Synthesis of Cytoplasmic Membrane during Growth and Division of *Escherichia coli*

DISPERSIVE BEHAVIOUR OF RESPIRATORY NITRATE REDUCTASE

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We have used the penicillin selection method of Autissier & Kepes [(1972) *Biochimie* 54, 93–101] to study the segregation of membrane-bound respiratory nitrate reductase (EC 1.9.6.1) in *Escherichia coli* for the three generations after cessation of nitrate reductase synthesis caused by withdrawal of nitrate from the growth medium. We also included a physical separation procedure that permitted direct assay for nitrate reductase activity among all fractions produced by the penicillin selection method. We conclude that the segregation of nitrate reductase after cell division is dispersive, and not semi-conservative as proposed by Autissier & Kepes (1972).

The cytoplasmic membrane of rod-shaped bacteria may in principle segregate among progeny after growth and division in three main ways (Fig. 1): semi-conservative, where synthesis of new membrane is localized at the central region; conservative, where synthesis is at one end of the cell; dispersive, where synthesis is either at many locations over the membrane surface or is at fewer locations from which new material may readily diffuse laterally throughout the membrane. There is no complete consensus on which of these modes of segregation occurs in the cytoplasmic membrane of *Escherichia coli*. Lin *et al.* (1971) concluded that synthesis is dispersive, as did Overath *et al.* (1971) and also Wilson & Fox (1971). Tsukagoshi *et al.* (1971) concluded that the largest piece of cytoplasmic membrane that could be conserved was about 50 nm long. Green & Schaechter (1972) proposed that the membrane segregates in about 250 conserved subunits each of area $4 \times 10^4 \text{ nm}^2$, which is not far removed from the estimate made by Tsukagoshi *et al.* (1971). By contrast, Kepes and his co-workers have examined the segregation of a number of markers for the cytoplasmic membrane in *E. coli*, and concluded that segregation is semi-conservative with a central growing zone (Autissier & Kepes, 1971, 1972; Autissier *et al.*, 1971). The experimental approach used by Autissier & Kepes (1971) required that the bacteria first be grown under conditions where synthesis of a given membrane marker was induced, followed by transfer to a medium where growth and

division continued but induction of the marker did not. The distribution of previously synthesized markers in the non-induced progeny was then measured with a penicillin lysis selection technique, whereby lysis was conditional on presence of the marker. It was found that the cells of the third generation after transfer to non-inducing conditions were only 50% sensitive to marker-dependent penicillin lysis, from which it was concluded that membrane synthesis was semi-conservative (Fig. 1).

The observations by Autissier & Kepes (1971, 1972) and Autissier *et al.* (1971) are not compatible with the idea that there is rather fast lateral mobility of proteins in the plane of the cytoplasmic membrane of *E. coli*. For instance, Sackmann *et al.* (1973) calculated from the rotational mobilities of phospholipids in the cytoplasmic membrane that the lateral diffusion coefficient of a protein of 5 nm diameter in that membrane would be about $3 \times 10^{-10} \text{ cm}^2/\text{s}$, which would permit the protein to travel laterally about $0.3 \mu\text{m}$ in 1 s. Furthermore, particle aggregation viewed in the freeze-fracture faces of *E. coli* membranes indicated that cytoplasmic membrane particles could rapidly move laterally in the membrane (Haest *et al.*, 1974).

Our own measurements of protein mobilities in the cytoplasmic membrane of *E. coli* are also indicative of rates incompatible with the lateral asymmetry required for semi-conservative segregation of newly synthesized membrane proteins. We find that the rotational relaxation time of cytochrome *o* (calculated from measurement of linear dichroism after polarized-laser-flash photolysis of the CO complex; see Junge & De Vault, 1975) is less than $100 \mu\text{s}$. Likewise

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Fig. 1. Patterns of segregation of a pre-existing membrane component (shown as dots) predicted by (a) conservative, (b) semi-conservative and (c) dispersive models of membrane growth

The example shown would be applicable to a synchronous culture starting where all the cells have just divided. For a non-synchronous culture approximately three generations are needed to achieve the distribution given by the two generations of semi-conservative growth shown above. Further details are given in Green & Schaechter (1972) and Autissier *et al.* (1971).

time-resolved temperature jumps and fast quenching for freeze-fracture studies indicated lateral diffusion coefficients of approx. $10^{-10} \text{ cm}^2 \cdot \text{s}^{-1}$ for cytoplasmic membrane particles at 37°C (M. T. Davison & P. B. Garland, unpublished work).

The methods leading to the conclusion of semi-conservative membrane segregation, and therefore of restricted lateral diffusion, are not directly comparable with the physical methods that have led to the opposite conclusion, namely of unrestricted lateral mobility of cytoplasmic membrane proteins. We have therefore examined the penicillin lysis procedure of Autissier & Kepes (1971, 1972), using respiratory nitrate reductase as a marker of the cytoplasmic membrane. To this procedure we have added a step that separates penicillin-lysed cells from unlysed cells. Our results lead us to conclude that the penicillin lysis procedure in our hands does not indicate semi-conservative synthesis and segregation of new membrane.

Experimental

Bacteria

All experiments were made with *E. coli* K12 Ymel, strain A1002 (*ato*⁻, *ilv*⁻, *metE*⁻, *lacI*⁻, *rha*⁻, *fadRc*),

supplied by Dr. B. A. Haddock of this Department. Stock cultures were kept as 4 ml suspensions in 15% (v/v) glycerol at -22°C ; 0.2 ml samples of these were used to start preliminary overnight cultures by inoculating 100 ml of a nutrient broth medium at 37°C aerobically.

Growth of cells under conditions for induction of nitrate reductase synthesis

Cells induced for nitrate reductase were obtained by inoculating 2.0 ml of a preliminary overnight culture (see above) into 500 ml of the minimal medium of Cohen & Rickenberg (1956) supplemented with KNO_3 (1%, w/v), glycerol (0.5%, v/v), casamino acids (casein hydrolysate) (0.1%, w/v), 10 mM- MgCl_2 , 1 μM -(NH_4)₆ Mo_7O_{24} , 1 μM - K_2SeO_3 and L-valine, L-isoleucine and L-methionine (each 20 $\mu\text{g}/\text{ml}$). Culture was at 37°C in fully filled conical flasks in an evacuated vacuum desiccator without stirring or agitation.

Control cells lacking nitrate reductase were grown by inoculating 200 ml of Cohen & Rickenberg (1956) minimal medium supplemented with D-glucose (1% w/v), casamino acids (0.1%, w/v), 10 mM- MgCl_2 , L-valine, L-isoleucine and L-methionine (each 20 $\mu\text{g}/\text{ml}$). The 200 ml of culture was in a 500 ml flask, not evacuated, and shaken at low speed.

Cells were harvested after 16 h (nitrate reductase cells) or 10 h (control cells) by centrifugation at 4500 rev./min for 15 min at $0-4^\circ\text{C}$ in the 6×750 ml head of an MSE Mistral 6L centrifuge. The cells were then washed twice with 50 mM- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.8, at $0-4^\circ\text{C}$, resuspended in 0.12 M-Tris/HCl buffer, pH 7.6, at a protein concentration of 4–6 mg/ml, and stored at $0-4^\circ\text{C}$ before experimentation on the same day.

Further growth of induced cells, but under non-inducing conditions

The penicillin selection procedure of Autissier & Kepes (1971) requires that cells induced for a given component of the cytoplasmic membrane should be allowed to continue growing for three more generations under conditions that are non-inducing for that component. According to the mode of membrane growth various predictions can be made about the distribution of the chosen component in cells of various generations after transfer from inducing to non-inducing conditions (see Fig. 1). Accordingly induced cells after harvesting were transferred to medium identical with that used for the growth of control (non-induced) cells. The progress of this culture was followed by measuring the apparent absorption at 600 or 420 nm, and samples removed at zero, one, two and three doublings of the original extinction value (i.e. that obtained immediately after transfer of induced cells to non-inducing medium) were equated with zero, one, two and three

generations of growth after cessation of the synthesis of nitrate reductase.

EDTA treatment and penicillin-induced lysis

Cells harvested from cultures by centrifugation were resuspended in 0.1 of their culture volume in 0.12M-Tris/HCl buffer, pH7.6, at 37°C containing 1 mM-EDTA, vigorously mixed for 1 min, then added to 9-fold excess of a medium composed of Cohen & Rickenberg (1956) minimal salts, glycerol (0.5%, v/v), 10 mM-MgCl₂, 1 μM-(NH₄)₆Mo₇O₂₄, 1 μM-K₂SeO₃, KNO₃ (1%, w/v), L-valine, L-isoleucine and L-methionine (each 20 μg/ml) and penicillin G (1000 units/ml). The culture was placed in 250 ml conical flasks with side arms, gassed for 10 min with O₂-free N₂ and kept at 37°C. Volumes were arranged such that the flasks were completely filled and gave an apparent A₆₀₀ value of 0.7 when measured through the side arm (diameter approx. 1 cm). A decrease in absorbance was equated with penicillin lysis.

Centrifugation on sucrose density gradients for separating lysed from non-lysed cells

Linear gradients of sucrose from 5% to 10% (v/v) in 23 ml-capacity polycarbonate centrifuge tubes were loaded with about 0.1–0.2 ml of a cell suspension containing about 3–4 mg of protein/ml, and centrifuged at room temperature for 15 min at approx. 500g in a swing-out rotor. After centrifugation 20-drop fractions (0.6 ml) were collected in test tubes by perforating the bottom of the polycarbonate centrifuge tube.

Assays

Protein was assayed as described by Lowry *et al.* (1951), with bovine serum albumin as standard. Nitrate reductase was assayed spectrophotometrically as described by Jones & Garland (1977), by using the radicals of Benzyl Viologen, Methyl Viologen and Diquat.

Reagents

Benzyl Viologen and Methyl Viologen were purchased from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Casamino acids and nutrient broth were from Oxoid, Basingstoke, Hants. RG24 6PW, U.K., and O₂-free N₂ was from British Oxygen Co., Worsley, Manchester M28 4UT, U.K. Diquat was generously given by the Plant Protection Division, ICI Ltd., Bracknell, Berks. RG12 6EY, U.K. Penicillin G (benzylpenicillin, sodium salt; 1650 units/mg) was purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

Results

Nitrate reductase activity after transfer of cells from inducing to non-inducing growth medium

If nitrate reductase activity was to be used for the

study of how a pre-existing membrane component might distribute among subsequent cell generations, it was first necessary to determine that nitrate reductase was no longer synthesized when growing cells were transferred from inducing to non-inducing growth medium. Fig. 2 shows how the specific activity of nitrate reductase (i.e. activity/mg of cell protein) declined after transfer of cells from inducing to non-inducing growth medium. Also shown is the theoretical curve for halving of the specific activity for each doubling of cell mass. We conclude from the data of Fig. 2 that synthesis of nitrate reductase did cease on transfer of cells from inducing to non-inducing conditions, and that the previously synthesized enzyme molecules were stable for at least three generations of subsequent cell growth. Benzyl Viologen radical rapidly penetrates to the cytoplasm of *E. coli*, and could donate electrons to enzymically active but proteolytically solubilized fragments of nitrate reductase (Jones & Garland, 1977). Such cytoplasmic fragments would not, of course, be representative of membrane molecules. However, identical results were obtained with Diquat radical (Fig. 2), which does not cross the cytoplasmic membrane and reduces the membrane-bound complex of cytochrome *b* and nitrate reductase at the periplasmic face of the membrane where the cytochrome *b* is

Fig. 2. Specific activity of nitrate reductase activity after transfer of cells from inducing to non-inducing growth medium

Growth conditions and assays were as described in the Experimental section. The electron donors for nitrate reduction were the radicals of Diquat (○), Benzyl Viologen (△) and Methyl Viologen (□). The lines are drawn with a slope that halves the specific activity each generation.

accessible (Jones & Garland, 1977). From this it seems reasonable to conclude that the nitrate reductase activity did not become cytoplasmic (as the result of some degradation process) after transfer from inducing to non-inducing growth conditions.

Sensitivity of cells to penicillin lysis

Lysis by penicillin requires cell growth for the synthesis of new and defective cell-wall material. Non-growing cells are not lysed. Thus a growth medium providing glycerol and nitrate anaerobically should support growth only of those cells possessing nitrate reductase. This point was tested by exposing to the penicillin lysis procedure cells that were either induced or not induced for nitrate reductase. As described by Autissier & Kepes (1971), we used a brief exposure to EDTA just before putting the cells into penicillin-containing medium. Fig. 3(a) shows that non-induced cells were not lysed, that induced cells were lysed and that a 1:1 mixture of induced and non-induced cells exhibited 50% lysis as measured spectrophotometrically. The absence of lysis in non-induced cells exposed to nitrate for up to 2 h indicates that nitrate reductase activity was not induced during the penicillin lysis procedure. Lysis was faster if casamino acids were included in the penicillin medium, but the non-induced cells were not lysed within 60 min, by which time the induced cells were maximally lysed. In all these experiments the lysis of induced cells was dependent on the presence of nitrate in the penicillin medium.

Separation of lysed and non-lysed cells on a density gradient

Penicillin lysis is an indirect assay of the presence

Fig. 3. Time course of lysis by penicillin of cells that were induced (Δ), not induced ($\bullet$) or mixed ($\circ$) in equal concentrations (A_{600} being used as a measure of cell concentration)

In (a) casamino acids (0.1%, w/v) were also added to the penicillin medium; in (b) they were not. The initial A_{600} value was approx. 0.7, with a 1 cm-light-path cuvette.

of nitrate reductase. To seek confirmation that, in a mixture of cells with and without nitrate reductase, penicillin did indeed select by lysis only the cells with the enzyme, we sought for a simple and rapid method of physically separating lysed from non-lysed cells. Following a suggestion from Dr. R. K. Poole, we developed a rate separation by centrifugation of cell lysates on a linear 5–10% (w/v) sucrose gradient. The separation of a mixture of lysed and non-lysed cells was easily visible by eye. Fig. 4 shows the distribution of 280 nm-absorbing and -scattering material along three separate gradients, where the inputs were respectively induced cells, non-induced cells and a 1:1 mixture, all after the penicillin lysis procedure. It is clear that centrifugation on the gradient separated non-lysed cells into a faster-sedimenting lower band and lysed cells into a slower-sedimenting upper band. The 1:1 mixture separated into two bands coinciding with the lysed and the non-lysed cells. The fractions coinciding with the peaks of 280 nm-absorbing material were assayed for protein and nitrate reductase activity with Benzyl Viologen radical as electron donor. The results (not shown) demonstrated that nitrate reductase activity was confined to the lysed cells (upper band).

Distribution of nitrate reductase among the progeny of cells after transfer from inducing to non-inducing growth medium

Our results so far show that the penicillin lysis procedure selected for cells containing nitrate

Fig. 4. Separation of lysed from non-lysed cells by centrifugation on a sucrose density gradient

The inputs to the three tubes shown were non-induced cells ($\bullet$), induced cells ($\circ$) or both, mixed in equal concentration (Δ), all after exposure to the penicillin lysis procedure for 1 h. The bottom of the gradient is on the left [10% (w/v) sucrose], the top on the right [5% (w/v) sucrose].

Table 1. *Specific activity of nitrate reductase in penicillin-lysed and non-lysed cells measured three generations after transfer to non-inducing growth medium*

Cells exposed to the penicillin lysis procedure three generations after transfer to non-inducing growth medium were sampled at points corresponding to 0%, 25%, 50% and 75% lysis (see Fig. 5) and separated on sucrose density gradients as described for Fig. 4. The fractions were measured for cellular material by their absorbance at 280nm, and the tubes at the peak of the lower band (intact cells) and upper band (lysed cells) were assayed for nitrate reductase activity, with Benzyl Viologen radical as donor, and for protein. The small increase of specific activity with increasing lysis is no greater than that expected from cell breakage, which normally increases the activity with Benzyl Viologen radical as donor by about 50–80% (Jones & Garland, 1977).

% lysis at which cells were sampled	Specific activity of nitrate reductase (μ mol of Benzyl Viologen radical oxidized/min per mg of protein)		
	Input	Lower band (intact cells)	Upper band (lysed cells)
0	0.045	0.049	<0.005
25	0.056	0.079	0.071
50	0.048	0.054	0.074
75	0.078	0.092	0.098

reductase, and should therefore be effective in discriminating among the alternative patterns of membrane segregation shown in Fig. 1. Autissier & Kepes (1972) reported that the nitrate-dependent lysis of cells sampled from growth in non-inducing media was rapid and complete, and did not change for up to two generations of growth after transfer to non-inducing medium, whereas cells of the third generation exhibited only a 50% lysis. Despite many attempts we were unable to confirm this observation for the third-generation cells. As shown in Fig. 5, the nitrate-dependent penicillin lysis of cells induced for nitrate reductase was much the same at zero, one, two and three generations after transfer to non-inducing medium. It might be argued that the relatively low rate of penicillin lysis obscured the biphasic behaviour of third-generation cells observed by Autissier & Kepes (1972), and that induction of nitrate reductase had occurred during the exposure to nitrate in the penicillin medium. We therefore sampled third-generation cells from the penicillin lysis procedure at points corresponding to 0%, 25%, 50% and 75% complete lysis, separated them into lysed and non-lysed fractions by density-gradient centrifugation and assayed the fractions for nitrate reductase. At no stage did we observe a selective separation of nitrate reductase into the band of lysed

Fig. 5. *Penicillin lysis of cells of various generations after transfer from inducing to non-inducing growth medium* Conditions are described in the Experimental section. Cells at the time of transfer are referred to as zero generation (▲), and thereafter cells were sampled from the non-inducing growth medium after one (○), two (□) and three generations (●). All these samples were exposed to the EDTA/penicillin lysis procedure, and lysis was followed spectrophotometrically at 420nm in cuvettes of 1 cm light path.

cell material (Table 1). These experiments therefore fail to show any evidence for heterogeneity of nitrate reductase distribution in the progeny of cells transferred from inducing to non-inducing growth conditions.

Discussion

Our results fail to support a semi-conservative segregation of nitrate reductase among the three cell generations after transfer to non-inducing growth conditions.

It is not clear why our results differ from those of Autissier & Kepes (1972). Difference of markers cannot be responsible, because similar results were reported irrespective of whether or not the cytoplasmic membrane marker was a permease or nitrate reductase. The semi-conservative model of membrane segregation favoured by these authors is difficult to reconcile with fast lateral mobility of proteins in the cytoplasmic membrane of *E. coli* (Haest *et al.*, 1974; Sackmann *et al.*, 1973). This difficulty does not arise with our own observations. Furthermore, the use of nitrate reductase as a marker

for membrane segregation allowed, in conjunction with the density-gradient separation, the direct assay of the marker in lysed and non-lysed cells. For these reasons we are inclined to favour our own observations and conclusions over those of Autissier & Kepes (1971).

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