brief communication

Deviation from homeoviscous adaptation in *Escherichia coli* membranes

Abraham H. Parola,* Mohammad Ibdah,‡ David Gill,§ and Arieh Zaritsky‡
Departments of *Chemistry, ‡Biology, and §Physics, Ben-Gurion University of the Negev, Beer Sheva 84105 Israel

ABSTRACT The process by which an organism changes the composition of its membranal fatty acids in response to growth temperature, so as to maintain optimal membrane functioning, is known as homeoviscous adaptation (HA). One expression of HA is the constancy of the fluorescence polarization (P) of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in membranes of cells grown at various temperatures. The P of DPH in the membranes of *Escherichia coli* was shown by us to be inversely proportional to bacterial growth rate on different carbon sources. This result, implying failure of HA, is now complemented by measurements of DPH lifetimes, which indicate that the dominant variables contributing to the drop in P are (*a*) the order parameter of the membrane, which goes down, and (*b*) the fluidity, which may slightly increase. These are then the changes induced by enhanced growth rate. Two additional effects, cell membrane permeability and sensitivity to thermal shock, determined by the diffusion of *o*-nitrophenyl-galactoside (ONPG) and by exposure to 52°C, respectively, are reported to increase with growth rate. We can now conclude that there is a deviation from the principle of HA in *E. coli* grown at various rates, brought about by controlling the growth media at constant temperatures.

INTRODUCTION

At physiological temperatures, the membrane bilayers of most organisms are liquidlike, a state characterized by a high degree of molecular motion (1). A sudden reduction of temperature results in a reversible phase transition to a hexagonal arrangement of the lipid fatty acids (2).

Membranes of thermophilic bacteria (adapted to grow optimally at high temperatures) contain more branched and saturated fatty acids than membranes of mesophils and psychrophils (which grow at medium and low temperatures, respectively) (3–5). The membranes of mesophilic bacteria, such as *Escherichia coli*, are richer in saturated fatty acids when grown at higher temperatures (6). Enrichment with unsaturated fatty acids is achieved at low temperatures by raising the proportion of phospholipids containing two unsaturated acyl chains (7). Such phospholipids have lower melting points and higher molecular flexibility than the analogues with saturated acyl chains (1, 2, 6, 8). High levels of unsaturated fatty acids thus compensate for the increased order and rigidity of lipids, caused by growth at low temperatures. Poikilothermic (ambient temperature) organisms have adaptive responses which compensate the direct effects of temperature changes and regulate the order and dynamics of membrane lipids for optimal functioning. This control mechanism, which preserves membrane order parameter by adaptive changes in lipid composition, is known as "homeoviscous adaptation" (HA) (9–11). Its general validity is taken as a working hypothesis.

In *E. coli*, temperature-induced changes in the activity of β-ketoacyl-acyl carrier protein synthase II were attributed to HA, mediated by lipid–protein interaction (12–14). The activity of this membranal enzyme seems to be modulated by the order and fluidity of the membrane itself (15). Such modulation is feasible: as was recently shown, differences in the properties of the integral protein ($Na^+ + K^+$)-ATPase of fish, birds, and mammals originate in HA of membrane order, rather than in large differences in the primary or secondary structure of the proteins (10).

Relationships between biosynthesis, lipid composition, order, and function in membranes are best revealed by correlating changes produced by dietary supplementation (11). The growth rate of *E. coli* at constant temperature can be varied by modifying the medium composition (16, 17). Faster growing cells must have faster uptake rates of building blocks per surface area to satisfy their anabolic requirements. Indeed, higher rates of passive diffusion, facilitated diffusion, and protein-mediated transport of e.g. glycerol correlate with a reduction in membrane order (18). The question thus arises, whether HA holds for *E. coli* grown at different rates at a constant temperature: One may expect that at faster growth rates (in nutritionally richer medium), membrane order will be lower, so as to enable faster uptake of nutrients.

Please address correspondence to Dr. Abraham H. Parola at the Department of Chemistry, Ben-Gurion University of the Negev, P.O. Box 653, Beer Sheva 84105 Israel.

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Enlarged diameters of *E. coli* cells grown in richer medium have been found to be inversely related to membrane order parameter (17), determined by fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene (DPH). This biophysical measurement indicates relative changes primarily in membrane order parameter and, to some extent, in membrane fluidity (10, 19). However, P also depends on the excited state lifetime (τ) (20). The higher values of P observed at slower growth rates (17) could thus reflect differences in membrane lipid order parameter or dynamics only if τ were not respectively reduced.

If indeed the order parameter of *E. coli* membranes would vary with the growth rate (15), the generality of HA would be challenged. To further substantiate this unusual finding, additional independent support is called for. Thus, two biological parameters, known to be affected by membrane order in bacteria, were examined: uptake rates of nutrients as well as sensitivity to heat (21, 22). Both were found to be higher in cells which have less ordered membranes.

We show here that the average lifetime of DPH, measured by phase modulation fluorometry (23), essentially does not vary when *E. coli* is grown at reduced rates in minimal media. In addition, the transmembrane diffusion rate of *o*-nitrophenyl-galactoside (ONPG) in *E. coli* cells and the sensitivity to heat of such cells are positively correlated with growth rate.

MATERIALS AND METHODS

Bacterial cultivation

Strain H266 of *E. coli* B/r (15) was cultivated at 37°C with vigorous shaking. Various growth rates (μ), measured either by optical density (OD) at 450 nm on a microsample spectrophotometer (model 300N, Gilford Instrument Laboratories, Inc., Oberlin, OH) or by turbidity with a #66 red filter on a Klett Summerson spectrophotometer (New York), were achieved by supplementing A + B minimal salts solution with different carbon sources as already detailed by us before (17); faster growth rate (μ = 3.0 h^{-1}) was obtained in Luria Broth (LB).

Cells (grown overnight in the various media) were diluted (at the logarithmic phase, OD ~ 0.4) 1:1,000 into a fresh (37°C) medium, and upon reaching again their logarithmic phase were harvested by centrifugation (10 min at 4°C, 1,200 *g*). Growth was terminated when the cells were washed twice in PBS (phosphate buffered saline, pH 7.4) and pellets (with similar cell densities and growth phase) were used for further studies.

Fluorescence lifetime measurements

Pellets were resuspended in PBS (OD 0.25 at 450 nm) and labeled (60 min at 37°C) in 3.10^{-5} M DPH, previously solubilized in THF (tetrahydrofuran; final concentration of 0.1%) (17).

Fluorescence lifetimes were measured on a model 4800 phase-modulation spectro-fluorometer (SLM Instruments, Inc., Urbana, IL), equipped with a depolarizer at the exit of the excitation monochromator and dichroic polarizers set at 55°. With excitation at 360 nm, fluorescence emission was passed through a Kodak Wratten sharp cut-off filter (Rochester, NY) at 400 nm. The phase reference was a zero-lifetime (scattering) solution (24). Five measurements (or more) of phase and modulation were taken at 6, 18, and 30 MHz in accumulation time windows of 25 s, and converted into τ_{phase} and τ_{mod} by SLM lifetime software on an Apple IIe microcomputer. Heterogeneity analysis of τ_{phase} and τ_{mod} data was carried out by least-squares analysis program ISSC6-87, assuming phase and modulation frequency-independent measurement errors, σ_{phase} = 0.2 and σ_{mod} = 0.004, respectively (25).

Uptake of ONPG and activity of β-galactosidase

ONPG is a derivative of β-galactoside which is transported through the membrane either by passive diffusion or by β-galactoside permease; within the cytoplasm, ONPG undergoes fast hydrolysis by β-galactosidase, releasing *o*-nitrophenol (ONP) which can be determined spectrophotometrically. Thus, the rate of ONP production represents the rate of total transport; by using thiodigalactoside (TDG), a competitive inhibitor to β-galactoside permease, the extent of nonmediated transport can also be determined.

The lactose operon, which encodes for both β-galactoside permease and β-galactosidase, was induced with 0.5 mM isopropylthiogalactoside (IPTG) in an overnight culture. Induction was maintained with IPTG when the culture was diluted 1,000-fold with fresh, prewarmed medium. Cultures were centrifuged at the logarithmic phase, resuspended in an equivalent volume of PBS containing 50 μg/ml of chloramphenicol (a bacteriostatic drug), and ONP production was determined by absorbance (at 420 nm). In vivo rates of ONPG hydrolysis and activities of total cellular β-galactosidase were determined in a total volume of 2 ml containing 2 mM substrate with cells equivalent to 4–6 KU (Klett units). After incubation at 28°C, the reactions were stopped with 1 ml of 1 M Na_2CO_3, and rates/activities were determined according to Miller (26), i.e., [β-galactosidase activity] = (OD_{420} − 1.75 OD_{550}) × 1,000/(time × volume × OD_{600}), where OD_{420} indicates the combined absorbance of ONP and the turbidity of the cells, 1.75 OD_{550} is the cell turbidity contribution at 420 nm, and OD_{600} is the turbidity of the sample; "time" is the duration of the reaction (min) and "volume" is that of the cell sample added to the reaction mixture (ml). These calculated values of activity are given in Figs. 1 and 2.

Passive permeability was measured in 6 mM thiodigalactoside (TDG; an active transport inhibitor). Under these conditions, ONPG is transferred exclusively by passive diffusion and is then split by the cytoplasmic enzyme β-galactosidase to galactose and ONP. The release of ONP is thus an excellent measure of the relatively slow passive diffusion of ONPG (shown in Fig. 1).

Mediated transport (shown in Fig. 2) was calculated by subtracting the passive permeability values from the in vivo ONPG hydrolysis values. For measuring total β-galactosidase activity in vitro, an unwashed cell sample (2 ml) was lysed with a drop of toluene and incubated at 37°C for 40 min. Production of ONP was then determined (26).

Heat sensitivity

Dennis and Yatvin reported a direct correlation between membrane order parameter and heat sensitivity of auxotrophic bacteria grown in various fatty acids (21). We looked for correlation between properties determined by the growth medium (e.g., membrane order) and survival after a heat shock. Accordingly, glycerol and LB cultures grown at the logarithmic phase were diluted 1,000-fold into 5 ml of fresh medium,

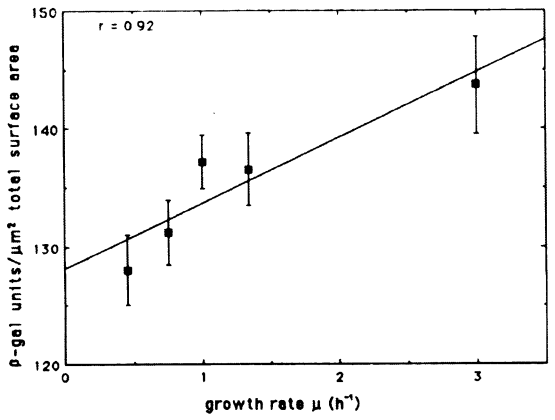

FIGURE 1 Relative rate of diffusion of ONPG through the membranes of *E. coli* grown on different sources of carbon, quantified in terms of β-galactosidase activity (see Materials and Methods; 26) per unit surface area (27).

prewarmed to 52°C. At intervals, 0.1- or 0.2-ml samples were taken and added to a similar medium at 37°C, then plated on LB or on glycerol minimal medium, each containing 1.5% (wt/vol) agar. The number of colonies was counted after overnight incubation at 37°C (Fig. 3).

RESULTS

Each pellet was resuspended in PBS, labeled with DPH and τ_{phase} and τ_{mod} were measured. The results were analyzed by heterogeneity analysis (25). Minimal χ^2 values were obtained (Table 1) for two lifetimes, averaged at 9.8 ± 0.8 (SD) and 1.92 ± 0.3 ns with approxi-

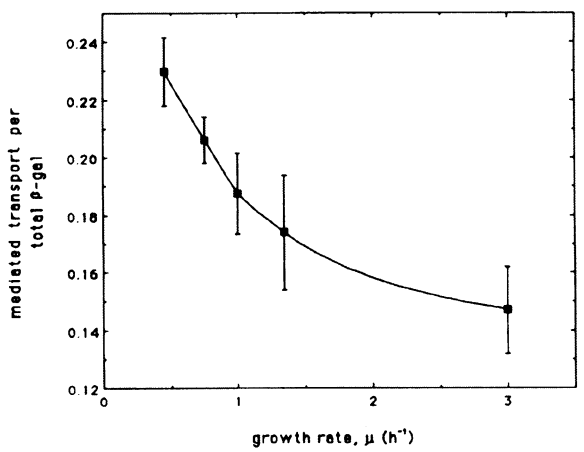

FIGURE 2 Dependence on growth rate of ONPG-mediated transport, determined by the ratio of β-galactosidase activity in intact cells to that in lysed cells (for details, see Materials and Methods).

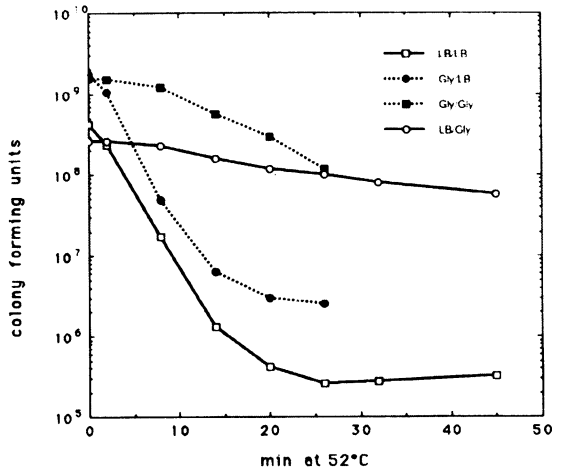

FIGURE 3 Survival curves of *E. coli* pregrown on medium X at 37°C, exposed to 52°C in X for *t* min (*x*-axis), removed to X at 37°C, and then plated on medium X or Y (with 1.5% [wt/vol] agar) and incubated at 37°C overnight. Code for media: X/Y, cells pregrown on medium X, plated on Y. Cells grown slowly on Gly media before and after the heat shock survived better.

mate fractional contributions (F) of 0.7 and 0.3, respectively; the calculated amplitude- and intensity-weighted mean lifetime values, $\langle\tau_a\rangle$ and $\langle\tau_F\rangle$, respectively, are shown as well (Table 1). Had $\langle\tau\rangle$ remained constant and independent of cell growth rate, then the rise in fluorescence polarization would represent a rise in membranal order parameter (17). The generally observed lengthening of $\langle\tau_a\rangle$ with reduced growth rate, or essentially unaltered $\langle\tau_F\rangle$ (Table 1) further supports this conclusion. Thus, our conclusion that HA fails when growth medium composition is modified is confirmed. Our measurements at only three modulation frequencies allowed fitting to biexponential decay with low χ^2.

Additional evidence of failing HA came from assaying three independent biological parameters that are affected by membrane order: activity of the membranal enzyme β-galactoside permease, membrane permeability to ONPG and heat sensitivity of the cells.

Assuming that the ratio between β-galactoside permease and β-galactosidase per cell remains constant, the mediated transport rates of ONPG were quantified in terms of the ratio (mediated transport) to (total β-galactosidase activity). This normalization to total β-galactosidase activity is essential because of the observed variation in enzyme levels in various cultures. Cells of *E. coli* B/r grown in different carbon sources have variable surface/volume ratio: faster growing cells have a smaller ratio (27). The simple diffusion of ONPG was thus expressed in terms of β-galactosidase activity per unit surface, and was found to rise with growth rate (Fig. 1).

TABLE 1 Fluorescence lifetimes of DPH in *E. coli* cultivated on different carbon sources

Medium	μ	τ*	α	$\langle\tau_n\rangle$‡	F§	$\langle\tau_F\rangle$‡	χ^2
	h^{-1}	*ns*		*ns*		*ns*	
LB	3.00	1.835 ± 0.018	0.673	4.3	0.289 ± 0.002	7.1	0.002
		9.243 ± 0.017	0.327		0.711 ± 0.002		
AB + glucose	1.46	1.815 ± 0.012	0.772	3.7	0.377 ± 0.002	7.0	0.006
		10.177 ± 0.034	0.228		0.623 ± 0.002		
AB + casein	1.36	1.568 ± 0.014	0.706	3.9	0.279 ± 0.002	7.4	0.003
		9.654 ± 0.015	0.294		0.721 ± 0.002		
AB + glycerol	1.00	1.843 ± 0.002	0.675	5.3	0.234 ± 0.008	10.0	45.52
		12.487 ± 0.195	0.325		0.766 ± 0.008		
AB + ala + pro	0.80	1.946 ± 0.023	0.668	4.5	0.291 ± 0.009	7.3	0.010
		9.557 ± 0.546	0.332		0.709 ± 0.009		
AB + succinate	0.59	2.305 ± 0.012	0.678	4.8	0.322 ± 0.002	7.5	0.002
		9.972 ± 0.018	0.322		0.668 ± 0.002		
AB + acetate	0.45	2.558 ± 0.021	0.664	5.1	0.335 ± 0.003	7.6	0.006
		10.066 ± 0.036	0.336		0.665 ± 0.003		

*In each pair of values, the top is τ_1 and the bottom is τ_2.
‡$\langle\tau_n\rangle = \langle\tau_{amplitude}\rangle = \alpha_1\tau_1 + \alpha_2\tau_2$.
$F_1 = \alpha_1\tau_1/\langle\tau_n\rangle$; $F_2 = \alpha_2\tau_2/\langle\tau_n\rangle$.
$\langle\tau_F\rangle = \langle\tau_{intensity}\rangle = F_1\tau_1 + F_2\tau_2$.
§In each pair of values, the top is F_1 and the bottom is F_2.

The mediated transport of ONPG per unit surface area is inversely related to growth rate (Fig. 2). These data may describe cells grown rapidly, having leaky membranes and reduced activity of a membranal enzyme.

Fig. 3 shows the recovery of *E. coli* cells after variable duration of exposure to 52°C. The broken lines are those of samples grown on glycerol minimal medium before the heat shock. They show higher recovery rates than the full lines, which represent cells grown in the rich LB medium before heating. This is of interest here because, when exposed to heat, the slowly grown cells had more ordered membranes. The two respective branches of the broken and the full lines in Fig. 3 represent the response to various media after the heat treatment. This branching indicates that cells recover better when grown slowly in the poor medium, probably because of the time given to repair vital functions.

DISCUSSION

Homeostasis of order in membranes of bacteria grown at different temperatures was demonstrated at both biochemical (11, 12) and biophysical (9, 10) levels. The change in growth temperature regulates the activities of envelope enzymes involved in fatty acids metabolism (12), so that HA is maintained. Thus, a rise in membrane order enhances the activity of enzymes associated with the biosynthesis of unsaturated fatty acids. Investigations of *E. coli* in vivo suggested that temperature was not the sole determinant of the ratio of unsaturated to saturated fatty acids (13).

The composition of the growth medium, applied at constant temperature, determines the bacterial growth rate and cell morphology (17, 27). We ask which other biological parameters are affected and whether HA is one of them. In our initial study (17), we reported that the fluorescence polarization of the lipophilic probe DPH decreased in membranes of *E. coli* grown at fast rate. This implied deviation from the HA principle (8, 9). The transition from P value to order parameter requires the use of the Perrin equation (19, 20), in which P values also depend on the excited state lifetime. We have shown here that the average lifetime values of DPH essentially did not vary in bacteria grown in richer media at higher rates (Table 1). Therefore, the associated reduction in P does reflect reduced membrane order, and the corresponding linear correlation between 1/P and cell diameter (17) expresses a real reduction of membrane order with growth rate. We can now conclude that there is a deviation from homeoviscous adaptation.

The double exponential decay of DPH fluorescence was reported before in other cell membranes (28) or even in artificial membranes of a pure single phospholipid (29). Such results can always be interpreted in terms of microheterogeneity. We suggest a nontrivial microheterogeneity in gram negative bacteria, such as *E. coli*, which have two membranes, outer and cytoplasmic. In the

present case, the short lifetime component has a larger preexponential value than in other live (28) or artificial (29) systems, perhaps pointing to a significant property.

Diffusion rate of ONPG is reduced when the bacterial membrane is more ordered (30). Hence, we measured the permeability of membranes to ONPG as an additional indicator of order. Because the surface/volume ratio also changes according to growth rate (27), we determined the relative permeability as the rate of ONPG diffusion per unit surface (Fig. 1) and concluded that in *E. coli*, the rate of ONPG diffusion per unit surface is proportional to growth rate.

In *E. coli* B/r, the ratio of (mediated transport) to (total β-galactosidase activity) inversely correlates with growth rate (Fig. 2). Thus, the faster rate of growth reduced the activity of β-galactoside permease and also lowered the order in the membrane. If indeed the activity of this enzyme responds to disorder in the membrane lipid phase, then it is an additional case of lipid–protein interaction. A similar correlation between the activity of β-galactoside permease (as well as other membranal enzymes, e.g., Mg^{+2}-ATPase [31]) and membrane order was already reported before (32). However, other membranal enzymes (e.g, protein-mediated transport of glycerol [18] and [$Na^+ + K^+$]-ATPase [30]) show the opposite response to lipid–protein interaction. In the case of Mg^{+2}-ATPase (31), the decrease in activity was attributed to vectorial displacement (33), modifying the exposure of the active site (24). The same hypothesis may account for the present results with β-galactoside permease. Thus, it was recently suggested that the molecule comprises two globular domains of similar size, separated by a solvent accessible cleft. The galactose binding site is very likely contained within this cleft (34). Such a location of the sugar binding site can be anticipated from earlier reports indicating that the monomer of lactose permease is active in galactoside binding. The bound substrate is in a low dielectric region within the folded polypeptide and yet the site is also connected to the membrane surface by an aqueous channel. Obviously lipid order could play a major role in varying the exposure of this binding site, presumably by the mechanism of vectorial displacement (33).

Another biological parameter associated with membrane order is bacterial heat sensitivity (21) because, with excessive thermal motion, a breakdown of the normal barrier properties of membranes (11) and perhaps the thermal denaturation of their enzymes may occur (10). For clarity, we distinguish between the history of the cells before and after heating. Cells, which had grown slowly on minimal medium (Gly) before heating, recovered better than those which had grown faster on rich medium (LB). Among other differences between the preheated samples, the first one had more ordered membranes and also was less damaged by the heating. During recovery from the heat shock, the dependence on the medium again subdivided each of the primary samples into a pair (Fig. 3). Though unrelated to the present work, the pairs of curves indicate best recovery when cell growth is slowed down by poor nutrition; The rate of repair seems to be inherently slow and a lag in growth is required.

In another set of experiments not reported here, the critical temperature of phase transition in faster growing cells (at a richer medium), monitored by temperature profiles of ONPG uptake, was also found to be lower. This is an additional evidence that the membranes are less ordered.

In conclusion, the patterns of ONPG uptake by the cells as well as their heat sensitivity confirm our initial suggestion that there is a deviation from the principle of homeoviscous adaptation in bacteria grown at various rates, brought about by modifying the growth media.

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