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Lipid-lipid interactions of *Escherichia coli* mimetic inner membrane at human physiological temperature

Javier Hoyo, Juan Torrent-Burgués and Tzanko Tzanov

Group of Molecular and Industrial Biotechnology, Department of Chemical Engineering, Universitat Politecnica de Catalunya, Terrasa, Spain

Abstract. The current strategies to eradicate bacteria require that the antimicrobial agent either penetrate or disrupt the bacterial membrane. In *Escherichia coli* (*E.coli)* as a model of Gram-negative strains, the antimicrobials have to cross two barriers – the outer and the inner membrane being the latter composed by ~ 77% phosphatidylethanolamine (PE), ~ 13% phosphatidylglycerol (PG) and ~ 10% cardiolipin (CL) lipids. Each one of these lipid families shares the same headgroup, but contains acyl chains with varying length and degree of unsaturation. Bacteria adapt their membrane lipid composition and metabolism in response to environmental signals, such as the temperature, resulting in different interactions with exogenous molecules, e.g. antibacterial agents. Herein, bacterial model membranes are prepared to evaluate the lipid-lipid interactions in Langmuir monolayers of binary mixtures at several molar ratios of PE and PG or CL at human physiological temperature (37°C). Both PE:PG and PE:CL monolayers were stable at 37°C and presented higher molecular areas (> 20 $Å^2$/molecule) than at 23°C. However, these lipid mixtures presented liquid-expanded state and rigidity (inverse of the compressibility modulus ~ 90 mN/m) slightly lower than at 23°C. Such athermalicity at biologically relevant temperatures may favour the preservation of the biological functions of *E.coli*.

Key words: Phosphatidylethanolamine — Phosphatidylglycerol — Cardiolipin — Lipid-lipid interactions — Bacterial mimetic membranes — Physiological temperature

Abbreviations: CL, cardiolipin; DPPG, dipalmitoylphosphatidylglycerol; *E.coli*, *Escherichia coli*; LB, Langmuir-Blodgett; LC, liquid condensed; LE, liquid expanded; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PLE, polar lipid extract; POPE, palmitoyloleoylphosphatidylethanolamine; POPG, palmitoyloleoylphosphatidylglycerol.

Introduction

Most recent strategies to eradicate bacteria, rely on disruption of the bacterial membrane with nano-formulated antimicrobial agents that ultimately causes cell death with less probability for resistance development (Ivanova et al. 2017; Ferreres et al. 2018; Hoyo et al. 2019a). However, the majority of clinically relevant antibiotics need to cross the membrane of Gram-negative bacteria such as *Escherichia coli* (*E.coli)* in order to exert their antibacterial effect. The outer membrane of *E.coli* is mainly composed of lipopolysaccharides, while the inner membrane contains mainly lipids (~ 77% phosphatidylethanolamine (PE), ~ 13% phosphatidylglycerol (PG), and ~ 10% cardiolipin (CL)) and proteins (Shokri and Larsson 2004; Yeagle 2016). Langmuir and Langmuir Blodgett (LB) films have been used to study *in vitro* the antibacterial mechanism of new agents (Fernandes et al. 2017; Ivanova et al. 2018). Simple and reproducible mammalian (Nichols-Smith et al. 2004; Domènech et al. 2006), thylakoid (Hoyo et al. 2012, 2015, 2016a, 2016b), and bacterial (Gidalevitz et al. 2003; Clausell et al. 2007; López-Montero et al. 2008; López-Montero et al. 2010; Michel et al. 2015, 2017) membrane models have been prepared using

Correspondence to: Javier Hoyo, Grup de Biotecnologia Molecular i Industrial, Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Rambla Sant Nebridi 22, 08222, Terrasa, Spain
E-mail: javier.hoyo@upc.edu

these techniques for studying the interactions of exogenous molecules, including antibacterial agents, with bacterial membranes. Previous works reported the application of inner bacterial membrane models composed of a single lipid, e.g. dipalmitoylphosphatidylglycerol (DPPG) (Gidalevitz et al. 2003) and palmitoyloleoylphosphatidylglycerol (POPG) (Gidalevitz et al. 2003; Clausell et al. 2007), while the outer bacterial membrane was mimicked by lipopolysaccharides (LPS) (Clausell et al. 2007). The validity of these models was compromised by the use of only one lipid and the absence of PE, the major lipid present in bacterial inner membranes (Yeagle 2016). Michel et al. (2015) mimicked separately the outer and inner leaflets by the use of LPS and a ternary mixture of monounsaturated bacterial phospholipids. Furthermore, the same authors prepared a similar model with a unique structure combining the LB and Langmuir Schaefer techniques (Michel et al. 2017). Both models lacked the use of natural lipid constituents of the bacterial membranes. López-Montero et al. (2008, 2010) solved this drawback using an *E.coli* polar lipid extract (PLE) comprised of a myriad of lipid structures sharing the same headgroup. Recently, we prepared biomimetic membrane models using also a myriad of lipid structures to evaluate the lipid-lipid interactions in binary mixtures composed of PE and PG or CL at 23°C. The models revealed that all tested ratios of lipids were in fluid state (liquid expanded, LE) and minor changes in terms of membrane rigidity were observed among the PE content in the mixture (Hoyo et al. 2019b).

Bacteria regulate their metabolism, membrane lipid composition and the degree of unsaturation of their hydrocarbon chains as a response to environmental signals (Larsson and Törnkvist 1996; Shokri and Larsson 2004). Several biomimetic membrane studies have shown relevant differences in the lipid-lipid (Suárez-Germà et al. 2011) or lipid-polymer (Krajewska et al. 2013a, 2013b) interactions upon increasing the temperature. Therefore, studying the role of temperature in bacterial mimetic inner membranes could anticipate the interactions between novel antibacterial agents and the membrane. Herein, our previous study of PE:PG and PE:CL monolayers at 23°C (Hoyo et al. 2019b) is reproduced at human physiological temperature of 37°C. The surface pressure-area isotherms of Langmuir films describe the physical states, rigidity and thermodynamic properties of the monolayers at the air/water interface (Fig. 1). The use of natural *E.coli* lipid extracts of the myriad of structures corresponding to each lipid, and the study of several binary mixtures of lipids, including the biologically relevant one, allows for a reliable evaluation of the lipid-lipid interactions in *E.coli* mimetic membranes to serve potentially as a model for validation of novel antibacterial agents.

Materials and Methods

Materials

Avanti Polar Lipids, Inc. provided PE (♯ 840027), PG (♯ 841188) and CL (♯ 841199) extracted from *E.coli*. Each lipid represents a myriad of structures that share the same headgroup, but differ in their degree and position of unsaturation. $CHCl_3$ and phosphate buffer solution (PBS) tablets were provided by Sigma-Aldrich (Spain). Ultrapure MilliQ

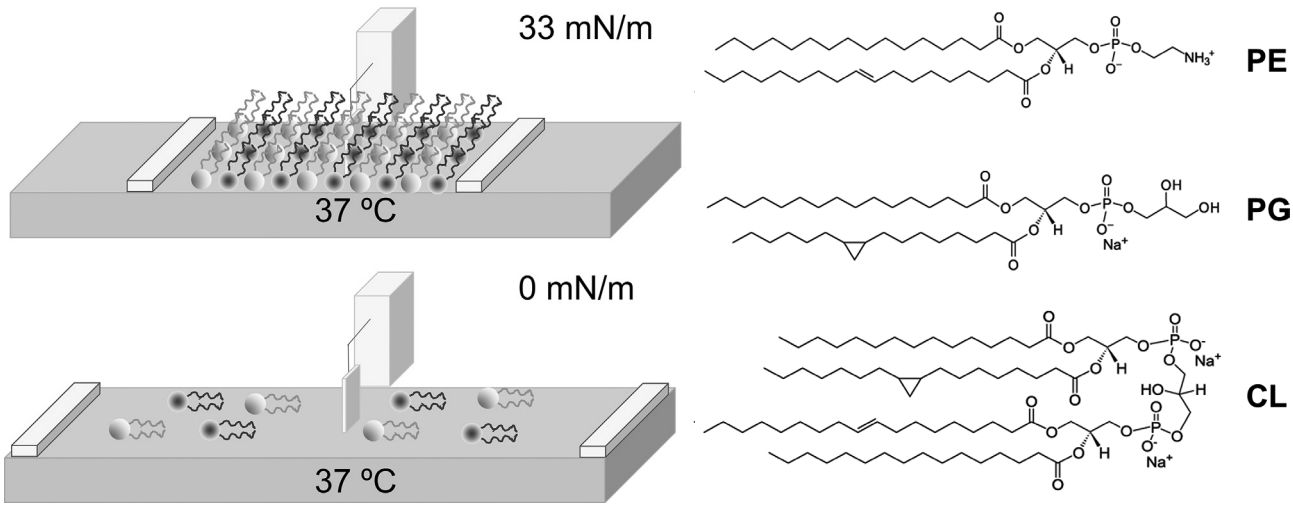

Figure 1. Scheme of a Langmuir monolayer formation and representative chemical structure of the myriad of lipid structures that shares the same headgroup: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) in lipid extracts from *E.coli.* (For more information, see section Materials and Methods).

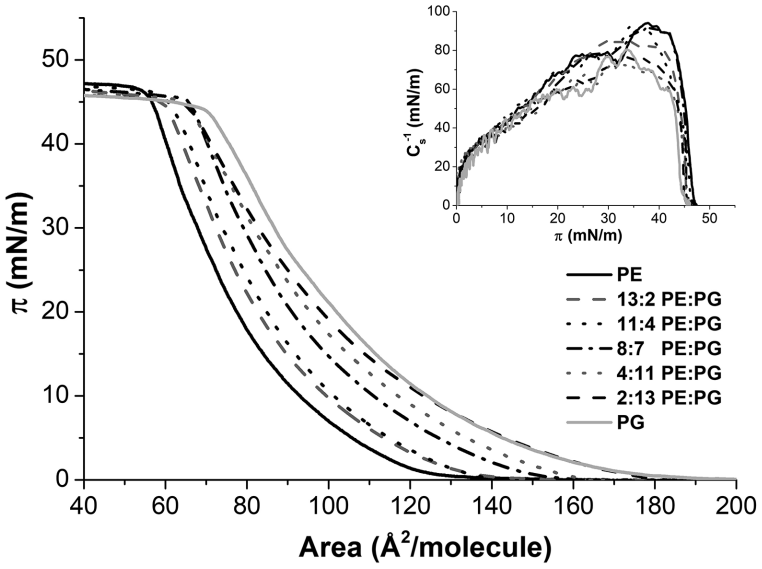

Figure 2. Surface pressure-area (π-A) isotherms for phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and PE:PG mixtures on PBS subphase at 37°C. Inset: Inverse of the compressibility modulus (C_s^{-1}) *vs.* surface pressure corresponding to the described π-A isotherms.

water with a resistivity of 18.2 MΩ/cm was used in cleaning procedures and for PBS at pH 7.4 preparation.

Surface pressure – area isotherms

PE, PG, and CL solutions in $CHCl_3$ (0.5 mg/ml) and the corresponding mixtures at different molar ratios were prepared by mixing each stock solution and were stored at –20°C until used. Surface pressure-area (π-A) isotherms were performed in a Langmuir trough equipped with two mobile barriers (KSV NIMA, model KN2002, Finland) with a total area of 273 cm^2 mounted on an antivibration table and housed in an insulation box at 37 ± 1°C. The temperature was maintained by connecting a thermostatic water bath to the inner circuit of the Langmuir trough and placing a temperature probe in the subphase. The Langmuir trough was cleaned with $CHCl_3$ and water. After subphase addition, the surface was further cleaned by suctioning. Immediately, 25 µl of the lipid or lipid mixture solution was added dropwise into the trough, and after 10 min evaporation of $CHCl_3$, the barriers were compressed at 25 cm^2/min. π-A isotherms were performed by triplicate.

Data analysis

Physical states

The inverse of the compressibility modulus C_s^{-1} was obtained from the π-A isotherms calculated according to Equation 1, where A is the mean area *per* molecule (Å^2/molecule), π the surface pressure (mN/m) and T the absolute temperature (K).

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)_T \quad (1)$$

Thermodynamic study

The mixing energy (ΔG_{mix}) of a mixture is obtained from the following equation in which A^E represents the excess area, A_1 and A_2 the area *per* molecule for the individual components, A_{12} the mean area *per* molecule for the mixture, x_1 and x_2 the molar fraction for each component, G^E the excess free energy of mixing, N_A the Avogadro's number, R the gas constant and T the absolute temperature.

$$A^E = A_{12} - (x_1 A_1 + x_2 A_2) \quad (2)$$

$$G^E = N_A \int_0^\pi A^E \, d\pi \quad (3)$$

$$\Delta G_{mix} = \Delta G_{id} + G^E \quad (4)$$

$$\Delta G_{id} = RT\,(x_1 \ln x_1 + x_2 \ln x_2) \quad (5)$$

Results

π-A isotherms, physical states and mixing behaviour of PE:PG system

The lift-off area for PE and PG at 37°C was observed at 135 and 185 Å^2/molecule and the collapse at π = 46 and 45 mN/m, respectively (Fig. 2). The isotherms were continuous and the C_s^{-1} curves reached their maximum ($C_s^{-1}{}_{max}$) at ≈ 94 and 80 mN/m (inset of Fig. 2) for PE and PG, respectively, confirming the LE (Vitovič et al. 2006) physical state of the system. The studied PE:PG mixtures show similar

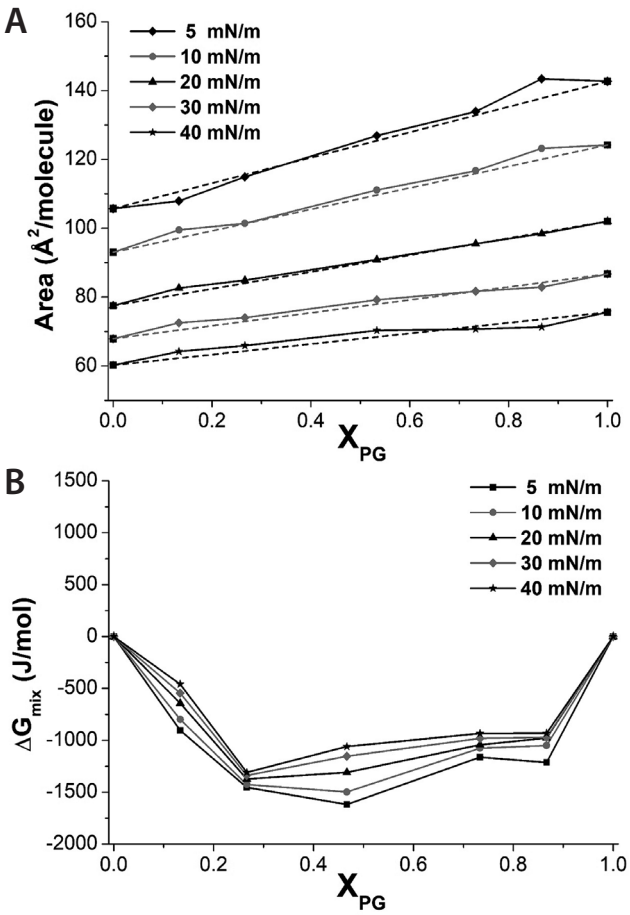

Figure 3. Mean area *per* molecule *vs.* molar fraction (X_{PG}; **A**) and mixing energy (ΔG_{mix}) *vs.* molar fraction (**B**) at several surface pressures for the PE:PG system at 37°C. Discontinuous straight line represents the ideal behaviour for each surface pressure.

isotherms and C_s^{-1} curves, mainly differing in the lift-off area that was higher upon increasing the amount of PG in the mixture. PE and PG showed similar size and shape. However, the PG headgroup is slightly larger than the headgroup of PE, explaining the differences observed in the lift-off area. The average molecular area (Fig. 3A) showed slight random deviations from the additivity rule (dashed line), confirming the ideal behaviour of the PE:PG Langmuir films regardless the PG content. The ΔG_{mix} curves (Fig. 3B) presented negative values for all tested mixtures, confirming their stability (Roche et al. 2006).

π-A isotherms, physical states and mixing behaviour of PE:CL system

The CL π-A isotherm at 37°C (Fig. 4) showed the lift-off area at 240 Å^2/molecule and the collapse of the film at π = 47 mN/m. The $C_s^{-1}{}_{max} \approx 94$ mN/m (inset of Fig. 4) indicates the LE physical state of the monolayer (Vitovič et al. 2006). The comparable hydrocarbon chains of PE and CL lead to similar isotherms and C_s^{-1} curves for their mixed monolayers. The increased molecular area observed upon increasing the CL content is correlated with the larger volume that occupy the four hydrocarbon chains in CL compared to the volume of two hydrocarbon chains in PE (Baumgärtner et al. 2007; Boyd et al. 2017).

The mean area *per* molecule *vs.* molar fraction curves (Fig. 5A) exhibited slightly positive deviations from the additivity rule (dashed line), corroborating that PE:CL system constitutes slightly non-ideal films at π > 5 mN/m. The negative ΔG_{mix} values (Fig. 5B) observed for all the mixtures indicate the stability (Roche et al. 2006) of the studied PE:CL mixtures.

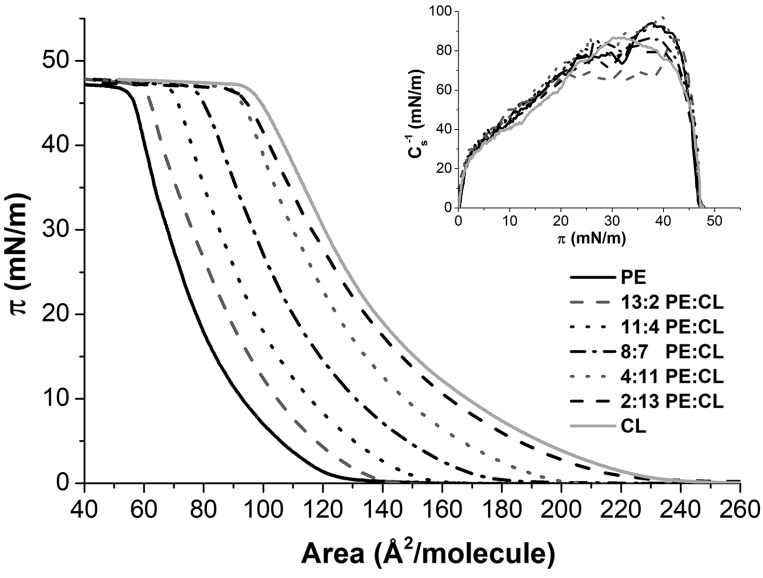

Figure 4. Surface pressure-area (π-A) isotherms for phosphatidylethanolamine (PE), cardiolipin (CL) and PE:CL mixtures on PBS subphase at 37°C. Inset: Inverse of the compressibility modulus (C_s^{-1}) *vs.* surface pressure corresponding to the described π-A isotherms.

Discussion

The current work used a myriad of natural lipid structures that share the same headgroup, but contain acyl chains with different length and degree of unsaturation, for preparing model bacterial membranes. PE:PG and PE:CL mixtures at 37°C showed higher lift-off areas (Fig. 2 and 4) than those observed at 23°C (Hoyo et al. 2019b). On the contrary, the collapse surface pressure was lowered upon the increase of temperature. Higher temperature favours the lipid vibrations and the dissociation of ionisable groups (Krajewska et al. 2013b) – PG and CL headgroups – inducing higher molecular areas. The PE:PG system yielded a higher lift-off area difference ~30 Å^2/molecule than PE:CL ~ 20 Å^2/molecule upon the temperature increase, attributed to the stronger interactions observed in the PE:PG system compared to PE:CL at 23°C (Hoyo et al. 2019b). Similarly, Suárez-Germà et al. (2011) observed that monolayers of lipids with PE headgroup presented higher lift-off area as the number of unsaturations of the hydrocarbon chains or the temperature was increased, according to observations for DPPG (Krajewska et al. 2013b) and dipalmitoylphosphatidylcholine (Krajewska et al. 2013a). The characteristics of the acyl chains (unsaturation and chains length) contributed to the steric hindrances between individual lipids resulting in a lower packing.

The $C_s^{-1}{}_{max}$ for the single lipids and their corresponding mixtures decreased below 100 mN/m, indicating that all studied systems present LE physical state in accordance to the results observed at 23°C (Hoyo et al. 2019b), where only PE lipid monolayer reached the liquid condensed (LC) state. The increase of the temperature favours the dissociation of ionisable groups (Krajewska et al. 2013b) and lowers the packing, thus yielding fluid phases. The rigidity of the studied systems at 23°C was slightly higher than at 37°C. The characteristics of the isotherms and the LE state observed for the binary PE:PG and PE:CL systems (Fig. 2 and 4) are in line with previous studies using a natural ternary mixture (Clausell et al. 2004; López-Montero et al. 2008). The presence of unsaturations promoted the formation of fluid phases in our PE:PG and PE:CL systems (inset of Fig. 2 and 4). Such phenomenon was also described (Suárez-Germà et al. 2011) for palmitoyloleoylphosphatidylethanolamine (POPE) and POPG that became less rigid upon increasing the number of unsaturations whereas the saturated dipalmitoylphosphatidylethanolamine reached solid state (S) (Hernández-Borrell and Domènech 2017). The PE:PG and PE:CL systems formed almost ideal monolayers at 37°C (Fig. 3A and 5A). In contrast, the PE:CL system showed non-ideal films at 23°C, inducing non-favoured mixtures when the amount of PE or CL was above 20% and $\pi \geq 30$ mN/m (Hoyo et al. 2019b). This observation is explained by the higher repulsion and vibration between lipids upon increasing the temperature.

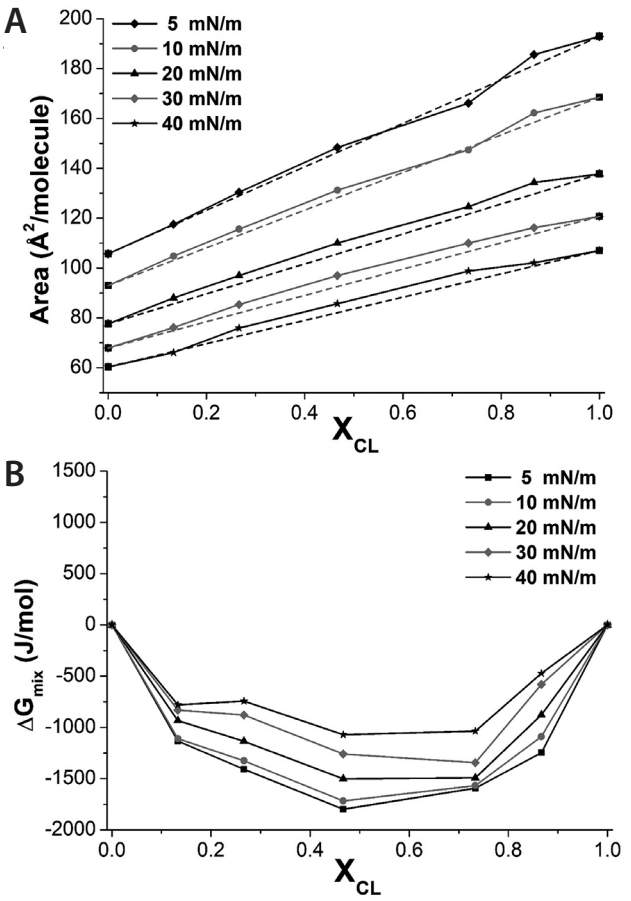

Figure 5. Mean area *per* molecule *vs.* molar fraction (X_{CL}; **A**) and mixing energy (ΔG_{mix}) *vs.* molar fraction (**B**) at several surface pressures for the PE:CL system at 37°C. Discontinuous straight line represents the ideal behaviour for each surface pressure.

The thermodynamic study showed negative ΔG_{mix} values for all studied systems (Fig. 3B and 5B), thus indicating the stability of the mixtures and the absence of phase separation (Gzyl-Malcher et al. 2008) domains for both PE:PG and PE:CL systems, regardless the PE:lipid ratio, contrarily to the PE:CL system unstable at 23°C. The formation of domains was observed for the POPE:CL (Domènech et al. 2006b) and POPE:POPG 3:1 (Seeger et al. 2009; Picas et al. 2010; Suárez-Germà et al. 2011) systems both in LB and supported planar bilayers – widely used for biomimetic membranes construction (Domènech et al. 2006b; Hoyo et al. 2013) – below and above the melting temperature. These observations were attributed to the tendency of POPE to form LC phase (Suárez-Germà et al. 2011) and the presence of Ca^{2+} ions that resulted in stronger interaction with POPG than with POPE (Picas et al. 2010), thus inducing phase separation. Oppositely to the POPE:POPG and POPE:CL systems, the thermodynamic studies of biomimetic membranes with

a natural myriad of bacterial lipids indicated the absence of phase separation domains at 37°C (López-Montero et al. 2008). Nevertheless, the same authors (López-Montero et al. 2008) observed their presence by epifluorescence microscopy, whereas Domènech et al. (2006a) did not detect such domains by atomic force microscopy (AFM), in line with our thermodynamic results (Fig. 3B and Fig. 5B). Most probably, the use of selective fluorescent dyes favors the visualization of such domains in biomimetic membranes (López-Montero et al. 2008), as previously observed in natural bacterial membranes (Mileykovskaya et al. 2001). These apparently contradictory results with some previous studies may be explained by the limitations of the thermodynamic study applied to a multicomponent system. Despite the fact that we have studied binary PE:PG and PE:CL systems at several molar ratios, each lipid named by its headgroup contains several structures, corresponding to different chain length and unsaturation, building a multicomponent system. Therefore the emergence of nanoscale lipid domains could be possible as observed in biological membranes (Jacobson et al. 2007).

The temperature of the bacterial membrane environment is an important parameter, since some of the lipid constituents of these membranes present phase transition close to the physiological human temperature (Silvius 1982). Therefore, the physical state influences both the permeability of the membrane and the interactions of the lipids with other membrane components, altering consequently their function (Barrera et al. 2012). Several mechanisms have been reported by which antibacterial agents target bacteria and alter their membrane properties (Epand et al. 2016). Therefore, the physical state and the fluidity of the bacterial membrane is of high relevance for the development of bactericidal agents. Several bacterial model membranes with different degree of similarity to the real membranes are described in the literature, but most of them are composed by only one or two lipid structures. Despite that some of these studies used unsaturated lipids to enhance the fluidity of the resulting membranes, the effect of the biological myriad of lipid structures was not evaluated, thus the reduction of fluidity was not considered neither the different interaction of the lipid structures with the antibacterial agent. In our work, the fluidity of the model membrane formed at 37°C was compared to the fluidity of a membrane formed at 23°C, concluding the athermalicity of the rigidity and physical state for PE:PG and PE:CL systems at biologically relevant temperatures. These results correlate with previous reports for other lipid systems at LE state (López-Montero et al. 2008) and may explain the ability of *E.coli* membranes to maintain their functionality across a broad temperature range (Remaut and Fronzes 2014) due to intermolecular hydrogen bonding between lipids and transmembrane proteins allowing the correct protein insertion and function in the membrane (Domènech et al. 2006b). The observed athermalicity suggested that the effect of antibacterial agents on model lipid membranes would be similar, regardless room or human physiological temperature.

Conclusions

E.coli model membranes were prepared using bacterium's natural lipid structures. These biomimetic membranes present almost athermal physical state and rigidity, which may be related to the ability of bacteria to maintain their functions at human physiological temperatures. These observations are of high relevance for the design of novel antimicrobial agents targeting the bacterial membranes. Similar behaviour of the bacterial membrane in the range of 23 to 37°C was detected, despite that the phase transition temperature of some lipid structures present in *E.coli* membranes is close to human physiological temperature. Independently from the different strategies that bacteria adopt to adapt their lipid membrane composition to the temperature of the environment, their bulk membrane behaviour in the studied temperature range was not significantly altered. Novel bactericidal agents targeting the bacterial membrane may be engineered considering the athermalicity of the membrane bulk properties. The simple and reliable bacterial membrane model described in this work may be further used for mechanistic studies *in vitro* of the interactions between bactericidal agents and bacteria.

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Conflict of interest. The authors declare no competing financial interest.

Author contributions. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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