the models of membrane assembly described in the accompanying paper, they do not exclude the possibility that membrane assembly might occur at a larger number of distinct loci. Work is currently in progress to determine an upper limit for the size of such loci (14).

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