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Lipid composition changes induced by tamoxifen in a bacterial model system

Cristina Luxo [a,b], Amália S. Jurado [b,*], Vítor M.C. Madeira [b]

[a] *Laboratório de Microbiologia, Faculdade de Farmácia, Universidade de Coimbra, 3000 Coimbra, Portugal*
[b] *Centro de Neurociências, Universidade de Coimbra, 3000 Coimbra, Portugal*

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Abstract

A putative relationship between growth impairment of *Bacillus stearothermophilus* by tamoxifen (TAM) and TAM-induced perturbation of the physical properties of bacterial membrane lipids has been observed. The supplementation of the growth medium with Ca^{2+} (a membrane stabilizer) partially relieves growth inhibition by TAM, allowing growth at TAM concentrations that fully impair growth in the basal medium. *B. stearothermophilus* modifies the membrane lipid composition in response to the addition of TAM to the growth medium and the response is sensitive to Ca^{2+}. Changes in lipid composition are observed in the acyl chains and in the polar head groups of phospholipids. The physical effects of alteration in these lipids was studied by fluorescence polarization of DPH and DPH-PA. Polar lipid dispersions from TAM-adapted cells grown in a Ca^{2+} medium show a shift of T_m to higher temperatures and a significant increase of the structural order as compared to lipids from control cells, suggesting that TAM-induced lipid composition changes compensate for the destabilizing effects of the cytostatic on membrane organization. The polar lipids from cells grown in the basal medium containing tamoxifen are also altered, but these alterations do not promote order increase of the bilayer in spite of a deviation of T_m to higher temperatures as detected by DPH. Data indicate that *B. stearothermophilus* controls the membrane lipid composition in response to tamoxifen, to compensate for TAM-promoted disordering in membranes and to provide an appropriate packing of phospholipid molecules in a stable bilayer, putatively disturbed by TAM incorporation.

Keywords: Tamoxifen; Bacterial model; Lipid composition change; Order membrane

1. Introduction

Tamoxifen is a nonsteroidal antiestrogen widely used in the treatment of human breast cancer because of its growth-inhibiting activity of tumor cells expressing estrogen receptors [1] and of estrogen receptor-negative breast cancer cells [2]. Thus, its antiproliferative action may involve mechanisms not restricted to the classical estrogen receptor binding system [3].

However, the early benefits of antiestrogen treatment tend to decrease with time due to the emergency of resistant tumor cells. In general, these cells become resistant to the drug with which they were initially treated and also to other structurally and pharmacologically distinct chemotherapeutic agents. The reason for this phenomenon, known as MDR (multidrug resistance), is still unknown. Often, the

* Corresponding author. Fax: 351 39 26798.

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resistance is characterized by decreased drug accumulation and overexpression of a 170 kDa membrane protein, known as P-glycoprotein [4], although some MDR cell lines and tumor cells do not, apparently, overexpress P-glycoproteins [5,6] suggesting that other mechanisms can be involved in drug-resistance of tumor cells. It has been suggested that the MDR phenotype of tumor cells involves a pleiotropic response including alteration in the molecular components [7,8] and in the dynamic properties of the cell membrane [9]. Therefore, the elucidation of the mechanisms which endow tumor cells with resistance to chemotherapeutic agents and the establishment of pathways which could contribute to circumvent or to reverse the MDR are of obvious interest. Modulation of the sensitivity of actively dividing tumor cells to cytostatics assumes also a significant clinical relevance. It has been shown that tumor cells, although provided with fatty acids synthesis ability, readily utilize exogenous fatty acids for membrane phospholipid synthesis [10,11]. On the other hand, it has been shown that growth and sensitivity of neoplastic cells to chemotherapeutic agents may be altered by the type of incorporated fatty acids [10–12]. Therefore, the manipulation of diet lipids may counteract tumor cells proliferation and enhance its sensitivity to chemotherapeutic treatments.

Biological membranes contain diverse lipid molecules, with different fatty acid composition and polar head groups, to achieve optimal lipid and protein packing into the bilayer and to guarantee membrane stability and functionality. With this concept in mind, we use here a prokaryotic cell known for its high ability of adaptative lipid composition changes, to further clarify the role of the plasma membrane on tamoxifen citotoxicity. Previously, we established that the interaction of tamoxifen with membrane lipids of *Bacillus stearothermophilus* induces physical effects similar to those described for other systems, either membrane models or native membranes [13]. Additionally, we achieved a good correlation between Ca^{2+} effects on the physical state of membrane lipids and its ability to modulate the sensitivity of bacterial cells to tamoxifen induced growth inhibition (unpublished data). Now, we show that *B. stearothermophilus* regulates the lipid composition in response to the addition of TAM to the growth medium supplemented or not with Ca^{2+}. Some of the lipid changes affecting the fatty acyl chain composition and the polar head groups could be predicted on basis of TAM perturbing effects on the physical properties of the lipid bilayer. However, other observed alterations may be putatively related to the preservation of a crucial balance between bilayer and non-bilayer phospholipids to preserve membrane functional viability. As suggested for other groups of lipophilic molecules, anesthetics and alcohols [14–16], it is possible that the physico-chemical basis of TAM (and other cytostatics) membrane interactions are related to bilayer lamellar stability, rather than to fluidity changes. Similar considerations may apply to evaluate lipid composition changes following acquisition of resistance to chemotherapeutic agents by tumor cells to establish clues to enhance neoplastic-cell sensitivity to chemotherapy by manipulation of the diet lipids.

2. Materials and methods

2.1. Chemicals

Tamoxifen and DPH were obtained from Sigma. DPH-PA was purchased from Molecular Probes.

2.2. Cultures

The strain of *B. stearothermophilus* and conditions of its maintenance and growth have been previously described [17]. Liquid cultures were started with an early stationary inoculum from a medium (dilute L-Broth) with a residual Ca^{2+} concentration (115 μM) and were grown in 1 l Erlenmeyer flasks containing 200 ml of the same medium supplemented (Ca^{2+}-medium) or not (basal medium) with Ca^{2+} to a final concentration of 2.5 mM. The flasks were shaken at 100 rpm in a New Brunswick water-bath shaker. Cultures with tamoxifen were grown in a medium (basal or Ca^{2+}-medium) to which tamoxifen from a concentrated ethanolic solution was added to obtain concentrations ranging from 2.5 to 10 μM; controls were grown in a medium without TAM, but with a volume of added ethanol corresponding to the maximal TAM concentration used in growth. Bacterial growth in media containing tamoxifen has been carried out in silanized Erlenmeyer flasks, since this drug strongly binds to glass material [18].

Growth was measured by turbidimetry at 610 nm in a Spectronic 201 spectrophotometer.

2.3. Extraction of polar lipids

Growth was stopped at the beginning of the stationary phase and the cells were harvested by centrifugation. Cells were then washed three times with buffer (10 mM Tris-Cl, pH 7.0). The lipids were extracted by the method of Bligh and Dyer [19], and quantified by measuring the amount of inorganic phosphate [20], after hydrolysis of the extracts at 180°C in 70% $HClO_4$ [21]. The polar lipids were isolated by preparative thin layer chromatography on 2 mm thick silica gel plates (Merck) developed in acetone and extracted with the mixture of solvents $CHCl_3 : CH_3OH : H_2O$ (45 : 45 : 10, by vol) followed by a phase separation, mixing the lipid extract with $CHCl_3 : H_2O$ (1 : 1, by vol). The lower phase was collected, evaporated to dryness on a rotary evaporator and the lipid residue was dissolved in a small volume of chloroform and stored under nitrogen atmosphere at −20°C.

2.4. Phospholipid analysis

The polar lipid classes were separated by thin-layer chromatography on plates (0.5 mm) of silica gel H, using the solvent mixture $CHCl_3 : CH_3OH : H_2O$ (65 : 25 : 4, by vol) for development. Separated classes were detected by iodine vapour reaction or with specific spray reagents: molybdate for lipids containing phosphate [22], ninhydrin for free aminogroups and α-naphtol for carbohydrates [23]. Identification of lipid classes has been performed with reference to standards run along with the samples on the TLC plates. The phospholipid bands were scrapped off from the plates and quantified by measuring the amount of inorganic phosphate [20], after hydrolysis at 180°C in 70% $HClO_4$ [21].

2.5. Preparation of fatty acid methyl esters

The polar lipid extracts were concentrated by a stream of nitrogen. Fatty acid methyl esters were prepared in 10 ml of 5% (w/v) H_2SO_4 in methanol. Tubes with PTFE-lined screw caps were flushed with N_2 and incubated at 70°C for 2 h. The tubes were cooled and extracted with addition of 10 ml hexane and 5 ml water. The upper phase was saved and the aqueous phase re-extracted with hexane. Anhydrous sodium sulphate was added to the combined hexane extracts which were allowed to stand for 30 min. The extracts were filtered through Whatman No. 1 filter paper, evaporated to dryness, and then dissolved in CS_2.

2.6. Identification and quantitative determination of fatty acids

The fatty acid methyl esters were analyzed with a gas–liquid chromatograph Varian Series 1400 equipped with a flame ionization detector. An open capillary SGE BP-20 (25 m, 0.53 mm) column was eluted with N_2 at 3 ml min^{-1} and a temperature programme was started at 145°C, held for 14 min, and then increased to 210°C at 4°C min^{-1}, and using 25 ml N_2 per minute as make-up gas in the detector system.

Peaks were identified by comparison of retention times with those of authentic standards (fatty acid methyl esters obtained from Supelco and Alltech).

2.7. Liposomes

Aliquots from lipid solutions in $CHCl_3$ (polar lipid extract), containing 1.34 mg of lipid were evaporated to dryness on a rotary evaporator. The dry residues were hydrated at 50°C under N_2 atmosphere, by gently shaking with 5 ml of 50 mM KCl, 10 mM Tris-maleate, pH 7.0. Then, the suspensions were vortexed for 1 min and stabilized overnight.

2.8. Fluorimetric measurements

Two fluidity probes were used: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-(4-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA). DPH in tetrahydrofuran and DPH-PA in dimethylformamide were incorporated into liposome suspensions (345 μM in phospholipid), as previously described [24] to give a lipid/probe molar ratio of ~400. After incubation at 55°C in the dark for 18–20 h, TAM was added from an ethanolic stock solution and the mixtures were allowed to equilibrate for 20 min at 37°C, before fluorescence measurements. The fluorimetric mea-

surements were performed with an Perkin–Elmer LS 50 computer controlled spectrofluorometer. The excitation was set at 336 nm and the emission at 450 nm (3 and 4 nm band pass). All the fluorescence measurements were corrected for the contribution of light scattering by using appropriate blanks without added probes. However, these corrections were generally negligible.

The degree of fluorescence polarization (P) was calculated according to Shinitzky and Barenholz [25] from the equation:

$$P = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of exciting beam. G is the correction factor for instrument polarization, given by the ratio of vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction.

Membrane ''fluidity'' is here used operationally as being inversely proportional to the polarization parameter. Although related, it is not identical with the physical fluidity involving isotropic motions in an isotropic medium very distinct from the anisotropic medium of the membrane. The polarization depends mainly on the angle of the probe rotational cone [26] and the rotational rate which are substantially influenced by the acyl chain dynamics of phospholipids. Fluorescence polarization monitors the average extent of chain motions or acyl chains relative orientational order. Therefore, probe polarization provides useful information about membrane order and can be used to monitor membrane order/disorder transitions [26].

3. Results

3.1. TAM effects on growth of B. stearothermophilus

B. stearothermophilus was grown at 65°C (centre of the optimal temperature range) in a complex medium containing 115 μM Ca^{2+} (basal medium) supplemented or not with additional Ca^{2+} to obtain a final concentration of 2.5 mM.

According to previous results [13], the addition of TAM to the growth medium prevents growth as a function of concentration, inducing a progressive increase of the lag time, a decrease of the specific growth rate and a decrease of the final cell density (Fig. 1). These effects also noticed in a Ca^{2+}-supplemented medium are more pronounced in the basal medium. Thus, 7.5 μM TAM completely inhibited growth in the basal medium but promoted a limited inhibition of growth in a Ca^{2+}-supplemented medium. The extension of growth in these conditions is similar to that induced by 2.5 mM TAM in the basal medium. Growth is still allowed at 10 μM TAM in the Ca^{2+} medium. To pursue the study of TAM effects on the lipid composition of bacterial membranes, cells were grown in the basal medium containing 0, 2.5 and 5 μM TAM and in a Ca^{2+}-supplemented medium containing 0, 7.5 and 10 μM TAM, being harvested in the beginning of the stationary phase.

3.2. TAM effects on the lipid composition of B. stearothermophilus

The polar lipids of bacterial membranes, accounting for 80–90% (w/w) of the total lipid extract [27], were analyzed to detect composition alterations induced by tamoxifen added to the growth medium. The minor lipid fraction composed of neutral lipids, including carotenoids, was not studied in this work. The polar lipids of bacterial cultures grown in the presence (TAM-cultures), and in the absence of TAM (control cultures), include the following main classes of phospholipids: phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and a phosphoglycolipid (PGL), in agreement with previous results [27]. Three minor unidentified phospholipids, α-naftol and ninhydrin negatives, were also detected. Two of them (X and Y) migrate close to PE (X) and to PG (Y) and their spots were scrapped together with those of the main phospholipids. The third minor component (Z) migrates as a well separated spot and was separately scrapped and quantified. Table 1 lists the proportions of the classes of phospholipids as affected by TAM. The effects of the addition of tamoxifen to bacterial cultures were studied by comparing the membrane lipid composition of cultures grown in the absence of drug (controls) and cultures grown with different TAM concentrations, in two different conditions: (a) – in a medium

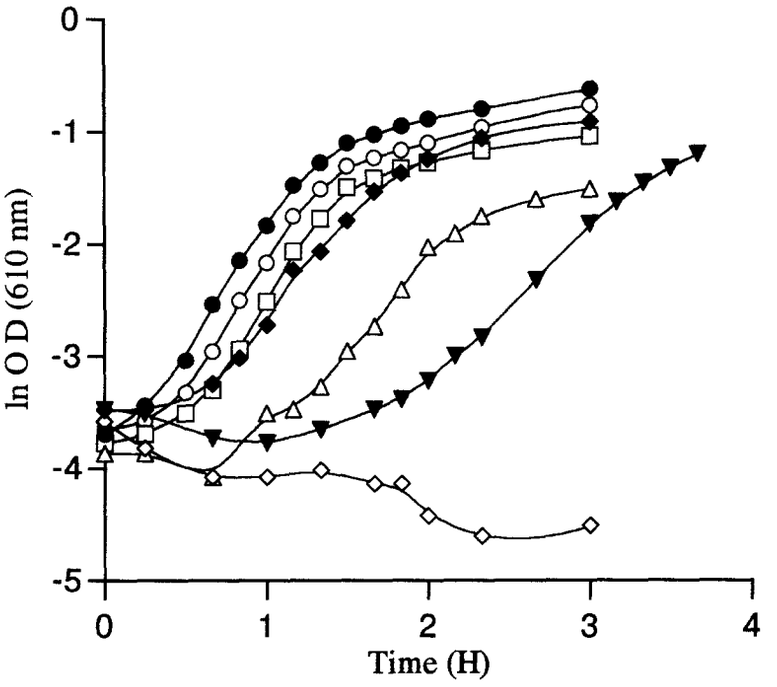


Fig. 1. Effect of tamoxifen and Ca^{2+} ions on the growth of cultures of *B. stearothermophilus* at 65°C. Cells were grown in a basal medium (open symbols) or in a Ca^{2+} supplemented medium (solid symbols), without added TAM (○, ●) and containing 2.5 μM (□), 5 μM (△), 7.5 μM (◇, ◆) and 10 μM (▼) TAM.

lacking a Ca^{2+}-supplement (−), i.e. in the basal medium, and (b) – in a Ca^{2+}-supplemented medium (+). For the sake of clarity, we will analyze separately TAM effects on cultures grown in the basal medium and in a Ca^{2+} medium. The addition of 2.5 μM TAM to the basal medium did not induce

Table 1
Phospholipid composition of *B. stearothermophilus*, as affected by TAM [a]

Additives to the growth medium		Phospholipid composition [b]				
Ca^{2+} (2.5 mM)	TAM (μM)	PE + *X* [c]	DPG	PG + *Y* [c]	PGL	Z [d]
−	0	61.9 ± 2.37	23 ± 1.97	6.4 ± 0.61	6 ± 0.18	1.3 ± 0.18
−	2.5	60.3 ± 0.95	24.4 ± 0.85	5.3 ± 0.35	7.6 ± 0.48 *	1.5 ± 0.35
−	5	47.8 ± 5 *,†	31.8 ± 1.9 †	8.3 ± 0.87 †	9.6 ± 1.76	1.4 ± 0.18
+	0	51.4 ± 1.82	21.2 ± 1.66	18.3 ± 0.63	5.9 ± 0.4	2.1 ± 0.9
+	7.5	56.8 ± 1.44 **	25.9 ± 1.19 *	6.6 ± 0.4 ***	8.3 ± 0.39 **	1.4 ± 0.62
+	10	54.6 ± 1 *	24.9 ± 0.77	6.9 ± 1.25 *	10.5 ± 0.58 ***	1.81 ± 0.61

[a] Values are given as mol% of phosphorus equivalent (total phospholipid phosphorus equals 100 mol%). Values of phospholipids contents were compared by Student's *t*-test, for the following paired observations: cultures grown in the basal medium (Ca^{2+}-) containing 0 μM TAM/2.5 μM TAM or 0 μM TAM/5 μM TAM; cultures grown in the Ca^{2+} medium (Ca^{2+} +) containing 0 μM TAM/7.5 μM TAM or 0 μM TAM/10 μM TAM (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$). The lipid contents of cultures grown in the basal medium (Ca^{2+}) containing 2.5 μM TAM/5.0 μM TAM and of cultures grown in the Ca^{2+} medium (Ca^{2+} +) containig 7.5 μM TAM/10 μM TAM were also compared in order to appraise the concentration dependence of TAM effects († $p < 0.05$).

[b] The values refer to pooled lipid extracts of 2–4 cultures at each growth condition from which 4 samples were independently used for TLC and phosphorus quantification. Means ± standard deviations are listed.

[c] *X* and *Y* are unidentified phospholipids which co-migrate with PE and PG, respectively (see text).

[d] *Z* is an unidentified phospholipid separated as a distinct spot in the chromatograms (see text).

Table 2

Fatty acyl chain composition of *B. stearothermophilus* lipids as affected by TAM[a]

Additives to the growth medium		Fatty acid composition[b]													
Ca^{2+} (2.5 mM)	TAM (μM)	*i*C14	*n*C14	*i*C15	*a*C15	*n*C15	*i*C16	*n*C16	C16:1	*i*C17	*a*C17	*n*C17	C17Δ	*n*C18	C18:1	C19:1 trans
–	0	0.67 ± 0.06	4 ± 0.41	19.8 ± 2.73	9.3 ± 0.65	1.3 ± 0.5	9.1 ± 0.2	25.2 ± 1.78	0.67 ± 0.34	8.7 ± 1	18.1 ± 1.85	0.42 ± 0.1	0.55 ± 0.13	0.68 ± 0.09	0.64 ± 0.04	0.83 ± 0.21
–	2.5	0.64 ± 0.1	3.8 ± 0.77	20.9 ± 1.17	8.3 ± 0.16 [c]	1.4 ± 0.48	9.1 ± 0.22	25.7 ± 1.6	0.53 ± 0.23	9.4 ± 1.1 [c,d,e]	16.9 ± 0.47	0.47 ± 0.18	0.63 ± 0.29	0.77 ± 0.22	0.57 ± 0.02 [c,e]	0.86 ± 0.27
–	5	0.69 ± 0.1	4.7 ± 0.84 [c,d,e]	21.8 ± 0.34	7.2 ± 0.13 [c,d,e]	1.1 ± 0.02	8.6 ± 0.16 [c,d,e]	29.6 ± 0.96 [c,d,e,f]	0.64 ± 0.25	9 ± 0.97 [c,d,e,f]	13.3 ± 1.09 [c,d,e]	0.4 ± 0.08	0.61 ± 0.3	0.82 ± 0.08 [c,e]	0.7 ± 0.09 [c]	0.86 ± 0.24
+	0	0.7 ± 0.05	4.49 ± 0.41	18.1 ± 1.01	10 ± 0.5	1.5 ± 0.75	8.8 ± 0.36	26.6 ± 1	0.32 ± 0.08	7.5 ± 0.17	18.9 ± 0.82	0.41 ± 0.16	0.52 ± 0.24	0.72 ± 0.1	0.54 ± 0.03	0.93 ± 0.27
+	7.5	0.64 ± 0.03 [a]	4.4 ± 0.34	20.5 ± 1.3 [a,c,e]	8.2 ± 0.68 [a]	1.6 ± 0.5	8.2 ± 0.12 [a]	27.8 ± 0.4	0.58 ± 0.3	9.1 ± 0.62 [a,e]	15.8 ± 1.7 [a,c,e]	0.52 ± 0.18 [a]	0.46 ± 0.15	0.79 ± 0.18	0.61 ± 0.05	0.78 ± 0.1
+	10	0.61 ± 0.18	4.7 ± 1.6	18.9 ± 1.04	7.6 ± 0.58 [a,c,d]	1.97 ± 0.47	7.77 ± 0.3 [a,d]	30.1 ± 1.62 [a,c,d,e,f]	0.76 ± 0.45	9.6 ± 2.3	14.6 ± 1.95 [a]	0.63 ± 0.47	0.55 ± 0.23	0.92 ± 0.08 [a]	0.68 ± 0.14	0.63 ± 0.31 [a]

[a] Values of fatty acids contents were compared by student's *t*-test, for the following paired observations: cultures grown in the basal medium (Ca^{2+} –) containing 0 μM TAM/2.5 μM TAM or 0 μM TAM/5 μM TAM; cultures grown in the Ca^{2+} medium (Ca^{2+} +) containing 0 μM TAM/7.5 μM TAM or 0 μM TAM/10 μM TAM ([a] $p < 0.05$; [c] $p < 0.005$; [e] $p < 0.0005$). The lipid contents of cultures grown in the basal medium (Ca^{2+} –) containing 2.5 μM TAM/5.0 μM TAM and of cultures grown in the Ca^{2+} medium (Ca^{2+} +) containing 7.5 μM TAM/10 μM TAM were also compared in order to appraise the concentration dependence of TAM effects ([d] $p < 0.05$; [f] $p < 0.0005$).

[b] The values refer to pooled lipid extracts of 2–4 cultures at each growth conditions from which 3 or 4 samples were collected to perform fatty acid analysis; means ± standard deviations are listed.

Table 3
General characteristics of fatty acyl chain distributions of *B. stearothermophilus* lipids as affected by TAM [a]

Additives to the growth medium		Fatty acid composition [b]			
Ca^{2+} (2.5 mM)	TAM (μM)	total straight	Total branched	total iso-acids	total anteiso-acids
−	0	33.8 ± 1.35	66.2 ± 1.38	38.2 ± 3.9	27.4 ± 2.4
−	2.5	34.1 ± 1.85	65.8 ± 1.86	40 ± 2	25.2 ± 0.5
−	5	38.8 ± 1.84 ***,†††	61.1 ± 1.85 ***,†††	40.1 ± 1.1	20.4 ± 1.1 **,†††
+	0	35.5 ± 0.75	64.5 ± 0.75	35.1 ± 1.4	28.9 ± 0.52
+	7.5	37 ± 0.55 *	62.8 ± 0.54 *	38.4 ± 1.85 ***	24 ± 2.2 **
+	10	40.4 ± 2.2 **,†	59.6 ± 2.2 **,†	36.8 ± 1.13 **,††	22.2 ± 1.4 ***

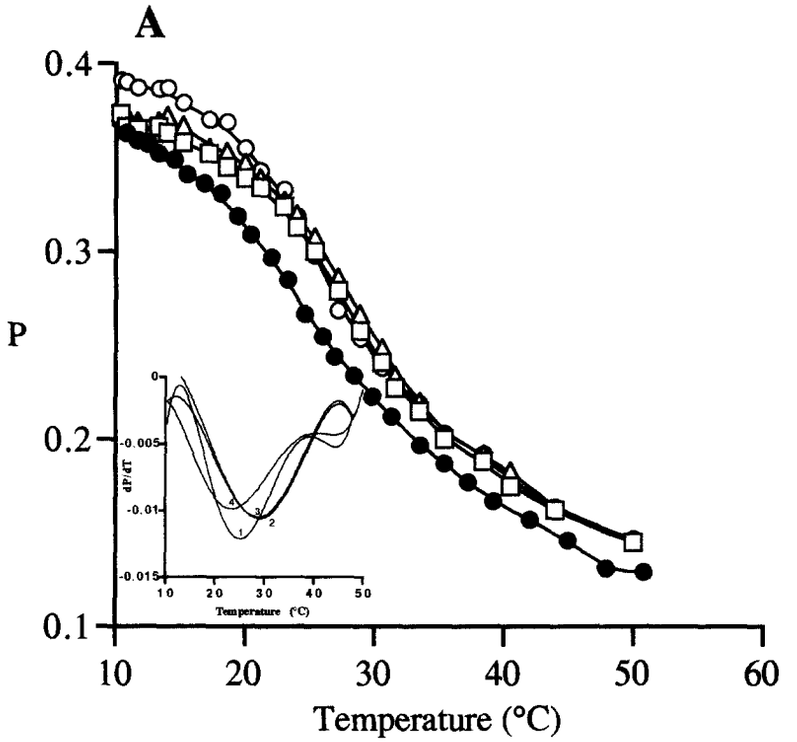
[a] Values of fatty acids contents were compared by Student's *t*-test, for the following paired observations cultures grown in the basal medium (Ca^{2+} -) containing 0 μM TAM/2.5 μM TAM or 0 μM TAM/5 M TAM; cultures grown in the Ca^{2+} medium (Ca^{2+} +) containing 0 μM TAM/7.5 μM TAM or 0 μM TAM/10 μM TAM (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$). The lipid contents of cultures grown in the basal medium ($Ca^{2+}-$) containing 2.5 μM TAM/5.0 μM TAM and of cultures grown in the Ca^{2+} medium ($Ca^{2+}+$) containig 7.5 μM TAM/10 μM TAM were also compared in order to appraise the concentration dependence of TAM effects ($^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.005$; $^{\dagger\dagger\dagger}p < 0.0005$).

[b] The values represent the sums of mol% of fatty acids represented in Table 2, arranged in chain categories.

important alterations of the phospholipid composition, except a statistically significant ($p < 0.05$) increase of the phosphoglycolipid content. However, the addition of a higher concentration of TAM (5.0 μM), besides potentiating the increase of the PGL content, induced a statistically significant ($p < 0.05$) decrease of the PE + *X* relative concentration associated with an increase of the DPG content. Comparing the lipid composition of cultures grown in the presence of 2.5 μM TAM with cultures grown with 5.0 μM TAM, statistically significant differences ($p < 0.05$) were found in the contents of PE + *X*, DPG and PG + *Y*. Regarding the lipid composition of cultures grown in a Ca^{2+}-supplemented medium, the addition of 7.5 μM TAM affects the contents of all main classes of phospholipids, with significant increases of PE + *X* and PGL contents ($p < 0.005$) and of DPG ($p < 0.05$); the more drastic alteration (statistically very significant, $p < 0.001$), however, was the decrease of PG + *Y* relative concentration by 63.9%. The addition of 10 μM TAM potentiates the increase of the PGL content but did not induce any additional modification of the contents of the other main classes of phospholipids as compared with 7.5 μM.

The acyl chain composition of *B. stearothermophilus* polar lipids, as affected by TAM is listed in Table 2. The dominant chains, representing 93.2–94.4% of the total, were myristic (*n*C14), 13-methyltetradecanoic (*i*C15), 12-methyltetradecanoic (*a*C15), 14-methylpentadecanoic (*i*C16), palmitic (*n*C16), 15-methylhexadecanoic (*i*C17) and 14-methylhexadecanoic acid (*a*C17). Branched chain species predominate (59.6–66.2 mol% of the total) in control and TAM-cultures, being the iso-acids more abundant than the anteiso acids (Table 3).

The addition of TAM to cultures in the basal medium or in a Ca^{2+}-supplemented medium induces a statistically very significant decrease of the total branched-chain fatty acids parallel to an increase of the total straight-chain fatty acids. In both growth media, the addition of TAM induces a non-linear concentration-dependent increase in the straight/branched chains ratio, although higher concentrations of the drug are required to produce a comparable change when the medium is supplemented with Ca^{2+}. Thus, in cultures grown in the basal medium, the addition of the 5.0 μM TAM induced very significant ($p < 0.0005$) alterations of the relative proportions of straight- and branched-chain fatty acids, the former increasing by 14.8% and the latter decreasing by 7.7%, corresponding to an increase of the straight/branched chains ratio by 25.5%. A double concentration of TAM (10 μM) was required in cultures grown in the Ca^{2+}-supplemented medium to induce similar fatty acid alterations (an


A
0.4
0.3
0.2
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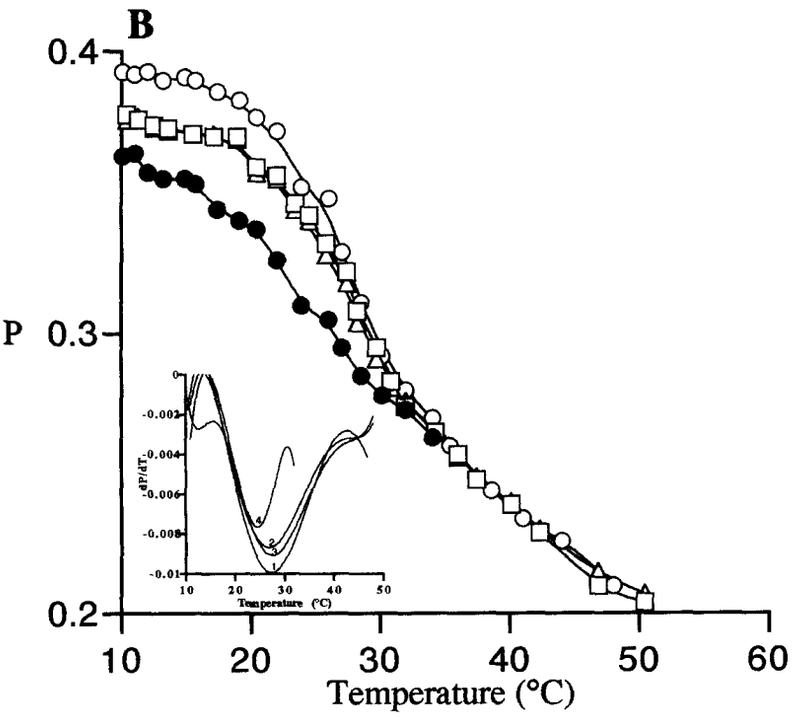
Temperature (°C)
dP/dT
-0.005
-0.01
-0.015
Temperature (°C)


B
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60
Temperature (°C)
dP/dT
-0.002
-0.004
-0.006
-0.008
-0.01
Temperature (°C)

increase of the straight chains by 13.8% and a decrease of the branched chains by 7.6%, yielding an increase of the straight/branched chains ratio by 23.6%). The addition of 7.5 μM TAM to this medium induced a limited effect, an increase of the total straight-chain fatty acids by 4.2% and a decrease of the total branched chains by 2.6%, yielding an increase of the straight/branched chains ratio by 7.3%. Within the branched-chain fatty acids, the anteiso-acids are mostly affected by TAM addition. The iso/anteiso ratio increases with the addition of TAM in the basal medium (by 41.7% with 5.0 μM TAM) as well as in a Ca^{2+}-supplemented medium (by 37.2% with 10 μM TAM).

The major shifts promoted by TAM are the decrease in the relative contents of *a*C15 (by 22.6% in the basal medium with 5.0 μM TAM and by 24% in the Ca^{2+}-supplemented medium with 10 μM TAM) and of *a*C17 (by 26.5% in the basal medium with 5.0μM TAM and by 22.8% in the Ca^{2+} medium with 10 μM TAM) and the increase of the contents of *n*C16 (by 17.5% in the the basal medium with 5.0 μM TAM and by 13.2% in the Ca^{2+} medium with 10 μM TAM) and of *i*C17, by 28%, in the Ca^{2+} medium with 10 μM TAM.

3.3. TAM effects on the physical behaviour of the polar lipids of B. stearothermophilus

The thermotropic properties of the bacterial lipids from cells grown in the absence of drug (control cultures) or in the presence of TAM, in a medium supplemented with Ca^{2+} or not, were studied by fluorescence polarization of DPH and its derivative DPH-PA. The effect of TAM on the physical state of bacterial lipid dispersions (multilayered liposomes) was studied in two different conditions:

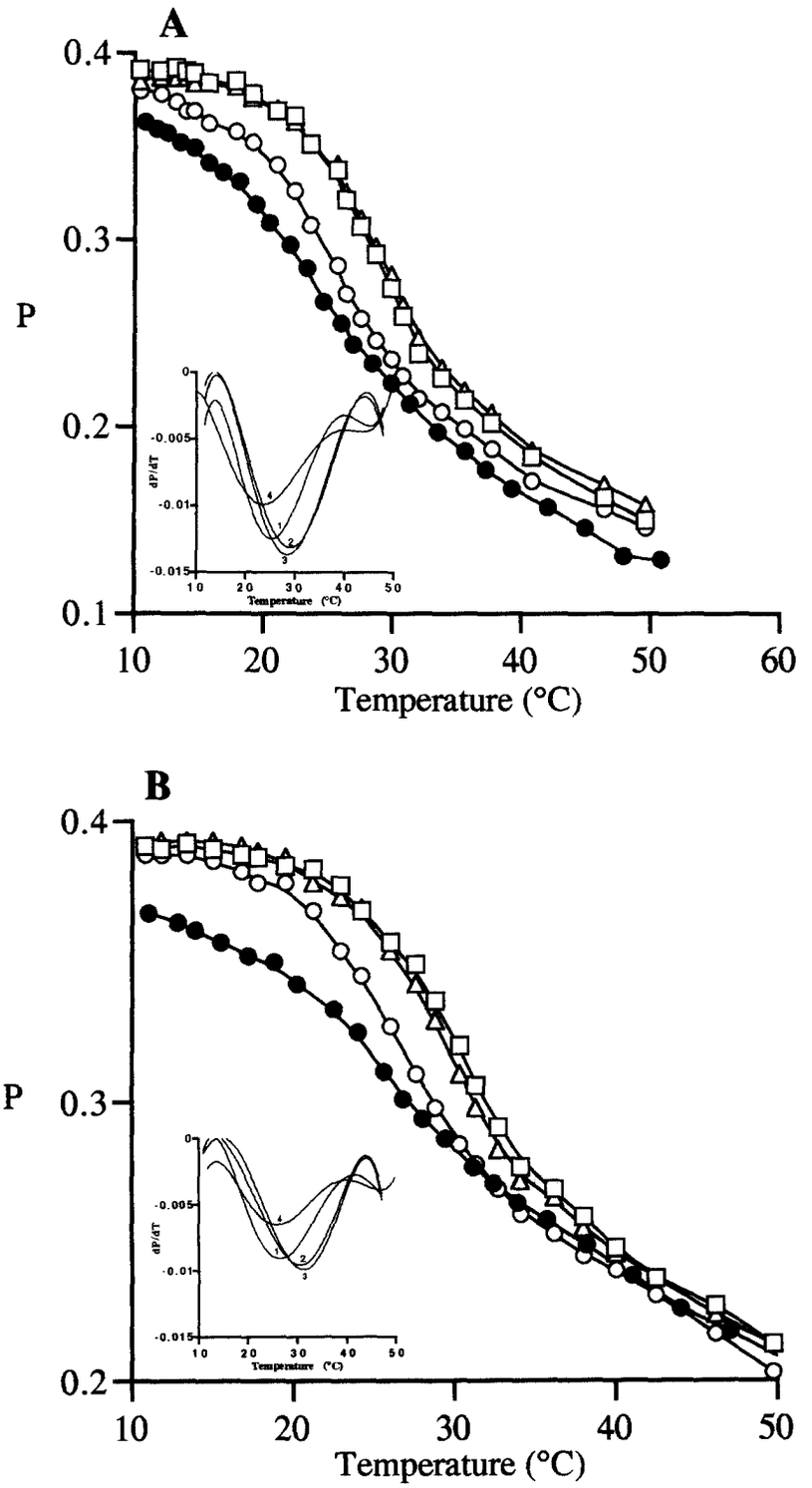
(a) as a result of lipid composition alterations, when TAM was added to the growth medium of bacterial cultures; and

(b) as a result of a direct TAM lipid interaction, when the drug was added to liposomes preparations.

The thermograms obtained by fluorescence polarization of DPH and DPH-PA (Figs. 2 and 3) with dispersions of the polar lipids from cells grown in different conditions exhibit a drastic decrease of the fluorescence polarization (P) over a range of about 20°C, indicating a broad transition in agreement with previous studies [13,27]. The accuracy of probe polarization to monitor a membrane order/disorder transition in these bacterial lipids was previously confirmed by differential scanning calorimetry (DSC). The DSC traces of bacterial polar lipids showed a broad endotherm over the same temperature range at which the decrease of P was observed [27]. The studies with the polar lipids of bacterial control cultures also yielded a reasonable agreement of the temperature defined by the midpoint of the linear decrease of P, corresponding to the peak of the first derivative of the polarization data (T_m), with the temperature of the endotherm peak [27]. Furthermore, the use of two fluorescent probes, DPH buried in the bilayer core [28] and DPH-PA anchored close to the interfacial region by its charged propionic group [29], allows comparison between the physical properties at different depths across the bilayer thickness. The thermotropic profiles of liposomes prepared with the polar lipids from cells grown in the basal medium containing 2.5 or 5.0 μM TAM show differences relative to liposomes prepared with the lipids from cultures grown in control conditions (Fig. 2). At temperatures below the phase transition, i.e. in a range from 10°C to 20°C, the dispersions of the polar lipids from bacterial cultures grown in a TAM spiked basal medium show a higher fluidity, in the hydrophobic core (Fig. 2(A)) and in the outer regions of the bilayer (Fig. 2(B)), in comparison with the bilayers prepared with the lipids from control cultures. As documented in Fig. 2 insets, the alteration of the

Fig. 2. Thermograms of fluorescence polarization (P) of (A) DPH and (B) DPH PA in liposomes prepared with the polar lipids of cells grown at 65°C in a basal medium without added TAM (control liposomes, (○, ●)) and containing (△) 2.5 μM and (□) 5 μM TAM. Control liposomes were incubated (○) without or (●) with 50 μM TAM. The insets represent the first derivatives of polarization data of the main plots, obtained by a computer graph program. Derivative curves 1, 2, 3 and 4 correspond to the main curves with the symbols ○, △, □ and ●, respectively. The thermotropic profiles are typical assays of three independent experiments. Polarization data are the average of three readings and error bars are not represented since, for most points, they are encompassed by the size of the symbols.

thermotropic profile of polar lipid dispersions induced by the addition of TAM to the basal medium is associated with an increase in the phase-transition temperature midpoint (T_m) as monitored by fluorescence polarization of DPH, but no alteration of T_m was detected by DPH-PA.



A distinct situation was observed with the lipids from cultures grown in a Ca^{2+}-supplemented medium (Fig. 3). Liposomes derived from cultures grown in the presence of tamoxifen exhibited values of fluorescence polarization of DPH (Fig. 3(A)) and DPH-PA (Fig. 3(B)), consistently higher than those of liposomes of control cultures, along the entire transition temperature range, indicating a significant decrease of fluidity over the entire thickness of the bilayer. A shift of the transition temperature midpoint to higher values was noticed by both probes in dispersions of polar lipids of TAM-cultures, as compared to controls (Fig. 3 insets).

The increase of TAM concentration from 2.5 to 5 μM in the basal medium, and the increase from 7.5 to 10 μM in the Ca^{2+} medium did not induce any additional alteration in the thermograms, which are practically coincident (Figs. 2 and 3).

Tamoxifen directly added to bacterial lipid dispersions promoted a concentration-dependent decrease in the polarization values of DPH and DPH-PA as reported in a previous work [13] and illustrated for a unique concentration in Figs. 2 and 3. According to calorimetric data (not shown), the direct interaction of TAM with liposomes promoted a shift of the phase transition to lower temperatures (Figs. 2 and 3, insets).

4. Discussion

B. stearothermophilus has been used as a model to clarify the antiproliferative action of tamoxifen putatively related with drug–membrane interaction [13]. The choice of this strain was motivated by the growth sensitivity to the physical state of membrane lipids [27]. Previous studies clearly indicate that interation of Ca^{2+} on the physical properties of bacterial membrane lipids improves growth at supra-optimal temperatures. On the other hand, bacterial cells change lipid composition to compensate for the thermal effects on membrane stability [27,30]. It is now established that a similar adaptation mechanism occurs in response to the destabilizing effects of tamoxifen. The significant lipid composition changes observed at TAM concentrations that slightly inhibit growth indicate that bacterial lipid regulatory system is highly precise and sensitive. It detects TAM-induced membrane disturbances at concentrations introducing no measurable physical alterations detected by general techniques of physical structure.

In agreement with previous data [13], it was observed that the addition of increased concentrations of TAM to the growth medium (basal or Ca^{2+}-supplemented) induced a progressively negative impact on all measured growth parameters, namely the lag time, the specific growth rate and the bacterial yield, indicating growth inhibition promoted by the cytostatic. The alteration of growth parameters is significantly extensive in a basal growth medium, as compared with Ca^{2+}-supplemented medium, reflecting a protection by Ca^{2+}. Stimulatory effects promoted by Ca^{2+} and Mg^{2+} have been also reported for a *Pseudomonas putida* strain growing in the presence of repressing solvents [31].

Owing to its high lipophilicity and strong partitioning in membranes [13,18], it might be expected that TAM induced changes in growth-reflecting perturbations at the membrane level. On the other hand, microbial cells are provided with efficient mechanisms of adaptation to environmental changes which disturb membrane structure and function, by controlling membrane lipid composition. Therefore, the study of the alterations in membrane phospholids classes and the acyl moieties, following the addition of TAM to the growth medium, is of obvious relevance. To estimate the role of Ca^{2+} ions on the improvement of tolerance to TAM, the study compares effects of TAM on cultures grown in basal and in Ca^{2+}-supplemented media. Lipid composition of cells grown in

Fig. 3. Thermograms of fluorescence polarization (P) of (A) DPH and (B) DPH-PA in liposomes prepared with the polar lipids of cells grown at 65°C in a Ca^{2+}-supplemented medium without added TAM (control liposomes, ○, ●) and containing (△) 7.5 mM and (□) 10 μM TAM. Control liposomes were incubated (○) without or (●) with 50 μM TAM. The insets represent first derivatives of polarization data of the main plots, obtained by a computer graph program. Derivative curves 1, 2, 3 and 4 correspond to the main curves with the symbols ○, △, □ and ●, respectively. The thermotropic profiles are typical assays of three independent experiments. Polarization data are the average of three readings and error bars are not represented since, for most points, they are encompassed by the size of the symbols.

control conditions (without drug) closely reproduces previous data [27,30]. In the basal medium, the addition of TAM decreases the ratio of zwitterionic (PE) to anionic phospholipids (PGL, PG, DPG). Thus, the PE/PGL + PG + DPG ratio decays from 1.75 (in control conditions) to 0.96 with the addition of 5.0 μM TAM to the basal medium, which might have a strong repercussion on the bilayer surface charge density and on the packing properties of the bilayer. A correlation between an increased anionic/zwitterionic phospholipids ratio and ethanol tolerance has been observed in *Escherichia coli* [32], *Bacillus subtilus* [33] and *Saccharomyces cerevisae* [34]. In our bacterial model, TAM affected the anionic/zwitterionic phospholipids ratio by increasing the DPG (and PGL) contents and decreasing PE. *Pseudomonas putida* modifies, in a similar way, the contents of PE and DPG in response to the presence of toluene, which has been explained in the light of a putative compensation for the propensity of the solvent to induce the formation of a hexagonal lipid phase [16]. The presumable distribution of TAM in the bilayer [13] is compatible with an increase in the hydrophobic volume, favouring the formation of high curvature structures, namely inverted hexagonal phases (H_{II}), particularly in a membrane with a high amount of PE, a lipid prone to non-bilayer phases [35]. Considering the increase of fluidity induced by TAM–lipid direct interaction, over the entire thickness of the bilayer, and the shift of the phase-transition temperature range to lower temperatures, as detected by DPH and DPH-PA (Figs. 2 and 3), it is expected that bacterial cells could react by lipid alterations envisaging a compensation of TAM perturbation. Lipid alterations induced by the addition of TAM to the basal medium, namely the enrichment of the membrane with straight-chain fatty acids and the increase of the iso/anteiso acids ratio, did not promote a decrease of fluidity of the polar lipids bilayers (Fig. 2) as expected, although a significant shift of T_m to higher values was detected by DPH. This finding strongly favours the hypothesis that these lipid alterations do not counteract TAM disordering effects on membranes; rather, the alterations may reflect a strategy to guarantee the stability of the lipid bilayer against a putative non-bilayer promoting ability of TAM molecules. The slightly increased fluidity of lipid bilayers from TAM-cultures as compared to controls, in the of 10–20°C range, more clearly detected in the outer regions than in the hydrophobic core of the bilayer, may be related to the decrease of PE and increase of charged lipids contents [36]. Lipid alterations in cultures grown in the basal medium, leading to an increase of the surface charge, may also reduce TAM partitioning into the bacterial membrane. Partition studies of TAM in liposomes, prepared from the polar lipids of bacterial control cultures [13], indicates that the high drug partition is affected by the lipid phase organization. Partition coeficients of TAM ($K_p = 8.2 \times 10^4$ at 25°C) were of the same order of magnitude as those reported for other membrane systems [18]. Therefore, drug incorporation into membrane–lipid dispersions from cultures grown with, or whithout TAM is possibly similar, although different drug concentrations could be predicted in discrete domains with different lipid phase organization. As suggested for other drugs [37,38], TAM may be unevenly distributed in biomembranes and the lipid composition changes induced by the drug in bacterial cells may serve the purpose of decreasing the partition coefficient of TAM in microdomains, particularly vulnerable to perturbations.

Let now consider TAM effects in a Ca^{2+}-supplemented growth medium. Relative to the control (lipids from cultures grown in a Ca^{2+}-medium without TAM), TAM induced an increase of PE, DPG and PGL relative concentrations and a very significant decrease of PG content. Any interpretation needs to take into account Ca^{2+}-induced alterations in the absence of drug. The most striking changes induced by Ca^{2+} were the decrease of PE (by 17%) and a threefold increase of PG. The aliphatic chains composition is not significantly sensitive to Ca^{2+}, apart from a slight increase of the ratio straight/branched chains. The thermograms obtained by fluorescence polarization with lipids from cultures in the Ca^{2+} medium are practically overlayed with those obtained with lipids from cultures in the basal medium, in the absence of drug, indicating that lipid alterations promoted by Ca^{2+} have little effects on the fluidity of the bilayers. Although not involved in a fluidity control, Ca^{2+} induced phospholipid alterations may disturb the balance between bilayer (cylindrical geometries) and non-bilayer (conical shaped) forming phospholipids, since the cation promotes conical ge-

ometries in lipid extracts rich in DPG, prone to adopt H_{II} phases [39]. Thus, the Ca^{2+} induced decrease of PE which, depending on the fatty acid composition, may also assume the conical shape [35], and the increase of the content of PG, a bilayer stabilizer [40], have obvious significance to preserve the stability of the bacterial membrane in the lamellar structure. On the other hand, the direct interaction of Ca^{2+} with acidic phospholipids promotes a decrease in fluidity of the bilayer with a significant shift of the main phase transition to higher temperatures [27]. This event may be related to the increased tolerance of bacterium to the addition of TAM to the growth medium. Calcium ions may compensate for the drug-induced disorder and T_m shift or may decrease the incorporation of TAM into the bilayer. Ca^{2+} induced shift of T_m may also result in a deviation to higher temperatures of the transition from a lamellar to a hexagonal phase. Therefore, the addition of TAM to cultures in the Ca^{2+}-supplemented medium, as compared to the basal medium, may have a less negative impact on the bilayer stability. Preliminary studies by ^{31}P-NMR (data not shown) indicate that the incubation of the polar lipids from bacterial control cultures with TAM did not, apparently, perturb the overall bilayer structure, yielding typical ^{31}P-NMR spectra of phospholipids in the lamellar phase, over the 20–70°C range, although a minor isotropic component is detected at temperatures > 45°C. However, stronger perturbations may occur in microdomains enriched with particular lipid species. Studies are currently underway to further clarify these possibilities.

At high concentrations ($> 7.5\,\mu$M), TAM disordering effects on the membrane might become more evident and the organism may be forced to modify the membrane–lipid composition to preserve the fluidity of the bilayer within limits compatible with effective function. Thus, the alterations induced by the addition of 7.5 and 10 μM TAM to cultures in the Ca^{2+}-supplemented medium may reflect a reinforcement of the membrane order. The decrease of PG, a phospholipid with lower T_m [36], may contribute to an increased lipid packing. Also, the increase of the phosphoglycolipid may serve a similar purpose. Glycolipids, because of their head groups with carbohydrate residues, may establish H-bonds with surrounding water, forming an ordered water interface that presumably attenuates the perturbations induced by the incorporation of alien lipophilic molecules in the hydrophobic core of the bilayer. In parallel, because of its bulk head group, the phosphoglycolipid may improve membrane packing by complementary interaction with cone-shaped components present in large amounts (PE and Ca^{2+}-DPG). The aliphatic-chains distribution of phospholipid species is also altered by the addition of TAM to cultures grown in a Ca^{2+}-supplemented medium. TAM induces an increase in linear fatty acids with a corresponding decrease in branched-chain species, and a decrease in anteiso-acids relatively to iso-acids. Since the branched chains, as a result of steric hindrances, are more loosely packed than straight chains in all-trans-conformation [41] and, among branched-chain species, the methyl anteiso-branched isomers promote a perturbation of the lipid order higher than methyl iso-branched species [42], the observed changes in fatty acid composition induced by TAM are of obvious adaptative significance. Thus, the fluidity of the lipid membranes from TAM-adapted cells was significantly decreased relatively to control cells, as detected by the probes of fluorescence polarization, DPH and DPH-PA, indicating a compensation mechanism against the disordering effects of TAM. Additionally, lipid changes induced by TAM shift the phase-transition temperature range of the bacterial polar lipids to higher values, as indicated by the derivative curves obtained from the fluorescence polarization data.

We realize that these data have to be cautiously interpreted, since the experiments were carried out with dispersions of the polar lipid extracts of bacterial membranes and the homogeneous dispersions of lipids we used in our experiments do not represent the real distribution of lipids in intact membranes. However, in spite of the inherent limitations, data of this work is of basic interest to further evaluate the potential significance of particular changes in membrane lipid composition of TAM resistant cells, namely the modulation of the lipid composition of tumor cells to enhance reactivity to TAM therapy.

5. Concluding remarks

The bacterial model used in this study alters lipid composition in response to the stress imposed by the

addition of TAM to the growth medium, at low concentrations. In agreement with findings for other microorganisms [16], the membrane lipid changes may suit preservation of fluidity within limits compatible with membrane functions or to restore the balance bilayer/non-bilayer geometries.

Since prokaryotic cells are provided with efficient mechanisms of regulation of polymorphic membrane lipid composition in response to environmental stress, in contrast to eukaryotic cells which normally function in homeostatic conditions and are not designed to rapidly change their lipid composition, bacteria are convenient models to evaluate membrane interactions of active drugs of pharmacolgical interest. Additionally, these studies may provide new insights to the problem of drug resistance in cancer chemotherapy.

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