The Mode of Segregation of the Bacterial Cell Membrane

(*E. coli*/minicell/phospholipids)

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ABSTRACT The distribution of phospholipids from labeled parental membranes to progeny cells was studied by autoradiography and a minicell system. The minicell experiments showed that, during growth, the parental membrane is diluted at the same rate in cells and in minicells, which indicates that ends of cells are not different from the cylindrical portions with regard to the distribution of parental molecules. The same result was obtained after labeling heme-containing proteins with δ -aminolevulinic acid. The autoradiographic experiments indicate that the membrane segregates in about 250 subunits 4×10^4 nm² in size. These subunits appear to be conserved during growth.

The membrane of bacteria may segregate in three possible ways; (a) semiconservation, where synthesis is restricted to the central region of the cell, (b) conservation, where synthesis is restricted to one or both ends of the cell, or (c) dispersion, where synthesis occurs in many locations over the cell surface. Lin, Hirota, and Jacob (12) showed that the cytoplasmic membrane of *Escherichia coli* segregates in a dispersive manner. By alternating a fatty-acid density label and radioactive amino-acid labels in *E. coli*, Tsukagoshi *et al.* (19, 20) showed that the largest piece that could be conserved is 50 nm in length. In some Gram-positive bacteria, segregation of the cell wall was semiconservative; (3, 4, 6, 7, 15, 18), while in Gram-negative bacteria, segregation was dispersive; (5, 8, 14, 16, 21).

We used [$2\text{-}^3\text{H}$]glycerol, specific for phospholipids, to follow the distribution of label from parental membrane to progeny cells by autoradiography, and to minicells by the dilution of label. We conclude that the phospholipid of the membrane is transmitted to progeny cells in about 256 conserved units.

MATERIALS AND METHODS

Strains. *E. coli* B/r was obtained from C. Helmstetter. *E. coli* BUG-6, a temperature-sensitive division mutant of *E. coli* AB1157 described by Reeve, Groves, and Clark (17) was a gift of the authors. χ -984, a minicell-forming strain of *E. coli* K-12 derived by R. Curtiss from the Adler minicell strain (1), was a gift of Stuart B. Levy.

Radioisotopes. [$2\text{-}^3\text{H}$]glycerol (8 Ci/mmol), [$3,5\text{-}^3\text{H}$] δ -aminolevulinic acid (1.6 Ci/mmol), and [14C]histidine (1.6 Ci/g) were obtained from New England Nuclear Corp., Boston, Mass.

Growth Conditions. For labeling the cell membrane, *E. coli* B/r was grown with [$2\text{-}^3\text{H}$]glycerol in C medium (containing per liter, 2 g NH_4Cl , 6 g Na_2HPO_4 , 3 g NaCl , 85 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 75 mg Na_2SO_4). After label was removed, cells were

resuspended in C medium plus 0.1% glucose and 0.5% unlabeled glycerol Penassay broth (Difco).

E. coli χ -984 was grown in C medium supplemented with 3% L broth, 1.0% glucose, and 100 $\mu\text{g/ml}$ each of adenine, pyridoxine, and histidine.

B. megaterium KM was grown on C medium plus 1.0% glucose or in Penassay broth.

Autoradiography. Autoradiography was performed as described by Lark (10). Cells were applied to slides by impression smears from agar blocks and allowed to dry. Slides were dipped in Kodak NTB-2 liquid emulsion at about 40°, drained, air dried, tightly sealed in black boxes containing Drierite, and stored at 0° until developed.

Slides were developed for 4 min at 16° in D-19 developer, then rinsed, placed in Kodak acid fixer for 5 min, washed twice, and allowed to dry. Cells were stained for 5 min with 0.02% Gentian violet, washed, and dried. For each time point, the number of grains per cell was counted for at least 500 cells; a light microscope was used.

Lipid Extractions. Lipids were extracted by the method of Kanfer and Kennedy (9) and counted in a Mark I liquid scintillation counter (Nuclear-Chicago). The radioactivity of the lipid extract was compared to that in an aliquot of whole cells after precipitation with Cl_3CCOOH . Recovery of more than 85% was usually obtained.

Minicell Purification. Minicells were separated from cells by differential centrifugation and further purified on 10–30% sucrose gradients according to the method of Levy (11). Cell contamination of minicells was estimated from viable counts and by microscopic observation. The ratio of minicells to cells was 10^4 – 10^5 .

Computations. All calculations were performed on a Digital Equipment PDP-9 computer fitted with a Calcomp Digital plotter. Algorithm design and coding for the various calculations was performed by Dr. James E. Green of the Department of Electrical Engineering of the Massachusetts Institute of Technology. Professor Arthur Koch of the University of Indiana wrote the theoretical equation and collaborated in writing the programs for this equation.

RESULTS

Autoradiography: Segregation of Labeled Parental Membrane. [$2\text{-}^3\text{H}$]glycerol was used as a specific label for *E. coli* phospholipids. *E. coli* B/r was grown in C glucose medium containing 32 Ci/liter of [$2\text{-}^3\text{H}$]glycerol at 37° for more than ten

FIG. 1. Growth curve and average number of grains/*E. coli* B/r cell pulse-labeled with [^3H]glycerol. Closed circles represent the growth of the *E. coli* B/r culture measured by optical density at 600 nm in a Zeiss spectrophotometer; Crossed circles represent the average number of grains per cell observed at the noted time after removal of label from the culture. These numbers were taken from slides developed after 91 days of incubation.

generations. Exponentially growing cells were filtered and washed with unlabeled, prewarmed C medium and incubated with shaking in Penassay broth. Samples were taken at intervals for lipid extraction and autoradiography.

Fig. 1 shows the growth curve (OD) and the average number of grains per cell ($\bar{X}$) as a function of time after the chase. Since the slopes of the curves are reciprocal within 5%, no turnover of phospholipids was measurable under these conditions. Table 1 contains the raw data for this autoradiographic experiment. Note that even after many generations the label is still in phospholipid.

Each of the models under consideration predicts a different pattern of grain distribution in autoradiograms. The number of grains per cell in a culture containing only labeled cells is distributed according to a Poisson distribution. When a class of unlabeled cells is superimposed upon a class of labeled cells, the total distribution becomes non-Poisson. For each of the models the transition from a Poisson to a non-Poisson distribution takes place at different times (Fig.

FIG. 2. Patterns of "old" and "new" membrane material predicted by the semiconservative, conservative, and dispersive growth models. The dark patches shown at zero time in this figure represent labeled membrane material. The drawings show the hypothetical distribution of label in the three models. The right side of the figure shows the fraction of labeled cells and the expected fit of the observed distribution to the Poisson distribution.

2): after one generation for the conservation model, after two generations for semiconservation, and not at all for dispersion.

Deviation from the Poisson distribution was assessed in the following way. In autoradiograms, cells with no grains include cells with no radioactivity and cells that represent the zero class of a Poisson distribution of labeled cells. To separate these two components the experimental data was plotted in a transform of the Poisson equation.

$$\ln(P_n)n! = n \ln \bar{x} - \bar{x}$$

$$\ln (\text{number of cells with } n \text{ grains} / \text{total number of cells} \cdot n!)$$

TABLE 1. Autoradiographic data from fully-labeled *E. coli* B/r cells

Minutes	No. of* generations	% Radioactivity† in lipids	Time of exposure (days)	No. of cells scored	Average (all cells) grains/cell	Average (grains/cell‡) (labeled cells)	Unlabeled§ cells
0	0	35	6	992	4.59	5.18	—
26	0.5	57	21	870	10.47	10.54	0.001
60	1.2	82	21	1157	7.75	7.78	0.004
135	3.0	106	21	1791	8.65	9.83	1.1
175	3.8	68	21	481	1.15	0.70	3.1
215	4.5	91	21	514	.37	1.50	6.0
260	5.6	105	70	1251	2.34	3.26	17.7
310	6.5	105	70	1372	1.51	2.74	28.3
360	7.4	—	70	888	0.98	2.81	45.3
428	8.5	78	70	1893	0.62	4.03	89.6
478	9.6	108	70	2956	0.11	3.19	87.6
535	10.6	105	70	2301	0.11	1.82	86.1

* Nearest one-tenth.

† (Radioactivity in lipid extract/Radioactivity in whole cells) $\times$ 100.

‡ Proportion of labeled cells is calculated from the intercept of the Poisson linear transformation.

§ Unlabeled cells = total cells with no grains — "Poisson" zeroes.

FIG. 3. Observed unlabeled cells (time zeros) at various times after removal of label. $[2\text{-}^3\text{H}]$ Glycerol (32 Ci/liter) was used to label phospholipids in each experiment. Open circles, fully labeled *E. coli* B/r grown in broth; closed triangles: pulse-labeled *E. coli* B/r grown in broth; open stars: *Bacillus megaterium* fully labeled and grown in broth; open diamonds: *E. coli* B/r fully labeled and grown in minimal medium (slow growing).

was plotted on the ordinate and n on the abscissa. A line was fitted to these points by the method of least squares, omitting the zero class, the class having one grain per cell, and the upper members of the distribution that comprise less than 5% of the total. This line represents the best estimate of the Poisson distribution; its slope and intercept can be used for determination of the value of $\bar{x}$. This derived $\bar{x}$ is used for calculation of the number of zero class cells belonging to the distribution. The observed number of zero class cells minus those calculated for the distribution equals the number of unlabeled cells, henceforth to be called "true zeros." The true zeros are plotted in Fig. 3 against time after the chase. The line is fitted by eye to these points. Other symbols in Fig. 3 represent a similar experiment in which the cells were pulse-labeled instead of steady-state labeled, an experiment done entirely in minimal medium, and an experiment with *B. megaterium* KM.

The finding that there were no unlabeled cells for the first six or so generations is in agreement with the data of Lin *et al.* (12) suggesting that *E. coli* membranes are partitioned

$$P(n) = \sum_{NBAR=50}^{100} \frac{C}{(NBAR)^2} \sum_{M=0}^{200} \left[\frac{XBAR}{NMAX} \right]^M \frac{n}{n!} \frac{e^{-NMAX} NMAX^n}{M!} \frac{NBAR^M e^{-NBAR}}{M!}$$

$$C \equiv \frac{1}{\sum_{NBAR=5}^{100} \frac{1}{NBAR^2}}$$

FIG. 4. Koch equation. NBAR, the number of conserved subunits in cells of various sizes. This varies from 50 to 100% of NMAX; M, the number of subunits; XBAR, the number of grains above the average cell in the class of cells just about to divide; NMAX, number of conserved subunits in those cells just about to divide; XBAR(M)/NMAX, average number of grains/cell in a cell with M subunits.

TABLE 2. Comparison of the experimental values to those predicted by the Koch equation for a steady-state autoradiographic experiment with *E. coli* B/r

Conserved subunits	% Zeros (theoretical)	% Zeros (experimental)	Generations
—	—	86.1	12
—	—	87.6	10
—	—	89.6	9
1	50.6	45.3	8
2	27.6	28.3	7
4	12.2	17.7	6
8	5.4	6.0	5
16	2.8	3.1	4
32	1.6	1.1	3
64	0.009	0.004	2
128	0.007	0.001	1

in a dispersive manner. The sharp increase in true zeros after about six generations suggests that the membrane phospholipids are not subdivided into molecules but are subdivided into subunits of finite size. For determination of the number of such subunits, it is necessary to know more precisely at what time the unlabeled class of cells appears.

Arthur Koch wrote an equation that takes into account the distribution of ages of cells in an exponential culture and the random decay of tritium, and makes the assumption that conserved subunits will be randomly distributed to daughter cells. This equation, which is shown in Fig. 4, was used for calculation of the distributions of label when the number of subunits per cell varies from 1 to 128. These distributions were used in the Poisson transform for calculation of the number of unlabeled cells. Table 2 shows an alignment of these theoretical numbers for the "true zeros" with the experimental numbers from Fig. 3. The time at which the average cell has one remaining parental subunit correlates best with the eighth generation of the experiments. Therefore, there were 256 (2^8) of these subunits originally present. We wish to emphasize that experimental uncertainties may introduce an error of a factor of 2, so that the actual number of original subunits is probably between 2^7 and 2^9 .

Autoradiography of Filament-Forming Mutants. BUG-6, a temperature-sensitive filament-forming mutant of *E. coli*, was used for investigation of the localization of membrane label. In a manner similar to the cell wall patterns seen in *Diplococcus pneumoniae* (3) and *Streptococcus pyogenes* (7), end localization of label may be demonstrated in filaments. Cells were grown in the presence of $[2\text{-}^3\text{H}]$ glycerol at the permissive temperature, 30°, and shifted to medium containing unlabeled glycerol at 42°, the nonpermissive temperature. In zonal growth, one or both ends should be heavily labeled, and the remaining material would be unlabeled after a few generations of growth. Filaments were observed for seven doublings of mass. No concentration of grains was seen either in filaments grown in liquid culture or in filaments grown on agar slides at the nonpermissive temperature after the label was removed. Fig. 5 is a photograph of a typical filament.

It is interesting to us that we were unable to grow filaments to greater than 2^8 cell units. At about the eighth generation cultures did one of three things in different

FIG. 5. A filament of *E. coli* BUG-6 that was pulse-labeled with $[2\text{-}^3\text{H}]\text{glycerol}$. Cells were labeled for 0.1 of a generation at 30° . Label was removed and cells were suspended in medium at 40° (see *Methods*) where they grew as filaments. No concentration of label was seen.

experiments. (a) Stopped growing abruptly and the optical density decreased, (b) filaments broke down to single cells and grew normally at 42° , or (c) filaments broke down partially and continued to increase in optical density at a reduced rate.

Minicell Experiments. *E. coli* χ -984, a minicell-forming mutant, permitted us to analyze the membrane of the ends of the cell. Minicells are formed at the ends of elongated *E. coli* cells. The basic experiment is presented in Fig. 6. A specific membrane ^3H -label is used, together with a steady-state $[^{14}\text{C}]\text{protein}$ label. At time zero the specific membrane label is removed and chased and the dilution of the specific label relative to the steady-state label is measured.

In either the central or end-growth models the ratios of the labels decrease at different rates in cells and minicells. In a fully conserved model minicells would be unlabeled if growth and septation occur at the same end, and fully labeled if they occur at opposite ends. Only in the dispersive model are the slopes of the cell and minicell lines the same. In an experiment with $[2\text{-}^3\text{H}]\text{glycerol}$ (Fig. 7), the two slopes decreased at the same rate.

δ -Aminolevulinic acid at low concentrations is a specific precursor for porphyrins. In *E. coli*, the heme-proteins are found in the cytoplasmic membrane (13). Fig. 8 shows the same experiment with δ -aminolevulinic acid as a tritium-

FIG. 6. Flow diagram of the pulse-sample experiment. A specific membrane tritium label was superimposed for a defined amount of time on a ^{14}C label of a generalized protein background. The specific label was removed and cells and minicells were separated. The cells were suspended in medium containing the generalized label and the specific membrane label was chased with unlabeled glycerol. At intervals of about one generation, cells and minicells were again separated and a portion of the cells was resuspended. The minicells collected at each generation were only those formed during that generation. The ratio of the specific to the generalized label was measured at each separation.

labeled precursor. Radioactivity of cells and minicells again changed at the same rate.

In an attempt to localize synthesis sites of membrane, cells were pulse-labeled with either $[^3\text{H}]\text{glycerol}$ or $[^3\text{H}]\delta$ -aminolevulinic acid, and the newly-formed minicells were examined at intervals of a quarter of a generation. If material is being made in one region and transported to another, the minicells and cells would show differences in their ratio of ^3H to ^{14}C at early times. The results seen after pulse labeling with either precursor were identical to those obtained from steady-state labeled cells. This indicates that if there is diffusion or trans-

FIG. 7. Dilution curves for cells and minicells labeled for 10 generations with $[2\text{-}^3\text{H}]\text{glycerol}$. The ^3H to ^{14}C ratio represents the ratio of specific membrane label present for 10 generations (^3H) removed at zero time, to generalized label (^{14}C) that is present throughout the experiment. The purpose here is to compare the slopes of the dilution lines for cells and minicells.

FIG. 8. Dilution curves of cells and minicells from a culture fully labeled (for 10 generations) with [^3H] δ -aminolevulinic acid. The ^3H to ^{14}C ratios here are as described in Fig. 7. Note that the difference between the minicell and cell values is less than with [$^2\text{-}^3\text{H}$]glycerol, although the slopes of the lines are identical.

port, it has reached equilibrium in less than one quarter of a generation.

DISCUSSION

We have shown that the distribution of labeled parental membrane to progeny cells is random for several generations, but then becomes bimodal with a class of unlabeled cells superimposed on a class of randomly labeled cells. This suggests that membrane is distributed dispersively in subunits of finite size. Analysis of the data suggested that there are about 250 of these subunits. An average *E. coli* is 1 μm in diameter and 3 μm in length and the area of each subunit is $4 \times 10^4 \text{ nm}^2$. The actual subunit area probably falls within a range of ± 2 of this value. This is in reasonable agreement with the figure of 50 nm in length published by Tsukagoshi and Fox (19, 20).

Unlabeled cells appeared at the same time whether cells were fully labeled or were labeled with short pulses of [$^2\text{-}^3\text{H}$]glycerol. This suggests that synthesis takes place at many sites simultaneously, rather than at a few sites sequentially. This result does not, however, answer the question of *where* on the cell surface the membrane subunits are made.

In "label-and-chase" experiments, we compared the dilution of the [$^2\text{-}^3\text{H}$]glycerol in minicells and whole cells. An exponential dilution at an identical rate, consistent with a dispersive mechanism, was seen in minicells and whole cells over the first five generations. Similar results were obtained with cells and minicells labeled with [^3H] δ -aminolevulinic acid, a specific precursor of porphyrins. Porphyrins are found in the electron transport enzymes of *E. coli* that are bound to the cytoplasmic membrane. In an analogous system, Wilson and Fox (23) obtained similar results with labeled membrane-specific proteins.

In enteric organisms phospholipids are found in nearly equal amounts in the cytoplasmic membrane and in the outer membrane (22). This would not affect the conclusion

of dispersive transmission in subunits but, if the two membranes segregate separately, it could affect the number of calculated subunits by a factor of two. In *Bacillus megaterium* KM (Fig. 1) the phospholipids are almost exclusively in the cytoplasmic membrane. Our experimental results with this organism appear identical to those with *E. coli* B/r. In the minicell experiment, the δ -aminolevulinic acid is found exclusively in the cytoplasmic membrane. Minicells labeled with [$^2\text{-}^3\text{H}$]glycerol.

The minicell experiments confirmed the dispersive transmission found with the autoradiographic experiments. These experiments set an upper limit of one-quarter generation on any rearrangement that may occur within the membrane. They do not, however, rule out the possibility that newly-made subunits may randomize by rapid transport within the membrane.

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