Molecular Microbiology (1999) **32**(6), 1173–1182

Transcription- and translation-dependent changes in membrane dynamics in bacteria: testing the transertion model for domain formation

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Summary

Cell cycle events have been proposed to be triggered by the formation of membrane domains in the process of coupled transcription, translation and insertion ('transertion') of nascent membrane and exported proteins. Disruption of domain structure should lead to changes in membrane dynamics. Membrane viscosity of *Escherichia coli* and *Bacillus subtilis* decreased after inhibition of protein synthesis by chloramphenicol or puromycin, or of RNA initiation by rifampicin, but not after inhibition of RNA elongation by streptolydigin or amino acid starvation of a stringent strain. The decrease caused by inhibitors of protein synthesis was prevented by streptolydigin if added simultaneously, but was not reversed if added later. The drug-induced decrease in membrane viscosity is energy dependent: it did not happen in KCN-treated cells. All treatments decreasing membrane viscosity also induced nucleoid compaction and fusion. Inhibition of macromolecular synthesis without membrane perturbation caused nucleoids to expand. Changes in membrane dynamics were also displayed during a nutritional shift-down transition that causes imbalance in macromolecular syntheses. The results are consistent with the transertion model, predicting dissipation of membrane domains by termination of protein synthesis or detachment of polysomes from DNA; domain structure is conserved if the transertion process is 'frozen'.

Introduction

The precise timing and spacing of major events in the bacterial cell cycle are most intriguing, yet complicated, phenomena in cell biology, despite the rapidly increasing number of proteins and genes claimed to participate in these processes. Generating new ideas and challenging them can thus be stimulating and productive. One such idea suggests (Norris, 1995a) that both DNA synthesis and septum formation are triggered by specific membrane domains formed as a result of the process of coupled transcription, translation and insertion (transertion) of membrane proteins. The transertion process can also explain nucleoid shape and partitioning (Woldringh *et al*., 1995).

The idea of membrane domains is based on several experimental facts. (i) Insertion of membrane proteins occurs simultaneously with the coupled transcription/translation in bacteria, hence anchoring respective genes to the membrane (Lynch and Wang, 1993). (ii) The frequency of transcription initiation for major membrane proteins is very high (Kennel and Reizman, 1977), with hundreds of copies synthesized simultaneously. (iii) Many integral membrane proteins (e.g. ATPase, NADH dehydrogenase, diacylglycerolglucosyltransferase) have specific lipid preferences (Dancey and Shapiro, 1977; Ksenzenko and Brusilow, 1993; Karlsson *et al*., 1997). Thus, high-level expression of a gene encoding a membrane protein may result in the formation of a compact region (domain) in the membrane, which is highly enriched by this protein and its preferred phospholipid (Fig. 1). Nascent polypeptides are kept together in such a domain by the dynamic structure of growing polysomes that are tethered to the same stretch of DNA. The last feature provides a simple prediction, that disruption of this gene–polysome structure leads to the dissipation of domains and detachment of the nucleoid from the membrane. This prediction is consistent with nucleoid compaction in the centre of the *Escherichia coli* cell and a reduced number of DNA–membrane attachment sites induced by rifampicin (Rif) or chloramphenicol (Cam) (Morgan *et al*., 1967; Dworsky and Schaechter, 1973; Zusman *et al*., 1973; Van Helvoort *et al*., 1998). Membrane patterns, displayed in *E. coli* stained with FM 4-64 (see Fishov and Woldringh, 1999) and presumably reflecting phospholipid domains, were also sensitive to Cam treatment. Dissipation of such homogeneous and ordered proteolipid domains would be detected by measuring average membrane properties such as viscosity (the inverse of fluidity), thus testing this prediction of the transertion model. Modulation of the transertion process may be achieved by

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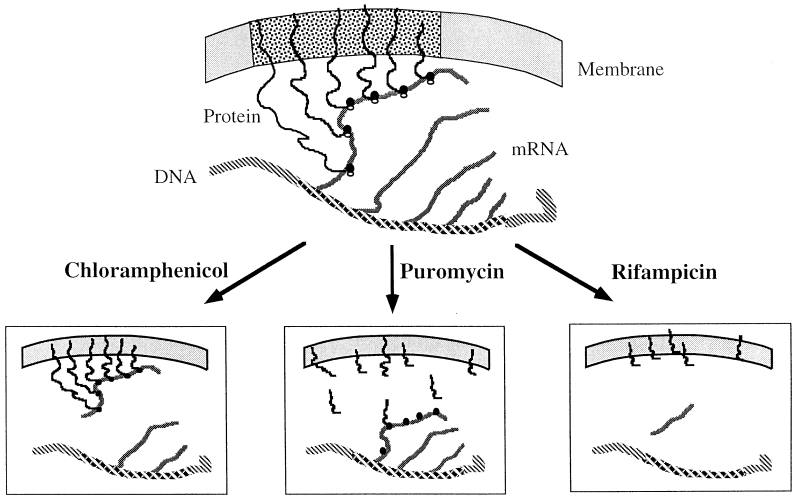

Fig. 1. Formation of a membrane domain (dotted area) as a result of coupled transcription, translation and insertion (transertion) of a membrane protein and predicted domain dissipation by drugs. For clarity, only one polysome is shown. Cam blocks protein synthesis by inhibiting peptidyl transferase activity, yet mRNA synthesis remains almost unchanged (Lark, 1972); Pur inhibits protein synthesis by premature release of nascent polypeptides (Smith *et al.*, 1965); Rif blocks the initiation of transcription, but does not affect elongation, which is already under way (Schleif, 1969; Lark, 1972). Nal, Stl and KCN are not expected to disintegrate the transertion (for explanation, see *Discussion*).

specific drugs that interfere with different stages of protein synthesis (Fig. 1) or by limiting energy and nutrient supply.

In this work, we show that membrane viscosity, as measured by anisotropy of fluorescence from *E. coli* cells labelled with 1,3-diphenyl-1,3,5-hexatriene (DPH), decreases remarkably upon addition of puromycin (Pur), Cam or Rif. We compare the drug effects with amino acid starvation in stringent and relaxed strains. The observed changes occur in the cytoplasmic membrane of *E. coli*, as DPH was found to stain this membrane preferentially (Fishov and Woldringh, 1999). This was confirmed further in the Gram-positive *Bacillus subtilis*; its additional advantage is that streptolydigin (Stl), which does not permeate through the outer membrane of *E. coli* (Schleif, 1969; Lark, 1972), can be used. This drug halts RNA elongation rather than initiation, which is inhibited by Rif (Schleif, 1969; Cassani *et al.*, 1971), thus leaving RNA polymerase (and polysomes) attached to the genes being transcribed (Cassani *et al.*, 1971). Hence, Stl addition may be considered as 'freezing' transertion. Disruption or freezing transertion was also reflected in the nucleoid morphology, as predicted by the model. Membrane viscosity, which decreases systematically with increased growth rate modified by medium composition (Zaritsky *et al.*, 1985; Parola *et al.*, 1990), did not change when growth rate was modulated by the availability of a single carbon source; it did decrease during the nutritional shift-down transition.

Results

Inhibition of macromolecular syntheses

To detect how nucleoid–membrane interactions are reflected in the membrane dynamics of *E. coli*, they were altered by inhibiting macromolecular syntheses: DNA replication was inhibited by nalidixic acid (Nal) (Goss *et al.*, 1965; Drlica, 1984), protein synthesis by Cam or Pur and RNA synthesis by Rif (Lark, 1972). Their effects on growth kinetics are well known and are not shown here. Mass growth stopped upon inhibition of protein or RNA synthesis and became linear in the presence of Nal, while in all cases, the rate of cell division descended to zero within about 20 min after drug addition. During steady-state growth of *E. coli* B/r H266 in glucose minimal medium, the value of DPH fluorescence anisotropy was stable at about 0.165 ± 0.004 for different cultures. Nal caused a slight increase in anisotropy; Cam, Pur or Rif induced a significant (12–15%) reduction in anisotropy (Fig. 2), which started after a reproducible lag period and was completed in about 30 min, despite the fact that growth ceased almost immediately. Final changes in anisotropy are presented in Table 1. The effect of Cam was fully reversible: anisotropy slowly returned to the initial value along with growth restoration after removal of the drug (Fig. 2).

All aspects of macromolecular synthesis are energy dependent and can thus be arrested by a shortage of ATP. A complete growth arrest by 3 mM KCN led to negligible changes in anisotropy; the addition of Cam had no further effect (Fig. 2, Table 1). Neither of these inhibitors changed DPH fluorescence anisotropy when added to fixed cells (data not shown).

The conserved DPH fluorescence lifetime at the lower anisotropy (Table 2) verifies that the decrease in membrane viscosity was the major reason for anisotropy changes induced by Cam (Shinitzky and Barenholz, 1978; and see *Experimental procedures*).

Stringent response

Protein synthesis can also be arrested by starvation for

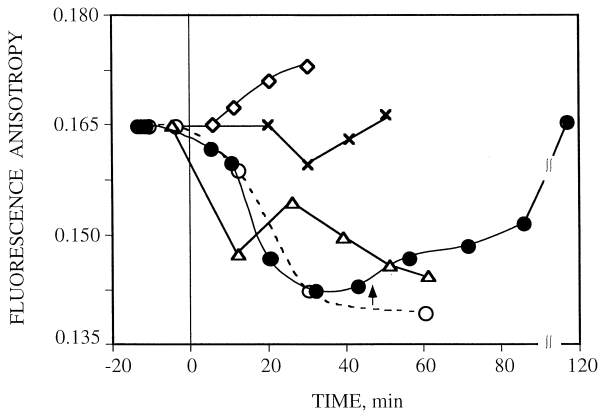

Fig. 2. DPH fluorescence anisotropy after the addition (at time zero) to glucose-grown culture of *E. coli* B/r of 10 μg ml^{-1} Nal (◇), 100 μg ml^{-1} Cam (●), 50 μg ml^{-1} Rif (△), 150 μg ml^{-1} Pur (○) or 3 mM KCN (×). Cam-treated cells were washed and resuspended in fresh prewarmed medium at 45 min (arrow). All data are shown as a percentage of a corresponding control, which was determined as an average anisotropy value measured in a steady-state culture.

essential amino acids. Taking into account that such starvation is coupled to the stringent response and pleiotropic effects of guanosine 3′,5′-bisdiphosphate (ppGpp) (Cashel *et al.*, 1996), membrane viscosity was determined in isogenic stringent RL331T (*relA*$^+$) and relaxed RL332T (*relA*) *E. coli* strains (Ryals *et al.*, 1982), and the effects of amino acid starvation were compared with those of Cam. A 10% reduction in anisotropy (from 0.153 ± 0.004 to 0.138 ± 0.004) was caused by Cam treatment in both strains, while only in the relaxed strain did it decrease under amino acid starvation to a similar extent (from 0.160 ± 0.007 to 0.146 ± 0.004). In addition to the unchanged membrane viscosity after amino acid depletion, the stringent strain differed in division run-out: about 70% of its cells completed division, whereas only 30% of the relaxed strain did (Fig. 3A); in both, mass increase stopped immediately (Fig. 3B). On the other hand, there was no difference in residual divisions (about 30%) after Cam treatment of both strains (Fig. 3A). As the time (D) between termination of the replication cycle and cell division is about 22 min (Helmstetter, 1996), it can be concluded that only the stringent cells in the D-period at the onset of amino acid starvation completed divisions under these growth conditions (doubling time of 28 min). In all the other three cases, the ability to complete all divisions was substantially reduced.

Inhibition of protein synthesis by either amino acid starvation, Cam or Rif also induces nucleoid fusion and compaction in filaments of *E. coli pbpB*, which is also a *relA1* mutant (Van Helvoort *et al.*, 1998). In the series of experiments described above, only the nucleoids of the amino acid-starved stringent cells look expanded and occupy the whole cell volume (Fig. 4C), even more than in an untreated control (Fig. 4A). On the other hand, Cam treatment of both strains as well as amino acid starvation of the relaxed strain induced the expected nucleoid compaction (Fig. 4B, E and F).

Mechanical disruption of cells

The mechanism and driving forces for membrane domain formation may be different and range from spontaneous dynamic microdomains to large 'patches' in the membrane 'fenced' mechanically by cytoskeleton structures (for reviews, see Bergelson *et al.*, 1995). The transertion model implies a dynamic mechanical nature of domains in bacterial membranes. Thus, simple physical disruption of the nucleoprotein structure constraining domains should lead to their dissipation, detectable by changes in membrane viscosity. The anisotropy value of DPH in control *E. coli* B/r H266 indeed decreased from 0.160 ± 0.004 to 0.143 ± 0.004 (−10%) as a result of exposure to disintegrating ultrasonic radiation (6 min with 0.5 min intervals). The same disintegration had no further effect in Cam-treated bacteria with the lowered anisotropy value (0.136 ± 0.002), indicating domain dissipation by Cam.

Table 1. Effects of different inhibitors of macromolecular syntheses on the membrane viscosity in *E. coli* or *B. subtilis*.

Inhibitor(s)	Protein synthesis[a]	DNA synthesis	RNA synthesis	ΔA (%)[b]
Nalidixic acid	+	0 (Elongation)	+	+5 ± 2
Chloramphenicol	0 (Elongation)	± (Initiation)	+	−15 ± 2
Puromycin	0 (Premature termination)	± (Initiation)	+	−15 ± 3
Rifampicin	±	± (Initiation)	0 (Initiation)	−15 ± 2
KCN	±	±	±	+4 ± 2
KCN + chloramphenicol	0	±	±	0
Streptolydigin	±	±	0 (Elongation)	0
Streptolydigin + chloramphenicol	0	±	0	0
Streptolydigin + puromycin	0	±	0	0

a. The effects of various drugs are classified as follows (see *Discussion* for details): 0, direct inhibition; ±, eventual inhibition; +, no inhibition.
b. Changes in viscosity, ΔA, are expressed as a percentage of DPH fluorescence anisotropy increase (positive) or decrease (negative) relative to the anisotropy value of untreated cells, which was 0.165 ± 0.004 (SD; n = 50) for *E. coli* and 0.150 ± 0.003 (SD; n = 25) for *B. subtilis*. The basic anisotropy value may vary slightly from culture to culture, but the relative effect of drugs was easily reproducible. An average of relative drug effects on individual cultures with standard error estimated from at least five experiments is shown.

Table 2. Fluorescence lifetimes of DPH in *E. coli* and *B. subtilis* cells subjected to different treatments.

Preparation	τ_1[a] ns	τ_2[a] ns	α_1[a]	α_2[a]	$\tau_{average}$[b] ns
E. coli (untreated)	8.07 ± 0.14	2.27 ± 0.06	0.77	0.23	6.75
E. coli + Cam	8.22 ± 0.28	2.43 ± 0.09	0.70	0.30	6.47
E. coli + Nal	7.43 ± 0.16	1.96 ± 0.10	0.77	0.23	6.20
B. subtilis (untreated)	6.67 ± 0.14	1.62 ± 0.05	0.71	0.29	5.21
B. subtilis + Stl	6.39 ± 0.15	1.49 ± 0.05	0.69	0.31	4.89

For experimental details, see *Experimental procedures* and corresponding figure legends.
a. Excitation lifetime values were measured in the frequency range 20–100 MHz and calculated for two-component lifetime: τ_1 and τ_2 with fractions α_1 and α_2.
b. $\tau_{average} = \tau_1 \times \alpha_1 + \tau_2 \times \alpha_2$.

Inhibition of RNA and protein syntheses in B. subtilis

Cam treatment of *B. subtilis* decreased DPH fluorescence anisotropy to the same extent as in *E. coli* (≈ 15%), but Stl, which also completely inhibited growth (not shown), did not affect the anisotropy (Fig. 5A; Table 1). Moreover, Stl prevented the effect of Cam, but was unable to reverse it if added after the anisotropy changes had already happened (Fig. 5A). Similar results were obtained with Pur (Fig. 5B); the anisotropy decreased to the same extent but with a slower rate at suboptimal Pur concentrations that do not totally inhibit growth. For Stl to cancel the effect of Pur completely, Stl had to be added a few minutes before Pur.

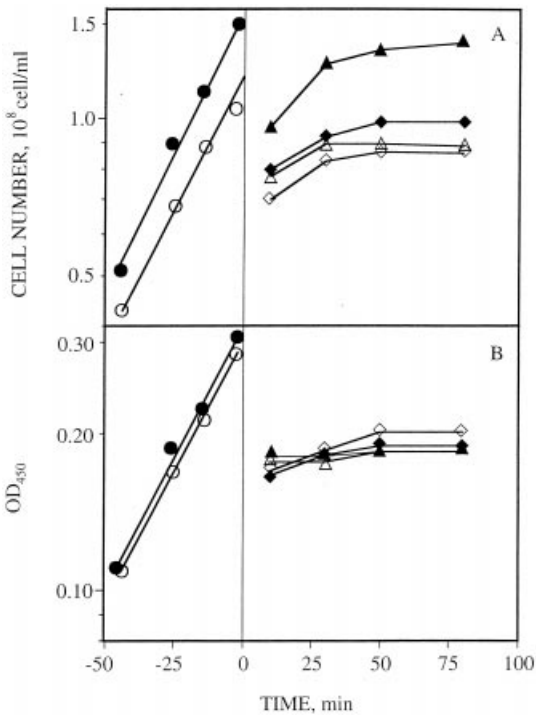

Fig. 3. Effects of Cam and amino acid starvation on cell divisions (A) and mass growth (B) in *E. coli* RL331T (*relA*$^+$) (closed symbols) and RL332T (*relA*) (open symbols). At time zero, portions of the cultures grown in a minimal medium supplemented with glucose and casamino acids (●, ○) were filtered and resuspended (1:2) in the same medium with the omission of casamino acids (▲, △) or in the full medium with Cam (◆, ◇).

As in *E. coli*, nucleoid fusion by Cam was also observed in *B. subtilis* (Fig. 6B); the average number of nucleoid bodies per cell decreased from 1.74 (197 cells) to 1.08 (164 cells). In contrast, the nucleoid area was spread along the whole cell after treatment with Stl alone or in combination with Cam (Fig. 6C and D). The division run-out after growth arrest by Cam or Stl was about 40% and 55% respectively (not shown).

Nutritional shift-down in E. coli

Structure–function relationships are often concealed in steady-state conditions, but may be discovered during transitional states. The well-defined transition, nutritional shift-down (Hansen *et al.*, 1975), was achieved by the addition of methyl α-D-glucoside (αMG) to a steady-state, glucose-growing culture. The mass growth rate (derived from slopes of the OD_{420} curves of Fig. 7) decreased from 1.45 h^{-1} in the unperturbed preshift culture to 0.48 h^{-1} immediately after the addition of αMG. Cell division rate (not shown), however, remained unchanged for about 1 h (Kjeldgaard *et al.*, 1958; Zaritsky and Helmstetter, 1992). A new steady state was established at about 70 min, with a growth rate of 0.85 h^{-1} and a correspondingly smaller cell size. Anisotropy fell early in the transition period (over 10% at 30 min), then overshot and reached a new steady-state value, similar to that observed before the transition at the same time as the growth rate (Fig. 7). No change in membrane viscosity was detected in glucose-grown *E. coli* when the steady-state growth rate was modulated by αMG (Fig. 7, inset). The systematic decrease in fluorescence anisotropy is correlated with an increase in growth rate when modulated by the quality of the carbon source (Zaritsky *et al.*, 1985).

Discussion

Bacterial membranes contain a wide variety of proteins and three major phospholipids with different fatty acids (e.g. Ames, 1968; Sweetman *et al.*, 1996). The membrane structure and dynamics depend on its composition, specific interactions between components (proteoprotein, proteolipid and lipid–lipid), functional state of proteins and

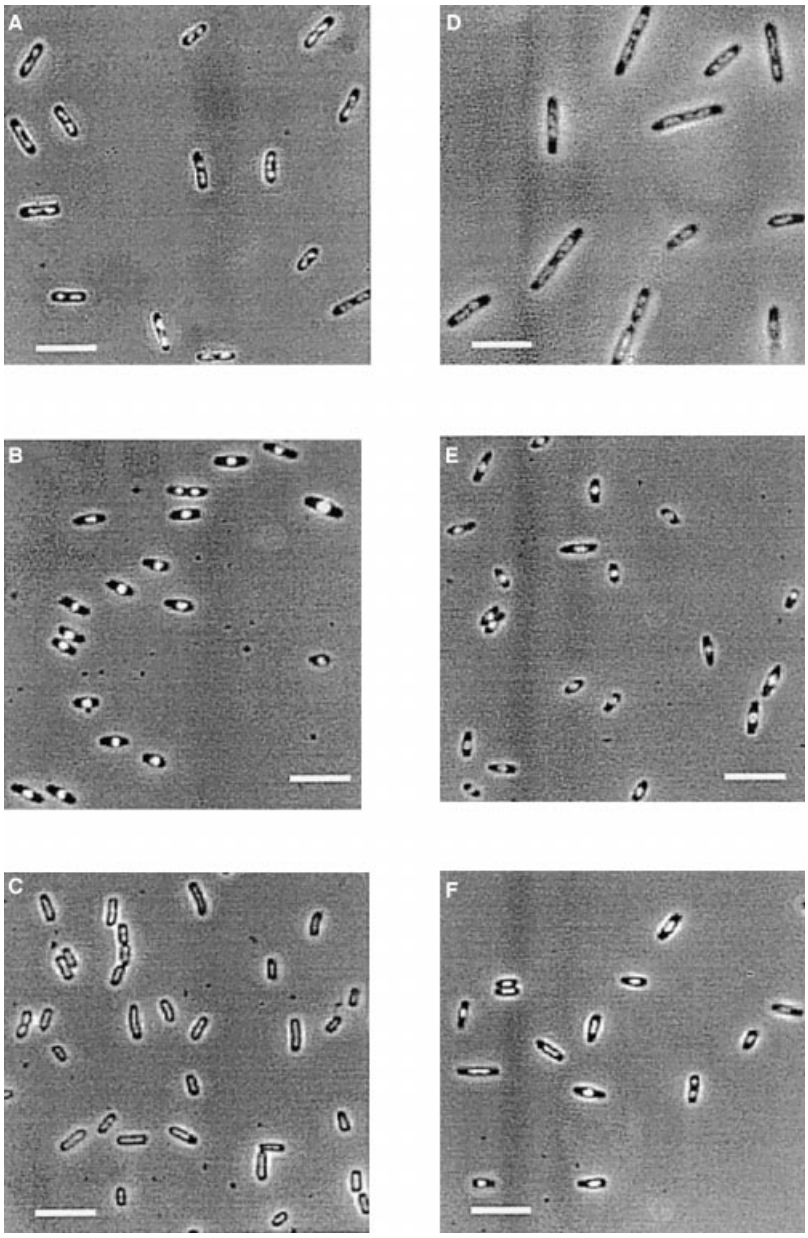

Fig. 4. Nucleoid morphology in *E. coli* RL331T (*relA*$^+$) (A–C) and RL332T (*relA*) (D–F). The samples were taken from steady-state cultures (A and D) and 60 min after treatment with Cam (B and E) or amino acid starvation (C and F in an experiment presented in Fig. 3. The cells were fixed with 0.25% formaldehyde, stained with DAPI (1 μg ml^{-1}), and images were taken by combined fluorescence and phase-contrast microscopy (Zeiss Axioplan 2 fluorescence microscope, equipped with Plan-Neofluar 100×/1.3 oil immersion lens and SPOT2 cooled CCD camera; Diagnostic Instruments). Bar = 5 μm.

electrochemical gradients across the membrane. To avoid any changes caused by enzymatic activities during labelling and measurement, formaldehyde fixation was used for sample preparation. The fluorescent probe is therefore expected to detect changes in phospholipid or protein composition and organization, implying that the measured viscosity is attributed to a non-active membrane without gradients. (See also *Experimental procedures* for the meaning of DPH fluorescence anisotropy.) Fixation itself had no effect on measured anisotropy, and possible direct interactions of drugs with the membrane were excluded by testing with fixed cells (data not shown).

Nal inhibits DNA gyrase and, thus, DNA replication (Drlica, 1984), but RNA and protein synthesis can proceed for a while (Goss *et al.*, 1965). The other drugs used here inhibit protein synthesis either directly or eventually (Table 1); in these cases, continued phospholipid synthesis might alter the lipid–protein ratio and perhaps explain the decrease in viscosity. However, this excess of phospholipid synthesis is a reflection of increased phospholipid turnover and excretion (Crowfoot *et al.*, 1972). Moreover, this would explain neither the absence of an effect of Stl nor the prevention of the Cam and Pur effects by Stl. The latter argument can also be used against the existence of a particular protein that would disappear when protein synthesis was blocked. The fact that membrane

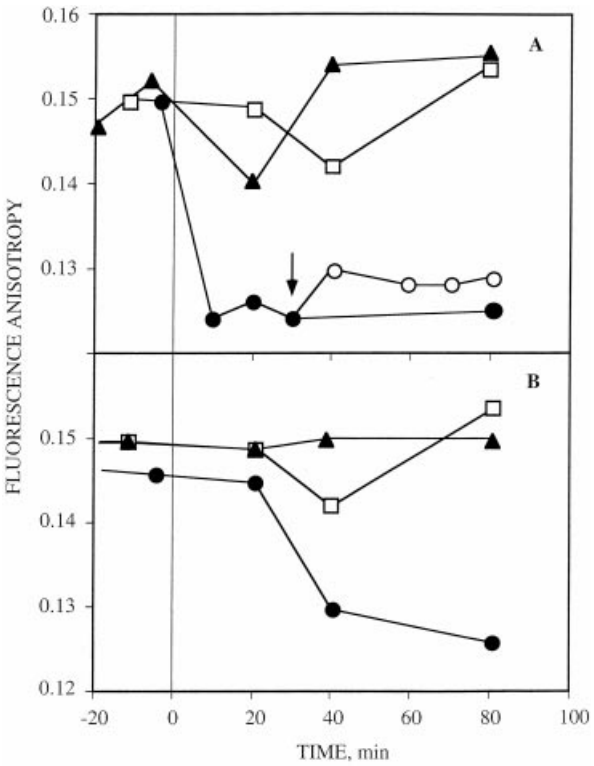

Fig. 5. Effects of inhibition of transcription and translation on DPH fluorescence anisotropy in steady-state grown *B. subtilis*.
A. Cam (100 μg ml^{-1}) and Stl (100 μg ml^{-1}) were added separately (● and □ respectively), simultaneously (▲) (at time zero) or consequently (○); the arrow indicates the time of addition of streptolydigin to chloramphenicol-treated bacteria.
B. Pur (150 μg ml^{-1}) and Stl were added separately (● and □ respectively) or simultaneously (▲) at time zero.

viscosity decreased in normal but not in Cam-treated cells upon ultrasonic disintegration also indicates the mechanical and not a compositional nature of these changes. Our explanation for the observed changes in membrane dynamics is based on the restriction of proteins to domains by a net of polysomes tethered to genes. The domains dissipate when these nets of polysomes are disrupted. This mechanism is illustrated in Fig. 1 and detailed below.

Continued RNA synthesis in the presence of Cam (Lark, 1972) permits mRNA termination. Cam-stabilized polysomes (Ennis and Sells, 1968) detach from the gene and are now free to diffuse in the membrane separately. Similarly, polysomes are detached from the gene (and then degraded) as a result of run-out of mRNA when further initiations are blocked by Rif. Premature termination of nascent protein chains by Pur (Smith *et al*., 1965) causes release of polysomes from the membrane (Smith *et al*., 1978; Marty-Mazars *et al*., 1983). These treatments destroy the structure created by transertion and result in the dissipation of domains, as revealed by the decreased average viscosity of the less ordered membrane (Table 1). Studies of the composition of membrane fractions (Marty-Mazars *et al*., 1983) are consistent with this interpretation: release of polysomes by treatment with Pur resulted in an increased ribosome-free membrane fraction, which contained proteins usually found in the ribosome-bearing fractions.

In contrast to the drugs that dissipate membrane domains, Stl does not; it inhibits transcription elongation without releasing RNA chains (Cassani *et al*., 1971). Consequently, protein synthesis ceases (Lark, 1972) with ribosomes stuck on the mRNA (Ennis and Sells, 1968). The entire transertion process, from genes to nascent peptides, becomes 'frozen'. The addition of Cam should obviously not change it. If transertion creates domains, freezing transertion should freeze domains, consistent with the absence of a change in viscosity in Stl-treated cells (Fig. 5A). Moreover, Pur can only be incorporated in polypeptide chains when protein synthesis is actually occurring. Hence, if it is added when transertion is frozen, it cannot cause chain release. In other words, Pur can only act before Stl takes effect. Reciprocally, Stl cannot reverse the effects of Cam or Pur once polysomes have been detached from the gene or membrane, and transertion is no longer there to be frozen (Fig. 5).

According to our explanation, the domain dissipation depends on the energy-requiring mRNA run-out or Pur incorporation and, thus, can be avoided by energy shortage. Indeed, KCN did not result in viscosity alterations and prevented the effect of Cam (Fig. 2, Table 1), although all macromolecular synthesis was also arrested.

Another case in which membrane viscosity did not change upon the arrest of protein synthesis was amino acid starvation of stringent, but not of relaxed, strains of *E. coli*. The stringent response is characterized by fast and strong accumulation of ppGpp, a phenomenon not seen in *relA* mutants (for review, see Cashel *et al*., 1996). High levels of ppGpp inhibit mRNA chain elongation (Sørensen *et al*., 1994; Vogel and Jensen, 1994), which, together with blocked protein synthesis, results in frozen transertion – similar to Stl. In a *relA* mutant, whose ppGpp pool decayed during starvation, the rate of mRNA chain elongation even increases (Vogel and Jensen, 1994), allowing polysome run-out as with Cam. In contrast, specific inhibition of DNA synthesis by Nal should lead to increased transcriptional density (Norris, 1995b) and, hence, increased viscosity, as observed (Table 1).

Nucleoid morphology was proposed as being determined by the same transertion process tethering DNA to the membrane (Woldringh *et al*., 1995) and may serve as an indicator of the extent of tethering. Nucleoid shape correlated perfectly with the membrane changes (Figs 4 and 6). It was expanded only in starved stringent *E. coli* and Stl-treated *B. subtilis* (and see the absence of KCN effect in Fig. 1E of Van Helvoort, 1996, p. 74), i.e. in

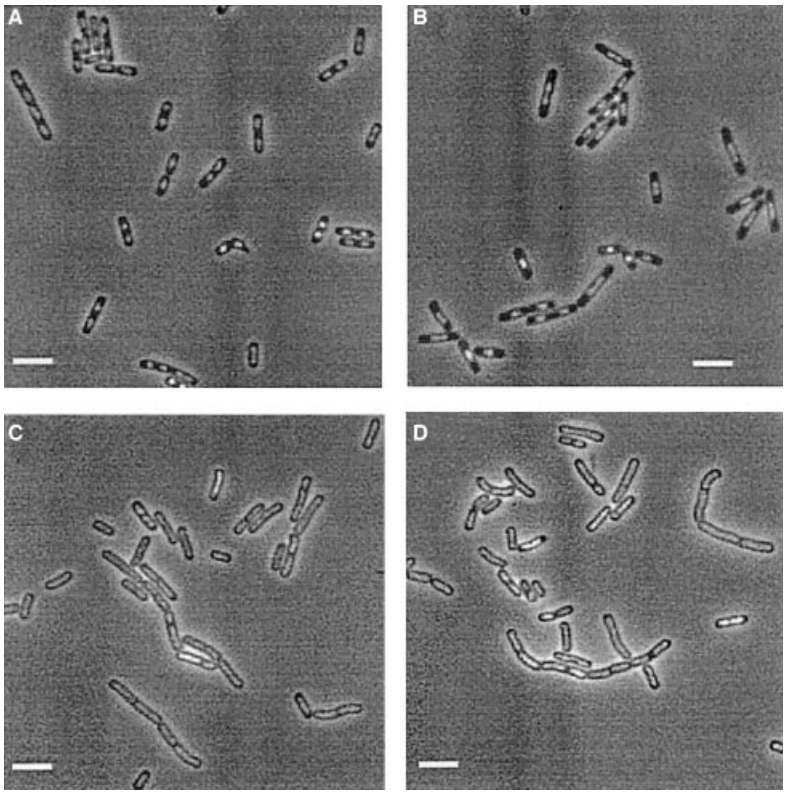

Fig. 6. Nucleoid morphology in *B. subtilis*: untreated control (A), 60 min after treatment with Cam (B), Stl (C) or both Stl and Cam (D). In the samples illustrated in (A) and (B), cells and nucleoids were scored to get an average number of nucleoids per cell (see *Results*). Staining and imaging were performed as described in the legend to Fig. 4. Bar = 5 μm.

bacteria with unperturbed membranes. This expansion, observed here for the first time to the best of our knowledge, is probably caused by relief from hypernegative supercoiling (the compaction force) when transertion of membrane proteins was inhibited without detachment from the membrane (Lynch and Wang, 1993; for explanation, see Woldringh *et al.*, 1995). The nucleoid was compacted and fused in all other cases in which the decrease in membrane viscosity was also observed. The combination of unsegregated nucleoids and the dissipation of domains, marking the division site, may be the reasons for the failure in division run-out (Fig. 3).

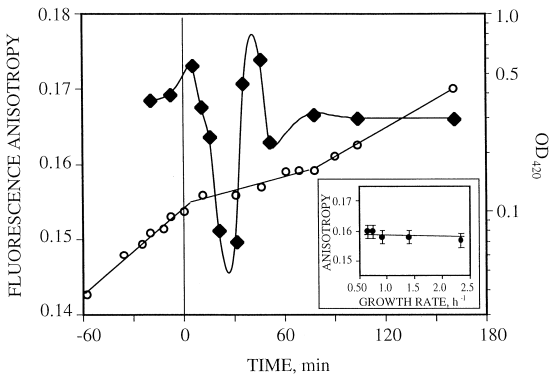

Fig. 7. DPH fluorescence anisotropy (♦) and culture biomass (○) during a nutritional shift-down (at time zero) to αMG/glucose = 10. The culture was diluted 1:2 by a fresh, prewarmed medium with the same αMG/glucose ratio at 60 min. The line drawn through anisotropy values is an interpolation fitting. Inset: DPH fluorescence anisotropy as a function of *E. coli* B/r steady-state growth rate, varied by αMG/glucose ratio (0, 5, 10 and 20) or the addition of casein hydrolysate (1%) to glucose minimal medium.

The substantial time (about 10 min) required for the cessation of RNA synthesis, e.g. after the inhibition of transcription initiation by Rif (Fig. 1a in Lark, 1972) presumably determines the lag period in the response of the membrane dynamics after drug addition (Fig. 2). The observed relatively slow further changes in membrane viscosity (Figs 2 and 5) may reflect low lateral diffusion rates of proteins in the dense cytoplasmic membrane. The effect of Cam, which preserves polysome structure (Ennis and Sells, 1968), suggests that the number of proteins inserted from a single polysome is not sufficient for domain formation; this process requires a number of polysomes held together.

Cam addition inhibits the initiation of DNA replication (Lark and Renger, 1969), which has been interpreted as a requirement for the synthesis of an unknown protein in the 15 min preceding initiation. An alternative explanation could be that Cam treatment dissipates membrane domain(s) needed for local DnaA activation by phospholipids (Castuma *et al.*, 1993; Mizushima *et al.*, 1996), as proposed by Norris (1995b).

RNA and protein accumulation cease abruptly when

glucose uptake is limited by αMG (Hansen *et al.*, 1975). At least up to 20 min after such a shift-down, the rate at which mature ribosomes accumulate is two- to threefold slower than the rates at which their components are being synthesized; between 30 and 60 min, the rate of accumulation of stable RNA and ribosomes increases slowly and, finally, the rates of all macromolecular synthesis events and macromolecular accumulation are matched again (Molin *et al.*, 1977). Hence, the transient decrease in membrane viscosity during nutritional shift-down (Fig. 7) may reflect the cessation and restoration of balanced protein and RNA synthesis or, in other words, dissipation and reassembly of transertion-formed membrane domains. The independence of membrane viscosity and the steady-state growth rate, modulated by αMG (Fig. 7, inset), may indicate that domains occupy a constant fraction of the membrane in cells of different sizes (in conditions of single carbon source limitation), as observed for cells of different lengths (ages) within a population (Fig. 2, inset, in Fishov and Woldringh, 1999).

Membrane patterns with apparent functional implications were observed in *E. coli* cells and filaments stained with FM 4-64 (Fishov and Woldringh, 1999). Their disappearance after CAM treatment and a concomitant decrease in DPH fluorescence anisotropy justify the assumption that the presumed membrane domains are more ordered (viscous). A 15% change in DPH anisotropy (Figs 2 and 5) corresponds to a small fraction (about 25%) of the envelope occupied by the visible areas interpreted as domains (Fig. 2 in Fishov and Woldringh, 1999). These and other data presented above are consistent with the transertion model.

It is noteworthy that evidence is accumulating for the existence of lipid domains in model and biological membranes and that these domains are active partners in regulating a variety of biological functions (e.g. Welti and Glaser, 1994; Bergelson *et al.*, 1995). Indications for membrane heterogeneity in bacteria may be found in the literature (e.g. de Leij and Witholt, 1977; Horiuchi *et al.*, 1983; Marty-Mazars *et al.*, 1983; Welby *et al.*, 1996; and cited in Norris, 1995a). Further biophysical and biochemical characterization of membrane domains in bacteria should be performed to comprehend their composition and function.

Experimental procedures

Bacterial strains, culture conditions and antibiotics

Strains of *E. coli* B/r H266 and *B. subtilis* OI1 (*ilvC1, leu-1*) were grown in M-9 minimal salt medium supplemented with 0.4% glucose and with glucose and 1% casein hydrolysate respectively. The *E. coli* steady-state growth rate (Fishov *et al.*, 1995) was modulated by changing the availability of a single carbon source to avoid differences in metabolic pathways and gene expression patterns. The non-metabolizable analogue, methyl α-D-glucoside (αMG), was used for competitive inhibition of glucose uptake (Hansen *et al.*, 1975). The bacterial strains RL331T (*relA*$^+$) and RL332T (*relA*) (Ryals *et al.*, 1982) were grown in M-9 minimal salt medium supplemented with 0.4% glucose and with 1% casein hydrolysate.

Bacteria were cultivated at 37°C with vigorous shaking (gyratory water bath shaker, model G76; New Brunswick Scientific). Absorbance (OD_{420}; Novaspec II; Pharmacia LKB), cell counts and size distributions (Coulter counter, model ZM, 30 μ orifice; Coulter Electronics) were used to follow growth and to characterize the physiological state.

Streptolydigin was a gift from Upjohn Laboratories; other drugs were purchased from Sigma. Antibiotics were added to the steady-state growing cultures at the following final concentrations: 100 μg ml^{-1} chloramphenicol; 100 μg ml^{-1} streptolydigin; 50 μg ml^{-1} rifampicin; 150 μg ml^{-1} puromycin; 10 μg ml^{-1} nalidixic acid.

Measurement of fluorescence anisotropy

This is a particularly useful method for studying the rotational dynamics of a fluorescent probe embedded within the lipid bilayer of a biological membrane (Shinitzky and Barenholz, 1978; Parola, 1993). On illuminating labelled cells with polarized light, the extent of emission anisotropy, determined as a ratio of polarized components to the total intensity, depends primarily on the angle between the absorption and emission dipole moments of the probe, its rotational Brownian motion during the excited state lifetime and its fluorescence lifetime. The magnitude of rotational Brownian motion depends on the size and shape of the probe molecule, its surrounding 'microviscosity' and temperature (Shinitzky and Barenholz, 1978). The probe's rotational mobility can be derived from the fluorescence anisotropy value and probe lifetime. Qualitatively, at constant temperature and probe lifetime, larger anisotropy reflects higher 'microviscosity'.

Anisotropy of DPH fluorescence was used to monitor changes in membrane dynamics, according to the general procedure described previously (Zaritsky *et al.*, 1985; Parola *et al.*, 1990), with some modifications. Briefly, samples of the bacterial culture (2–5 ml) were fixed by formaldehyde (0.25% final concentration), collected on polycarbonate membrane filters (0.2 μm pore size; Poretics) and washed with PBS (pH 7.4). Filters were frozen in liquid nitrogen. For labelling, filters were thawed, bacteria resuspended in PBS (OD_{450} = 0.25) and incubated for 45 min at 37°C in the presence of 10^{-7} M DPH (added as 10^{-4} M solution in tetrahydrofuran). An unlabelled portion of the sample, incubated under the same conditions, served as a scattering reference (usually less than 3% of the intensity of the labelled sample). Steady-state fluorescence anisotropy was measured at 37°C using a Perkin-Elmer LS50B spectrofluorometer with excitation at 360 nm and emission at 430 nm, 2.5 nm and 7 nm slits, respectively, in the 'Read' mode, with 3 s integration time. Five or six readings were taken for each sample, providing an estimated instrumental error of ≈ 1%. An SLM-4800 spectrofluorometer, modified by ISS to a Gregg-MM multifrequency phase modulation spectrofluorometer, was used for measuring lifetimes (Parola *et al.*, 1990; Parola, 1993).

Anisotropy changes are interpreted here in terms of variation in viscosity, verifying that DPH lifetimes are constant under the conditions studied, implying corresponding variations in the membrane structure, composition or organization, generally termed membrane dynamics. It is important to note that small changes (of about 10%) in fluorescence anisotropy may reflect pronounced changes (of about 25%) in membrane 'microviscosity' (Shinitzky and Barenholz, 1978). Moreover, measured anisotropy is a weighted average of the overall cell membrane and of all cell ages in the culture; the local changes may be much larger and cell age dependent.

Acknowledgements

We acknowledge Professor Vic Norris for fruitful discussions and enthusiasm, and Emilia Klyman for technical help. The microscopy service of the Institute for Applied Biosciences (BGU) assisted by Dr V. Erukhimovitch is appreciated. We thank Upjohn Laboratories, Kalamazoo, MI, USA, for the kind gift of streptolydigin. This work was supported by a Guastalla Fellowship of the Foundation Raschi (to I.F.) and in part by grant no. 91-00190/2 from the US–Israel Binational Science Foundation (BSF), Jerusalem, Israel (to A.Z.) and grant no. N00014-89-J-1625 of the Office of Naval Research (to A.H.P.).

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