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Influence of membrane-lipid composition on translocation of nascent proteins in heated *Escherichia coli*

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In studies using *Escherichia coli* we have shown that new protein species appear in the outer membrane fraction with concomitant losses of nascent proteins from the soluble and inner membrane fractions following heat exposure. Of the various explanations for this phenomenon, temperature-induced membrane disorganization appeared the most likely. It is suggested that heat mimics the action of the signal sequence of a protein on the lipid bilayer allowing non-signal-sequence-containing proteins to be translocated. To test this hypothesis we grew *E. coli* K1060 cells, an unsaturated fatty acid requiring auxotroph, supplemented during growth with fatty acids of varying chain length in an attempt to determine whether biological membranes of varying ability to maintain their bilayer configuration could be constructed. The rationale being that such membranes would allow us to determine whether differences in translocation would occur in cells grown at the same temperature supplemented with either 16:1 or 20:1 unsaturated fatty acids when the cells were subjected to a series of thermal insults. Protein translocation occurred to a greater extent and at lower temperatures in cells supplemented with the longer chain fatty acid. Treatment of outer membranes with either 1 M salt, 6 M urea or high pH and studies determining fluorescent polarization values by scanning up and down through a series of temperatures ranging from 15 to 49 °C indicate that the proteins translocated by heat to the outer membrane are integral. Protein translocation may represent an adaptive response to an altered environment enabling the cell to respond to stress by stabilizing its outer membrane.

Introduction

In a recent study we have shown that new protein species appear in the outer membrane fractions with concomitant losses of nascent proteins from the soluble and inner membrane fractions of heated *E. coli* [1]. Several phenomena could underlie this translocation of protein. One possible explanation is that the new outer membrane proteins are heat-stress proteins [2–4]. The data obtained using a heat shock minus strain or rifampicin-treated *E. coli* mitigate strongly against that explanation [1].

A second possibility is that the phenomenon is explainable by heat-induced conformational changes (denaturation) of cytoplasmic proteins which causes them to adhere to the outer membrane during disruption and fractionation of cells.

Abbreviations: 16:1, palmitoleic acid; 18:1, oleic acid; 20:1, eicosenoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; EDTA, ethylenediamine tetracetic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DEGS, diethylene glycol succinate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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That is highly unlikely based on the results of pulse-chase experiments which clearly demonstrate that only nascent proteins undergo heat-induced translocation [1]. Furthermore, the fact that there are no heat-induced changes in the Coomassie blue staining patterns of proteins already in the soluble and inner membrane fractions after 5 min at 48 °C also argues against this non-selective mechanism [1].

A third possibility is that elevated temperature increases membrane disorganization and promotes a phase transition of the membrane lipids of *E. coli* resulting in an increase in zones of adhesion [5] or interlipidic connections between the inner and outer membranes [6–8]. Strong support for the model that zones of adhesion provide pathways for protein translocation to the outer membrane is forthcoming from the work of Smit and Nakaido [9]. They showed that newly synthesized porins appeared on the cell surface as discrete patches uniformly distributed over zones of adhesion sites. Whether these zones of adhesion are emphemeral structures which are greatly increased by local perturbations such as hyperthermia remains to be determined.

The current study strongly supports the theory that heat is acting non-specifically to trigger changes in membrane lipids resulting in a flow of the majority of newly synthesized proteins to the outer membrane. We determined whether translocation occurred to a greater or lesser extent in cells grown at the same temperature, supplemented with either 16 : 1 or 20 : 1 unsaturated fatty acids, and subjected to a series of thermal insults. The evidence presented is consistent with the idea that variable amounts of non-bilayer states normally exist in biological membranes and are likely to be important in maintaining normal function in cells exposed to changing ambient conditions.

Materials and Methods

E. coli K1060 (R. Overath strain, CGSC0-5040, Yale University) were grown in Cohen–Rickenberg medium [10] supplemented with either palmitoleic acid (16 : 1) or *N*-eicosenoic acid (20 : 1). Cells were grown overnight to stationary phase at 39 °C, 25 ml were transferred to flasks containing 500 ml of supplemented medium and grown to an absorbance of 0.8 ± 0.1 at 650 nm (midlog phase) for experiments.

Cells were harvested by centrifugation ($1000 \times g$), washed two times with Cohen–Rickenberg buffer, and resuspended in Cohen–Rickenberg buffer that had been preheated in a waterbath to either 39 °C or 42–48 °C. Amino acid incorporation was assayed using L-[^{35}S]methionine (specific activity, 800 Ci/mmol; New England Nuclear, Boston, MA), 1 μCi/ml was added to the incubation buffer prior to the addition of *E. coli*.

When outer, inner and soluble cell fractions were desired cells were handled in the following way. They were harvested by centrifugation at $5000 \times g$ for 10 min and washed two times with 100 ml of Hepes buffer (10 mM Hepes/1 mM EDTA/5 mM 2-mercaptoethanol/10% glycerol, pH 7.4). The final pellet was suspended in 20 ml Hepes buffer and cells disrupted in a French press at 83 MP_a using two passes. Unbroken cells and cell debris were removed by centrifugation and the supernatant reserved for cellular fractionation. Total membranes of disrupted cells were pelleted by centrifugation at $149\,000 \times g$ for 60 min at 4 °C. The supernatant (soluble fraction) containing both the cytoplasmic and periplasmic fractions was reserved for electrophoresis and the pellet was resuspended in 2 ml of Hepes buffer.

The procedure of Janoff et al. [11] was used to isolate inner and outer membranes. Protein was determined by the method of Lowry [12] and depending on the experiment either equal amounts of protein or radioactivity were loaded on SDS-polyacrylamide gel electrophoresis gels.

To obtain the periplasmic fraction, the method of Neu and Heppel [13] was used. The cell pellet was resuspended in 45 ml of a solution containing 10 mM Tris base, 0.4 mM EDTA, and 20% sucrose (pH 7.1). After incubation for 10 min at room temperature with gentle stirring, the bacteria were centrifuged for 10 min at $12\,800 \times g$ at 4 °C. The supernatant was removed and the pellet resuspended by vigorous in 10 ml of 0.1 mM magnesium chloride at 4 °C. This suspension was incubated in an ice bath for 10 min with occasional stirring and then centrifuged at $12\,800 \times g$ for 10 min. The supernatant, which constituted the periplasmic fraction, was collected. The pellet, which

constituted the outer membrane, inner membrane and the cytoplasmic fraction, were suspended in the Hepes buffer and the fractions were collected as described above.

The cytoplasmic enzyme 2-galactosidase was assayed by the procedure of Miller [14].

SDS-polyacrylamide gel electrophoresis was run according to the method of Laemmli [15] with separating gels of 10% acrylamide and 4.5% acrylamide stacking gels. One-dimensional gels were stained in 0.125% Coomassie blue. Molecular weights were estimated from the following standards: bovine serum albumin (M_r = 66 000), ovalbumin (M_r = 45 000), glyceraldehyde-3-*P* dehydrogenase (M_r = 36 000), carbonic anhydrase (M_r = 29 000) and trypsinogen (M_r = 24 000). Acrylamide, bis-acrylamide, SDS, Coomassie blue and all protein standards were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorography, according to the method of Bonner and Lasky [16] was used to detect radioactive protein bands on gels which were dried in vacuo. Two-dimensional gel electrophoresis was performed by the Kendrick Laboratory (Madison, WI) according to the method of O'Farrell [17]. Isoelectric focusing in glass tubes, using 1.6% (pH 5–7) and 0.4% (pH 3.5–10) ampholines (LKB Instruments, Baltimore, MD), was carried out at 400 for 12 h, followed by 800 V for 30 min. The final tube gel pH gradient extended from pH 3.8–8.3 as estimated from acetylated cytochrome p*I* markers (Cal-Biochem-Behring, La Jolla, CA). Purified vitamin D dependent calcium-binding protein (M_r = 27 000; p*I* = 4.2) was the internal standard. Protein standards used were Myocin (M_r = 20 000), phosphorylase A (M_r = 94 000), catalase (M_r = 60 000), actin (43 000 Mr) and lysozyme (M_r = 14 000). They appeared in fine horizontal lines on silver-stained [18], 10% acrylamide slab gels. Flurorography was done as described above.

Fatty acid analysis was performed on a gas chromatograph (Tracer, Model 220) using methyl esters of the fatty acis. Lipids were extracted from isolated membranes by a modification of the methods of Folch et al. and Bligh and Dyer [19,20]. Membrane samples (3 ml) in Hepes buffer were used for lipid extraction. The lipids were then resuspended in 6 ml of 0.5 M methanolic HCl (Supelco) and refluxed for 1–2 h at 75°C to produce methyl esters of the fatty acids. The lipids were cooled and extracted twice with 10 ml of petroleum ether and concentrated by evaporation. A 10% DEGS on 80/100 chromsorb W column programmed from 150 to 195°C at 3°C/min following an initial 3 min hold on 150°C was used for separation of the fatty acid methyl esters. Fatty acids were identified by relative retention times of known standards and peak area determinations by integration with a Varian (Model 4270) integrator.

Phospholipids were analyzed as described by Jacobson and Yatvin [21]. Authentic standards were used to identify the various phospholipids. After identification, phospholipids were scraped from the TLC plates, extracted and quantitated by determining inorganic phosphorus [22].

1,6-Diphenyl-1,3,5-hexatriene was the probe used for membrane fluorescence polarization determinations. A stock solution of DPH ($3.2 \cdot 10^{-3}$ M) in tetrahydrofuran was stored at 4°C, and 1 μl was added per ml of membrane suspension while rapidly vortexing, and the polarization *P*-values were determined using an Elscint MV1-a microviscosimeter. Samples were diluted until the *P*-value did not change to avoid errors introduced by light scattering. Then an additional 0.25 μl DPH per ml of membrane suspension was added while rapidly vortexing. *P*-value determinations were done in triplicate over a temperature range of 7–59°C. Samples were kept in the dark after DPH was added [23].

The outer membrane fraction of cells grown in oleic acid and incubated for 10 min at 39 or 48°C were each diluted in 4 ml of Hepes buffer. Each temperature group was then divided into four 1 ml aliquots. The samples were incubated at 21° after the addition of either 1 ml Hepes buffer, 1 ml of 0.03 M sodium hydroxide (final pH, 11.4), 1 ml 2 M sodium chloride (final concentration, 1 M sodium chloride) or 1 ml 12 M urea (final concentration, 6 M urea), respectively. Following a 20 min incubation period, the samples were diluted to 20 ml with Hepes buffer and centrifuged at 190 000 × *g* for 1 h at 4°C in a 70 Ti rotor. The control, high pH, high saline and 6 M urea-treated outer membranes were resuspended in 1 ml of Hepes buffer, assayed for protein using the Lowry procedure and subjected to gel electrophoresis as described above.

Results

Escherichia coli K1060 supplemented with either 16 : 1 or 20 : 1 unsaturated fatty acids and grown as described above were incubated for 10 min with L-[^{35}S]-methionine at 39, 42 and 43° C with no measurable loss in cell viability. Subcellular fractions were isolated and proteins resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The fluorograms of the one-dimensional gels reveal that there is a quantitative increase in nascent proteins of the outer membranes from 20 : 1 supplemented cells exposed to either 42 or 43° C, compared to those exposed to 39° C, the growth temperature (Fig. 1). In addition, the 20 : 1 supplemented cells at 43° C had both greater qualitative and quantitative increases in outer-membrane-labelled proteins compared to either similarly grown cells heated to 42° C or those supplemented with 16 : 1 and exposed to 43° C (Fig. 1). By 47° C (data not shown) translocation of nascent protein was quite similar in the outer membranes of 16 : 1 and 20 : 1 supplemented cells.

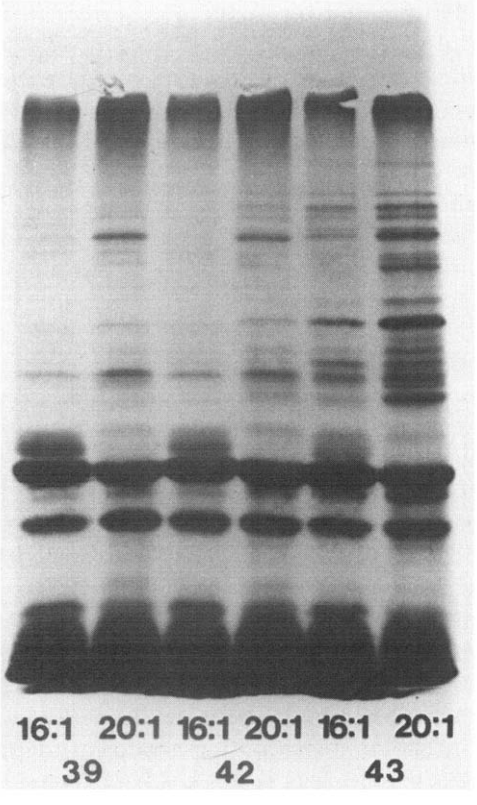

Fig. 1. Fluorogram of *E. coli* K1060 outer-membrane protein patterns of cells supplemented during growth with 16 : 1 or 20 : 1 unsaturated fatty acids and grown at 39° C. Cells were exposed for 10 min to 39, 42 and 43° C.

When two dimensional gel electrophoresis was performed on the outer membrane fraction of control (39° C) and heated (43° C) K1060 cells, the differences in the patterns of protein translocation to the outer membrane appear even more striking than the one-dimensional gels. Fig. 2a and b compare fluorograms of control (39° C) 16 : 1 and 20 : 1 treated cells. As with the one-dimensional gels, the differences between fluorograms of these differently supplemented unsaturated fatty-acid-grown cells are minimal, but nevertheless real. More striking differences are seen when one compares the fluorograms depicted in Fig. 2c and d. As in the 1D gel patterns, many more proteins have been translocated to the outer membrane in 20 : 1 supplemented cells exposed to 43° C, compared to similarly heat-treated 16 : 1 grown cells. Fig. 3 is a histogram of total units of 2-galactosidase obtained from membranes of equal numbers of cells grown in medium supplemented with either 16 : 1 or 20 : 1 and subjected to 39, 45 or 48° C. It can be seen that slightly more 2-galactosidase exists at growth temperature in the 20 : 1 supplemented cells and at 45° C the increase above control is almost twice that noted in the 16 : 1 grown cells (11-fold increase compared to a 7-fold increase). By 48° C, although the differential is minimal, in the 20 : 1 grown cells it is 22-times background compared to 21-times background in 16 : 1 supplemented cells (Fig. 3).

Table I depicts the ability of these bacteria to alter their fatty acid composition in response to the unsaturated fatty acid supplied in the medium. There are differences in the fatty acid distribution between total, inner and outer membranes within each supplemented group. The most striking feature of the membranes from 20 : 1 supplemented cells is the relatively small amount of 20 : 1 incorporated and overall a lack of uniformity in acyl chain length. In the 16 : 1 supplemented cells the acyl chain lengths were quite uniform with more than 90% of the membrane fatty acids were 16 carbons in length.

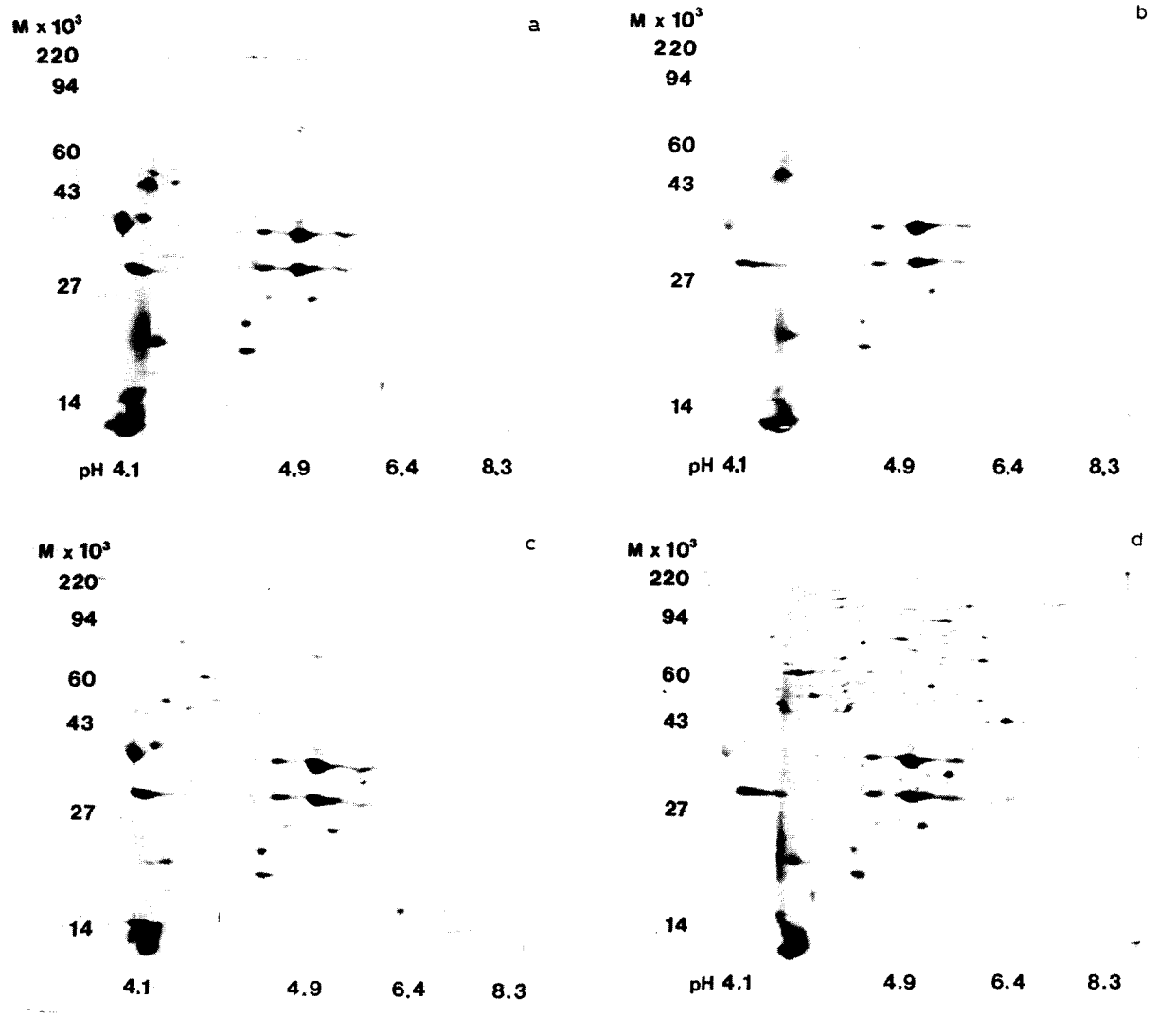

Fig. 2. Fluorogram of *E. coli* K1060 outer- membranes. (a) Two-dimensional gel electrophoresis of outer-membrane proteins proteins of cells grown at 39 ° C supplemented with 16 : 1 fatty acid and exposed to 39 ° C for 10 min. (b) Two-dimensional gel electrophoresis of outer-membrane of cells grown at 39 ° C supplemented with 20 : 1 fatty acid and exposed to 39 ° C for 10 min. (c) Two-dimensional gel electrophoresis of outer-membrane proteins of cells grown at 39 ° C supplemented with 16 : 1 fatty acid and exposed to 43 ° C for 10 min. (d) Two-dimensional gel electrophoresis of outer-membrane proteins of cells grown at 39 ° C supplemented with 20 : 1 fatty acid and exposed to 43 ° C for 10 min.

As shown in Table II in both 16 : 1 and 20 : 1 grown cells cardiolipin content is markedly increased relative to 18 : 1 grown cells, whereas the phosphatidylglycerol, phosphatidylethanolamine contents are reduced. How the altered distribution of phospholipids contributes to the differences in translocation in heated 16 : 1 and 20 : 1 grown cells is not known.

The results, shown in Fig. 4a and b, demonstrate that the new proteins observed in the outer membrane, if not integral, are certainly tightly bound. This assumption is predicated on one-dimensional SDS-polyacrylamide gel electrophorisis patterns obtained from the treatment of 37 and 48 ° C outer membranes isolated from cells supplemented with oleic acid. Aliquots of these mem-

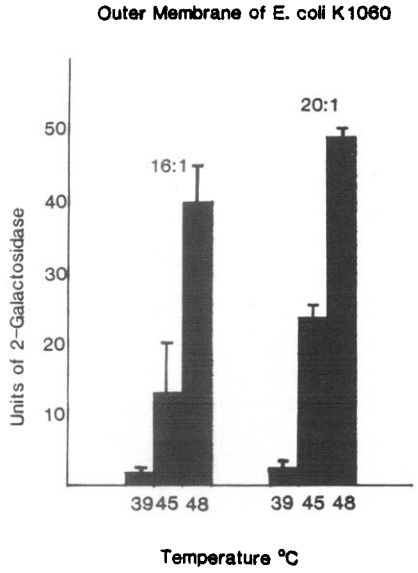

Fig. 3. Outer membrane 2-galactosidase activity. The effect of heat (10 min) on total 2-galactosidase activity (units/10^{10} cells) of outer membranes of *E. coli* K1060 grown at 39° C and supplemented with either 16:1 ($n = 5$) or 20:1 ($n = 4$) fatty acids is depicted. A significant one-tailed t-test ($\alpha = 0.05$) was obtained at 45° C.

TABLE I

FATTY-ACID COMPOSITION OF *ESCHERICHIA COLI* K1060 TOTAL, INNER AND OUTER MEMBRANE FRACTIONS

Fatty-acid supplement	12:0	14:0	16:0	16:1	20:1
Total membranes					
20:1	6.6 [a]	36.6	38.7		18.1
16:1		9.3	48.8	41.9	
Inner membranes					
20:1	1.1	32.8	34.9		31.2
16:1		6.0	52.4	41.6	
Outer membranes					
20:1		55.1	31.6		13.3
16:1		7.6	54.1	38.3	

[a] % of total fatty acid.

TABLE II

PHOSPHOLIPID HEADGROUP ANALYSIS OF *ESCHERICHIA COLI* K1060 TOTAL MEMBRANES

Fatty-acid supplement	Phosphatidyl ethanolamine	Phosphatidyl glycerol	Cardiolipin
16:1	77.0 [a]	3.8	19.1
18:1	87.0	6.1	6.9
20:1	68.6	3.0	28.3

[a] % of total phospholipids.

branes received 20 min treatments at 21° C in either Hepes buffer at pH 7.4, 1 M NaCl, 6 M urea or Hepes buffer at pH 11.4. As can be seen

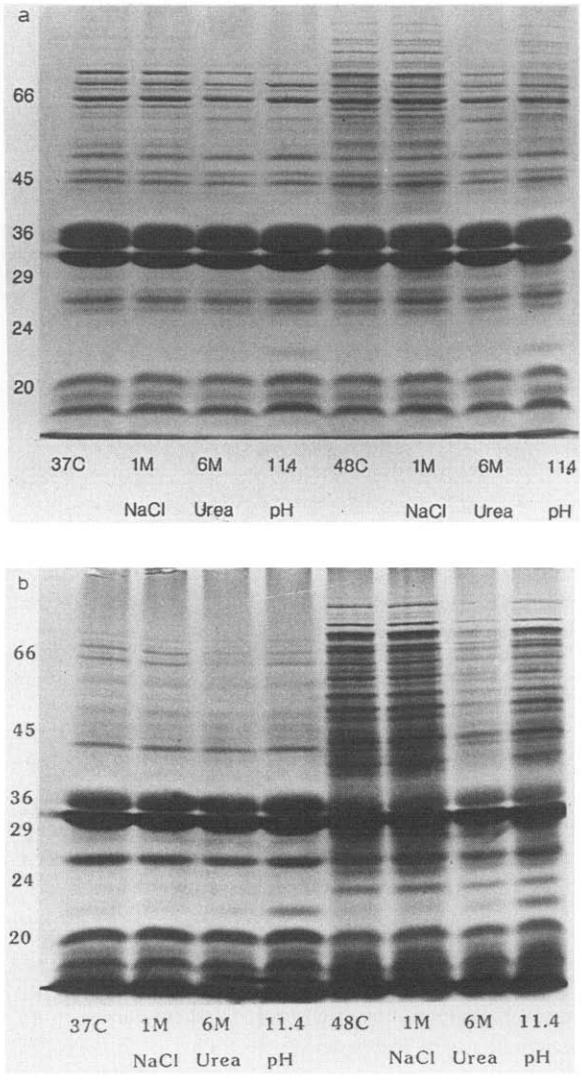

Fig. 4. (a) Effects of saline, pH and urea and banding patterns of *E. coli* K1060 outer-membrane proteins. Patterns 1–4 are aliquots of an outer-membrane fraction collected from the same sample cells grown at 37° C supplemented with oleic acid (18:1). To obtain 5–8 an aliquot of the above-grown cells was heated to 48° C for 10 min and the outer-membrane fraction collected. Fractions 1 and 5 were exposed to Hepes buffer only, 2 and 6 were exposed to 1 M NaCl, 3 and 7 were exposed to 6 M urea, and 4 and 8 were exposed to pH 11.4 Hepes buffer. All samples were exposed for 20 min at 21° C. Equal amounts of proteins were run (150 μg/well). (b) Fluorogram of (a).

from the Coomassie blue staining patterns shown in Fig. 4a the outer membranes treated with 6 M urea lose protein and there is also a slight decrease in outer membrane proteins of cells treated with pH 11.4 buffer. Proteins normally found in the outer membrane of 37°C exposed cells were also susceptible to 6 M urea at pH 11.4 buffer. The fluorographs revealed a decrease in labelled protein after treatment with 6 M urea, without a complete loss of any individual protein bands. The loss was more marked in the fluorogram of 48°C outer membrane. An increase in a low molecular weight protein seen in the pH 11.4 treated membrane may be a degradation product (Fig. 4b).

Depicted in Fig. 5 are the fluorescent polarization values of the outer membranes collected from cells grown at 39°C in medium supplemented with 20 : 1 which were then heat treated for 20 min at 39 or 48°C. The cells exposed to 48°C have a consistently higher polarization value beginning between 30°C and 35°C. Similar increases in polarization values are obtained with outer membranes of 16 : 1 and 18 : 1 supplemented cells subjected to 48°C heating compared to controls (data not shown).

Discussion

In an earlier study, we reported the appearance of new protein species that are non-signal sequence containing in the outer membrane fractions of heat-treated cells and the loss of proteins from the soluble and inner membrane fractions of *E. coli* K1060 cells. Using rifampicin treated cells and a strain of *E. coli* that does not show the heat shock response, heat-induced nascent protein translocation was still observed [1]. To explain our observation we proposed that elevated temperature increases membrane disorganization and promotes a phase transition, resulting in an increase in either zones of adhesion [5] or interlipidic connections between the inner and outer membranes such as occur in multilamellar vesicles [6–8]. To test this hypothesis cells were grown supplemented with 16 : 1 and 20 : 1 fatty acids in an attempt to produce membranes with lipids showing variable responses to heat.

The simplest explanation for the data shown in Fig. 1 and 2a–d is that the formation of either such zones of adhesion, non-bilayer intermediates, or interlipidic connections may occur in heated cells which facilitate translocation of the majority of newly synthesized protein from the cytoplasm to the outer membrane. From the data in Figs. 1 and 2a–d it is apparent that translocation of protein is greater at 42 and 43°C in the 20 : 1 grown cells than in the 16 : 1 grown cells.

There is evidence in studies using model membranes composed of phospholipids with fatty acids of similar chain lengths, that such membranes exhibit greater stability than when wide variation exists in acyl chain length [26,27]. As seen in Table I, the fatty-acid chain lengths of the 16 : 1 grown cells are much more uniform (90% of the acyl chains are 16 carbons long) than those grown in medium supplemented with 20 : 1 unsaturated fatty acid. Therefore, one possible explanation of the differences in translocation between these bacterium supplemented with the two different fatty acids (Figs. 1 and 2) is that the greater stability obtained in the 16 : 1 grown cells apparently reduces the extent of heat induced interlipid junctions.

Another possible explanation is based on the work of Seddon et al. [28] who demonstrated that the hexagonal phase transition T_H values measured for saturated di-*n*-acyl and alkylphosphatidylethanolamine rise sharply as the acyl chain length is decreased. Similar T_H values for phosphatidylethanolamines containing unsaturated acyl chain have also been reported (Tilcock and Cullis, 1982; Gagne et al. 1985; and Silvius et al. 1985) [29–31]. As the temperature is increased above 46°C, the differences in translocation rate between 16 : 1 and 20 : 1 grown cells rapidly disappear (data not shown). A further indication of the disappearing differences as exposure temperature is increased between differently supplemented cells is found in Fig. 3. It can be seen that differences exist at 45°C in the total 2-galactosidase content, but by 48°C these differences no longer are significant (Fig. 3). The data in Fig. 3 support the conclusion that at the higher (48°C) temperature sufficient membrane instability had occurred to allow essentially maximum translocation in both membrane systems.

In our earlier work [1] the evidence as to whether the proteins translocated at elevated temperatures

are integral membrane proteins or simply associated with the outer membrane was strong but not conclusive. This issue has been further addressed by treatment of isolated outer membranes obtained from cells exposed to either 37 or 48° C with 1 M salt, 6 M urea or high pH (Fig. 4a and b). Although there are major differences between the outer membrane patterns of 37 and 48° C treated cells, within both groups 6 M urea had the same effect of depleting proteins from the gel banding patterns. We interpret this as supporting the contention that the translocated proteins, if not integral membrane proteins, are tightly bound.

Even though many of these newly translocated proteins (2-galactosidase, etc.) are not normally membrane bound, recent work with cytochrome b_5 suggests that highly polar proteins can be inserted into membranes by mechanisms involving non-specific destabilization of membranes [32]. One such mechanism is heat which increases the propensity of both phospholipids [33] and lipopolysaccharides [34,35] of *E. coli* membranes to form non-bilayer structures. When polarization values of outer membranes from cells supplemented with 20 : 1 fatty acid and exposed to 39 and 48° C were determined over a wide temperature range (7 to 59° C), repeated heating and cooling of the sample through this temperature range did not alter the polarization pattern. This was, in fact, true even when the sample was repeatedly cycled up and down through the temperature range on subsequent days. Since the protein-induced increase in membrane microviscosity was retained, it can be concluded that the new proteins were tightly bound to the membrane.

At growth temperatures, it has been suggested that proteins are translocated by a mechanism involving hydrophobic sequence insertion into lipid membranes [36–39]. Implicit in this theory is the postulate that proteins which are translocated are synthesized on membrane-bound polyribosomes. The binding can occur as a result of signal sequence interaction, either with a signal channel protein [36] or with membrane phospholipids [37–39]. A further attachment site on the membrane is postulated via a ribophorin-ribosome interaction [38].

Based on the present study and our earlier work [1] of heat-induced protein translocation it would appear that the majority of soluble proteins in *E. coli* are in fact synthesized on membrane-associated polyribosomes, since during heating the majority of newly synthesized proteins are translocated to the outer membrane. The determination of what proteins are synthesized by free or membrane-bound polyribosomes has been very difficult to resolve. Cancedda and Schlesinger [41] in a study aimed at resolving this issue in *E. coli* for secreted and cytoplasmic proteins concluded that their thin section electron micrograph and their biochemical data did not permit them to resolve the issue. Interestingly, *E. coli* heat-stress proteins do not translocate during heating. Whether their falure to translocate is related to their site of synthesis or other factors is unclear [40]. Furthermore, an explanation of why apparently only nascent proteins are translocated needs to be elucidated. One possibility is that once the nascent peptide is released from the polyribosomes and assumes its mature configuration, translocation can no longer occur. A number of factors could explain this lack of translocation of mature proteins, including nascent peptides forming multisubunit protein or being modified by post-translational change including proteolytic processing, altering their shape and hydrophobicity.

A recent report suggests that one must view compartmentalization of *E. coli* proteins with caution. Tommasson et al. [42] found differences in distribution of hydrophobic proteins of *E. coli* which were determined by either cell fraction or immunocytochemical techniques. Their concerns apply to the distribution of hydrophobic proteins and probably not relevant to our studies, since most of the proteins we see translocated normally reside in the cytoplasmic and periplasmic fractions and are therefore hydrophilic. Furthermore, we have demonstrated that heated labelled cytosolic proteins do not associate (bind) to outer membranes [1].

The most likely explanation for the triggering event in heat-induced non-signal sequence protein translocation is a heat-induced disordering of membrane lipid structures which permits the transfer of nascent protein to the outer membrane. The use of fatty acids of varying chain lengths during growth of the unsaturated fatty acid requiring auxotroph *E. coli* K1060 provides support that

the triggering mechanism for protein translocation in heated cells is membrane bilayer reorganization during heating. While membrane fluidity per se could be the critical factor in outer membrane and periplasmic proteins reaching their final compartment [43], it would appear that cells grown in the presence of medium supplemented with 16 : 1 fatty acids produce more heat stable membrane bilayers (at lower temperatures) than the same cells grown in medium supplemented with 20 : 1 fatty acid.

In addition to heat, other conditions such as changes in pH, ion concentration, or other local perturbations might alter the physical state of the lipid portion of the membrane. We postulate that such changes could result in altered protein translocation and would provide cells such as *E. coli* a rapid mechanism to respond to environmental stress by stabilizing the outer membrane by increasing lipid order. Furthermore, the temperatures and heating times employed in these studies are non-lethal and the resulting protein translocation may represent an adaptive response to an altered environment. Such protein translocations occur rapidly (within 10 s of heat exposure) in the absence of RNA synthesis and could be part of the trigger mechanism the cell utilizes to increase membrane lipid order as part of its initiation of adaptive responses to a variety of stresses including heat [40] (e.g., homeoviscous adaptation [44], stress protein induction, etc.).

It is still an open question, however, as to whether adding protein to a bilayer results in an increase in lipid order. The fluorescent probe used in this study (DPH) indicates increased order when protein is translocated to the outer membrane (Fig. 5). It has been argued that although DPH is supposed to mimic the lipid molecule or chain, the probe molecule is rigid and much larger than the C–C bonds of a lipid chain and may not be sensitive to any small disorder of the CH_2 groups [45]. Furthermore, data from NMR studies have been interpreted to suggest that added protein ('high' protein-lipid molar ratios) would have a disordering effect on lipids [46]. Whether the approximate doubling of outer membrane protein we observed fits the criteria of 'high' protein-lipid molar ratios is unclear. Nevertheless, regardless of how it affects lipid order most probably the new protein in the outer membrane of heated cells

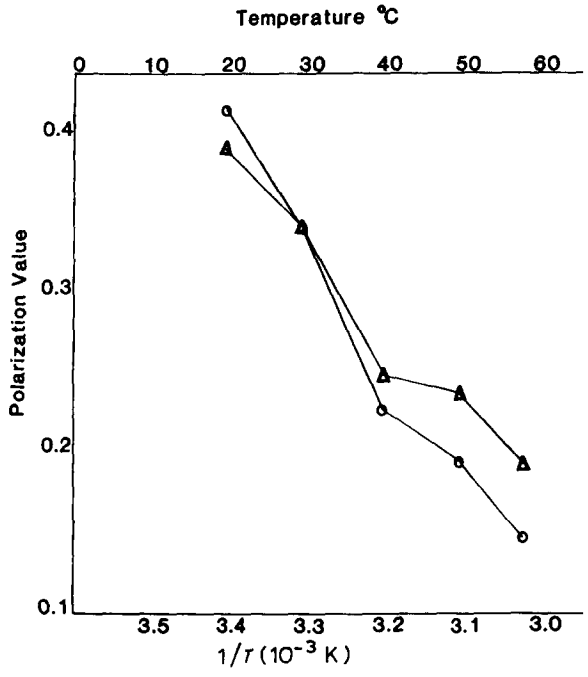

Fig. 5. Fluorescent polarization values of *E. coli* K1060 outer membranes. Cells were grown at 39 °C supplemented with 20 : 1 fatty acid. Outer membranes from cells exposed to 39 (○) and 48 °C (△) for 10 min were mixed with DPH and fluorescent polarization values determined over a temperature range of 7–59 °C.

contribute to their adaptive response.

The three major findings in this report are (1) that the fatty-acid composition of the membrane influences heat-induced translocation of non-signal-sequenc-containing protein; (2) this occurs as a result of heat mimicking the effect of a signal sequence on the lipid bilayer; and (3) that the translocated proteins are integral outer membrane proteins.

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