Biosynthesis and Turnover of Outer-Membrane Proteins in *Escherichia coli* ML308-225

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Isolated outer membranes and outer-membrane extracts from *Escherichia coli* ML308-225 in the early-exponential growth phase contain more protein than do corresponding preparations from late-exponential- or stationary-phase bacteria. Isotope-dilution experiments show that this is due to a loss of protein from the membrane during the exponential growth phase. Inhibition of bacterial growth and protein synthesis stabilizes the outer-membrane-protein concentration. Protein synthesis in the absence of bacterial growth results in higher concentrations of protein in the outer membrane.

Proteins in the outer membrane of *Escherichia coli* were first reported by Miura & Mizushima (1968) and were characterized by Schnaitman (1970). The importance of outer-membrane proteins to the permeability of the outer membrane was demonstrated by Nakae & Nikaido (1975). Structural roles for these proteins in the cell envelope include covalent linkage to the cell wall by Braun's lipoprotein (Braun & Bosch, 1972), interaction with lipopolysaccharide (Schweizer *et al.*, 1978) and formation of a protein matrix by the major outer-membrane protein (Rosenbusch, 1974). Minor outer-membrane proteins have also been implicated in the control of DNA replication (Gudas *et al.*, 1976) and of cell division (James, 1975). In view of the apparent importance of these proteins to the structure and function of *E. coli*, we have examined the biosynthesis and turnover of these proteins during the bacterial growth cycle.

Outer-membrane proteins by definition were originally characterized after the separation of bacterial membranes by sucrose-density-gradient centrifugation (Miura & Mizushima, 1968; Schnaitman, 1970). Subsequent isolation techniques have exploited the differential solubility of the proteins in a crude membrane fraction. Procedures based on the solubility of the cytoplasmic-membrane proteins in buffered Triton X-100 and of the outer-membrane proteins in buffered SDS have been devised (Schnaitman, 1973; Hindennach & Henning, 1975). Although such techniques are convenient for multiple or large-scale extractions, they may be deceptive in studying outer-membrane-protein biosynthesis or turnover, as many factors may influence the yield of extractable protein. Our observations based on the extraction of proteins from crude membrane preparations have been confirmed with purified outer-membrane preparations.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and Methods

Bacterial growth

Escherichia coli ML308-225 was a gift from Dr. W. A. Hamilton of the Department of Biochemistry, University of Aberdeen. It was maintained on nutrient agar slopes. Liquid cultures were grown at 37°C with forced aeration in minimal-salts medium containing 0.5% (w/v) D-glucose and 0.2% (w/v) L-leucine. For radioactivity-incorporation experiments, L-[³H]leucine (The Radiochemical Centre, Amersham, Bucks., U.K.) was added to give a final specific radioactivity of between 35 and 45 µCi/g.

Turbidity at 650 nm was used to measure bacterial growth. Culture samples were also examined by phase-contrast microscopy. Bacteria were harvested by centrifugation for 30000g-min. In experiments on protein biosynthesis, culture samples were made 100 µg/ml with respect to chloramphenicol before centrifugation. Quantification of outer-membrane-protein extraction was done with cold-acetone-dried and weighed bacteria.

Preparation of membranes

Preliminary experiments showed that membranes from bacteria lysed by ultrasonication were unsuitable for fractionation; an observation suggests that fusion of the outer and cytoplasmic bacterial membranes occurs during sonication (De Leij & Witholt, 1977). Accordingly, bacterial protoplasts were prepared by the method of Weiss (1976). Duplicate samples of early-exponential- and stationary-phase bacteria were prepared. In each case one sample was used for protoplast preparation and the other was acetone-dried to give the dry weight of bacteria used in the membrane fractionation. Protoplasts were lysed by dilution into 10 vol. of cold water. The crude membrane fraction was sedimented by centrifugation for 2000000g-min. A

2.0 ml suspension of this fraction was layered on to a 35 ml discontinuous sucrose gradient as described by Schnaitman (1970). After centrifugation for 7700000g-min in the Beckman SW27 rotor, the gradient was collected in fractions of 2.0 ml from the bottom of the tube. Fractions were assayed for protein by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Extraction of outer-membrane protein

The method used was based on those of Schnaitman (1973) and of Hindennach & Henning (1975). Freshly harvested or acetone-dried bacteria from a 10 litre culture was resuspended in 50 ml of assay buffer and sonicated for successive 30 s intervals on an MSE Sonicator (fine probe, maximum amplitude) until microscope examination revealed that less than 10% of the cells remained intact. The suspension was centrifuged for 30000g-min to remove whole cells and then for 2000000g-min to sediment the crude membrane fraction.

This precipitate was resuspended in 15 ml of 10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 1.5% (v/v) Triton X-100 and 0.05% (v/v) 2-mercapto-ethanol. The suspension was incubated at room temperature for 60 min and was then centrifuged for 2000000g-min. The precipitate was resuspended in 5 ml of 15 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 2% (w/v) SDS and 0.05% 2-mercapto-ethanol (SDS extraction buffer). This suspension was incubated and centrifuged as described above and the supernatant was retained as the outer-membrane-protein fraction. Protein content was measured by the method of Lowry *et al.* (1951), with bovine serum albumin dissolved in SDS extraction buffer as a standard. Fractions were analysed by SDS/polyacrylamide-gel electrophoresis by using the continuous- and the discontinuous-buffer methods described by Bragg & Hou (1972). Protein samples were heated to 100°C for 3 min before electrophoresis. Stained gels were scanned with an ISCO 1310 densitometer.

For radioactivity-incorporation experiments, culture samples were processed as described above, but by using one-tenth of the volumes. In these experiments the supernatants obtained from the bacterial lysates were retained and the soluble protein was precipitated by incubation at 4°C with equal volumes of 10% (w/v) trichloroacetic acid. After 60 min, the mixtures were centrifuged for 200000g-min and supernatants were decanted. The precipitates were vigorously mixed with SDS extraction buffer. After incubation at 20°C for 60 min, they were again centrifuged for 2000000g-min and the protein concentrations of the supernatants were measured as described above.

For measurement of radioactivity, 50 µl portions of the extracts were dried on 21 mm-diameter glass-

fibre discs (Whatman GFC). The discs were put into standard scintillation vials and 1 ml of toluene containing 0.27% (w/v) 2,5-diphenyloxazole was added to each. Radioactivity was measured over 10 min intervals in a Packard Tri-Carb liquid-scintillation spectrometer. Background radioactivity was measured with portions of extraction buffer dried on to glass-fibre discs. The efficiency of radioactivity counting was measured by using samples of a ³H standard (Packard) dried on glass-fibre discs, and was found to be 32%.

The effects of inhibition of protein synthesis and of bacterial growth on the outer-membrane-protein concentration were measured in cultures treated with 0.05 g of chloramphenicol/litre or 0.01 g of nalidixic acid/litre (both from Sigma, St. Louis, MO, U.S.A.). The cultures were processed as described for the radioactivity-incorporation experiments.

Results and Discussion

The experiment summarized in Table 1 shows that the yields of outer-membrane proteins extracted from *E. coli* ML308-225 vary with the growth phase of the culture. They represent about 7–8% of the total bacterial protein in the early-exponential phase of growth, but fall to about 3–4% in the late-exponential and stationary phases. These results were confirmed by the analysis of bacterial membranes fractionated by sucrose-density-gradient centrifugation. The outer-membrane fraction of late-exponential-phase bacteria contained about half of the protein present in the corresponding fraction from early-exponential-phase bacteria. Protein contents of the inner-membrane fractions were similar.

Fig. 1 shows the results of SDS/polyacrylamide-gel electrophoresis. Extracts made from cultures at different phases of growth are comparable by continuous-gel electrophoresis, and show a single major band. Discontinuous-gel electrophoresis (Bragg & Hou, 1972) resolves the major band into at least three components. Both electrophoresis techniques also show a component of low mobility and hence of high molecular weight. A similar profile has

Table 1. *Extraction of outer-membrane proteins*
The total protein present in outer-membrane-protein extracts was determined as a percentage of the total bacterial protein.

<i>A</i> ₆₅₀ of culture when harvested	0.18	0.25	0.90	1.4
Total outer-membrane protein extracted (mg)	46.0	39.5	16.0	26.0
Outer-membrane protein (% of total bacterial protein)	7.8	7.1	2.8	4.2

Fig. 1. Densitometric records of polyacrylamide-gel electrophoresis

The top of the gel is at the left-hand side in each case. For full experimental details see the Materials and Methods section. (a) Early-exponential-phase outer-membrane proteins, continuous electrophoresis. (b) Late-exponential-phase outer-membrane proteins, continuous electrophoresis. (c) Early-exponential-phase outer-membrane proteins, discontinuous electrophoresis. (d) Late-exponential-phase outer-membrane proteins, discontinuous electrophoresis. (e) Early exponential-phase outer membrane, discontinuous electrophoresis. (f) Concentrated culture supernatant, discontinuous electrophoresis.

been observed by Schnaitman (1974) for *E. coli* ML308, by using discontinuous-gel electrophoresis. A sample of outer membrane prepared from exponential-phase bacteria by sucrose-density-gradient centrifugation shows an electrophoretic profile that is similar to those of the extracts.

It can be concluded that the extraction technique used gives a protein preparation that is very like the protein present in isolated outer membranes. Furthermore, the observed fall in outer-membrane-protein concentration that accompanies bacterial growth is not due to an alteration in the efficiency of extraction. The overall amount of these proteins in a culture increases as growth proceeds, but there is less protein in the outer membrane of late-exponential- and stationary-phase bacteria than in the outer membrane of early-exponential-phase organisms. The membrane-fractionation experiment showed similar amounts of protein in the inner-membrane fractions. This suggests that, for a given weight of bacteria, the amount of membrane does not vary with growth, as would occur if bacterial size or shape varied. Phase-contrast microscopy confirmed that there was no significant variation in the size or shape of bacteria within the growth range used in these experiments.

There are two explanations that would account for these observations: the biosynthesis of outer-membrane proteins may be retarded or stopped, or

the proteins may be lost from the membrane as growth proceeds. These explanations were tested by an isotope-dilution experiment. A culture containing [^3H]leucine was grown into the early-exponential phase and was then rapidly harvested by centrifugation. Most of the cells were used for membrane extraction and the remainder were resuspended in fresh unlabelled medium at 37°C and the same cell density as before harvesting. Samples were then taken at intervals for membrane extraction. The protein concentration and radioactivity of each extract were used to calculate the specific radioactivity of outer-membrane protein at each stage of growth. In addition, a similar value was calculated for bacterial cytoplasmic protein at each stage.

The initial specific radioactivity of the outer-membrane proteins was 1.7 $\mu\text{Ci/g}$; subsequent specific radioactivities are shown in Fig. 2 as a percentage of the initial value. Fig. 2 also shows corresponding specific radioactivities for cytoplasmic protein, expressed as a percentage of the initial value,

Fig. 2. Specific radioactivity of outer-membrane and cytoplasmic protein during the bacterial growth cycle. A 4 litre culture containing 0.2% (w/v) L-[^3H]leucine (35 $\mu\text{Ci/g}$) was harvested at $A_{650}^{15} = 0.15$. An outer-membrane-protein extract was made from 60% of the cells; the remainder were resuspended in non-radioactive medium and incubated at 37°C. Extracts were made of samples taken at intervals and the specific radioactivity of each was calculated and plotted as a percentage of the specific radioactivity of the first extract (1.7 $\mu\text{Ci/g}$). Cell lysates were used to derive specific radioactivities for soluble cytoplasmic protein, also expressed as a percentage of the initial value (4.9 $\mu\text{Ci/g}$). ○, Specific radioactivity of outer-membrane protein; ●, specific radioactivity of cytoplasmic protein; ▲, bacterial growth. The arrow indicates the point at which the radioactive medium was removed.

4.9 $\mu\text{Ci/g}$. The specific radioactivity of outer-membrane-protein fraction declines at a slightly faster rate than that of the cytoplasmic fraction during the period of exponential growth. This indicates either a higher rate of synthesis of outer-membrane protein or a loss of protein from the outer membrane. The latter alternative is in agreement with the previous results.

The specific radioactivity of the outer-membrane-protein fraction continues to fall during the stationary phase, which suggests that the synthesis of outer-membrane proteins continues after net cytoplasmic protein synthesis has ceased. This result was confirmed by an experiment in which a late-exponential-phase bacterial culture was transferred into medium containing [^3H]leucine that had previously been used to grow a similar culture. Samples from this culture were processed as in the previous experiment and the results are shown in Table 2. The specific radioactivities are expressed as percentages of the final specific radioactivities of the outer-membrane- and cytoplasmic-protein extracts: these were 2.2 and 4.5 $\mu\text{Ci/g}$. The specific radioactivity of the cytoplasmic-protein fraction rises rapidly during the final stages of bacterial growth, but remains almost constant throughout the stationary phase. In contrast, the specific radioactivity of the outer-membrane-protein fraction continues to rise throughout the stationary phase. There is no evidence for an early termination of the production of outer-membrane proteins; their incorporation into the membrane continues into the stationary phase, although they may have been synthesized at an earlier stage.

The rates of synthesis of the two protein fractions were also measured in the early stages of exponential growth. Fig. 3 summarizes the results of this experiment, in which the radioactive leucine was added to

the culture 30 min before taking the first sample, and the specific radioactivities of the protein extracts were determined at 30 min intervals as described above. The final specific radioactivities were 1.2 and 3.4 $\mu\text{Ci/g}$ for the outer-membrane- and cytoplasmic-protein extracts respectively. It is apparent that the specific radioactivities of both series of extracts rise at a similar rate and it can be concluded that, in the early-exponential growth phase, the rates of production of outer-membrane and cytoplasmic proteins are similar. Thus the more rapid decline in specific radioactivity of the outer-membrane proteins reported in Fig. 2 is due to a loss of proteins from the membrane, rather than to a lower rate of synthesis and incorporation into the membrane.

The prolonged incorporation of proteins into the outer membrane reported in Table 2 is presumably responsible for the slight rise in protein concentration in the final extraction outlined in Table 1. The loss of proteins from the membrane must have ceased for this rise in concentration to occur. Thus proteins are only lost from the outer membrane during the exponential period of growth, suggesting that this phenomenon is caused by rapid growth or cell division. The release of outer-membrane fragments from exponentially grown *E. coli* JC 411 has been reported

Fig. 3. Specific radioactivity of outer-membrane and cytoplasmic protein during the early stages of the bacterial growth cycle

A 4 litre bacterial culture was inoculated. At the time indicated by the arrow, L-[^3H]leucine was added to give a specific radioactivity of 4.5 $\mu\text{Ci/g}$. Samples were taken at intervals and outer-membrane-protein extracts were prepared. The specific radioactivity of each was calculated and plotted as a percentage of the specific radioactivity of the final extract (1.2 $\mu\text{Ci/g}$). Cell lysates were used to derive specific radioactivities for soluble cytoplasmic protein, also expressed as a percentage of the final value (3.4 $\mu\text{Ci/g}$). ○, Specific radioactivity of outer-membrane protein; ●, specific radioactivity of cytoplasmic protein; ▲, bacterial growth. The arrow indicates the point at which the [^3H]leucine was added.

Table 2. Specific radioactivity of outer-membrane and cytoplasmic protein during the late stages of the bacterial growth cycle

A 1 litre culture containing 0.2% (w/v) [^3H]leucine (45 $\mu\text{Ci/g}$) was harvested at $A_{650} = 1.0$. The supernatant was used to resuspend cells from a non-radioactive but otherwise identical culture. Samples (300 ml) were harvested to make outer-membrane-protein extracts and the specific radioactivity of each was calculated. Cell lysates were used to derive specific radioactivities for soluble cytoplasmic protein.

Time after addition of radioactive medium (h)	1	3	19
A_{650} of culture when harvested	1.17	1.45	1.48
Specific radioactivity of outer-membrane proteins ($\mu\text{Ci/g}$)	0.7	1.2	2.2
Specific radioactivity of cytoplasmic proteins ($\mu\text{Ci/g}$)	2.7	4.2	4.5

(Mug-Opstelten & Witholt, 1978), confirming a previous observation (Rothfield & Pearlman-Kothencz, 1969).

This deduction is confirmed by the experiments summarized in Fig. 4. Addition of either chloramphenicol or nalidixic acid results in the inhibition of bacterial growth, and in stable amounts of outer-membrane proteins. These values are low (approx. 4% of bacterial dry weight) when protein synthesis is directly inhibited by chloramphenicol, but are much higher (approx. 11% of bacterial dry weight) when nalidixic acid inhibits DNA synthesis and bacterial growth. Thus, when protein synthesis continues in the absence of bacterial growth, the protein concentration in the outer membrane rises to twice the maximum value observed in normally grown cells.

We have attempted to demonstrate outer-membrane proteins in bacterial culture supernatants. The supernatant from a 250 ml late-exponential-phase culture was dialysed against water at 4°C for 24 h and then freeze-dried. The residue was dissolved in 0.3 ml of SDS extraction buffer. Polyacrylamide-gel electrophoresis (Fig. 1f) showed very faint protein bands corresponding to those of an outer-membrane-protein extract, with a strong band of lower-molecular-weight material. The protein concentration of the solution was too low to be accurately measured by

the Lowry *et al.* (1951) assay, but the total protein recovery was estimated to be less than 20 µg.

Thus only small quantities of outer-membrane proteins were detected in the culture supernatant. It is possible that proteins released from the membrane may be degraded by extracellular proteinases. The existence of cell-envelope proteinases in *E. coli* (Murgier *et al.*, 1977) suggests the possibility of proteolytic degradation within the membrane giving rise to diffusible degradation products.

The physiological significance of these observations is unclear. The release of outer-membrane fragments may be an inevitable consequence of the incorporation of newly synthesized membrane material (Mug-Opstelten & Witholt, 1978), although this phenomenon results in the preferential release of newly synthesized outer-membrane protein. In view of the functional importance of several outer-membrane proteins, the turnover of these proteins may be desirable for the control of cell growth and division. Our results could account for the turnover of the outer-membrane-associated functions of the *E. coli* tonB gene (Kadner & McElhaney, 1978). Flexibility in the amounts of functional proteins in the outer membranes may only be obtained at the cost of partial loss of structural proteins. Viable mutants lacking all the major outer-membrane proteins have been described (Henning & Haller, 1975), so it is unlikely that the partial loss of these proteins is deleterious.

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Fig. 4. Effect of antibiotics on the biosynthesis and incorporation of protein into the outer membrane

In the control experiment, 2 litre samples were taken from a 10 litre culture at 1 h intervals. The cells in each sample were acetone-dried and the outer-membrane-protein concentration was determined as a percentage of the bacterial dry weight. Similar experiments were performed in which chloramphenicol (0.05 g/litre) or nalidixic acid (0.01 g/litre) was added 30 min before the first sample was taken, as indicated by the arrow. ○, Control culture; ●, chloramphenicol-treated culture; ▲, nalidixic acid-treated culture.

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