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Pseudomonas aeruginosa produces phosphatidyltris(hydroxymethyl)aminomethane and derivatives when grown in Tris-buffered medium

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ABSTRACT

For optimal growth of a microorganism, the pH of the culture medium should be set at an optimum value. For that reason, growth media require buffering agents. We show in this study that, when grown in a medium supplemented with tris(hydroxymethyl)aminomethane (Tris), *Pseudomonas aeruginosa* is able to use this organic compound to produce new phospholipids. We thus pointed out that phosphatidyltris(hydroxymethyl)aminomethane as well as diphosphatidyltris(hydroxymethyl)aminomethane was detected in membrane lipid extracts of bacteria grown in Tris-buffered medium. Moreover, the amounts of lysoglycerophospholipids in the lipidome of *P. aeruginosa* grown in Tris-buffered medium increased leading to the presence of lysophosphatidylglycerol and lysophosphatidyltris(hydroxymethyl)aminomethane as well as other lysophospholipid derivatives. Finally, we investigated the effect of the presence of these exogenous phospholipids on the susceptibility of *P. aeruginosa* to some antibiotics. We observed a decrease of the minimal inhibitory concentrations of different antibiotic families, *i.e.*, fluoroquinolones, aminoglycosides, ß-lactams and polymyxins, proving the importance of the buffer choice for growth medium and its impact on the lipidome.

1. Introduction

Microorganisms can grow in a wide range of environments. Consequently, the bacterial growth can be influenced by a wide variety of physical factors including: temperature, osmolarity, oxygen concentration and pH. For an example, *Pseudomonas aeruginosa*, an opportunistic Gram-negative pathogen, is well known for its successful adaptation to several environmental niches [1] and its natural aquatic and terrestic habitats can span a pH range from 4.5 to 9.5 [2]. This species is also characterized by its ability to cause a wide spectrum of nosocomial infections in immunocompromised patients [3]. In particular, *P. aeruginosa* causes chronic lung infections that are the most significant cause of morbidity and mortality among cystic fibrosis patients [4]. For these reasons, *P. aeruginosa* represents one of the most studied bacteria in laboratory experiments. In most studies, obtaining the optimal pH for growth requires the use of buffering agents. Phosphate, acetate, citrate, zwitterion compounds are examples of buffering agents that may be added to culture media. However, the ability of bacteria to adjust their lipidic composition in response to environmental changes (pH, salinity, temperature, …) [5,6] questions the effect of growth media and notably of the nature of the buffering agent on the lipid composition of the bacterial membrane.

Inner membranes (IMs) of *Pseudomonadaceae* mainly consist of the zwitterionic phosphatidylethanolamine (PE) as well as negatively charged phosphatidylglycerol (PG) and diphosphatidylglycerol (dPG) [7,8]. In particular, when grown in choline-buffered medium, *P. aeruginosa* IM is constituted of: PE, 71%; lysophosphatidylethanolamine

Abbreviations: IM, inner membrane; FA, fatty acid; FFA, free fatty acid; dLPE, dilysophosphatidylethanolamine; dLPG, dilysophosphatidylglycerol; dLPtris, dilysophosphatidyltris(hydroxymethyl)aminomethane; dPG, diphosphatidylglycerol; dPTris, diphosphatidyltris(hydroxymethyl)aminomethane; LPE, lysophos phatidylethanolamine; LPG, lysophosphatidylglycerol; LPC, lysophosphatidylcholine; LPTris, lysophosphatidyltris(hydroxymethyl)aminomethane; MGM, minimum glucose medium; MHB, Muller-Hinton broth; MIC, minimum inhibitory concentrations; NAPS, N-acylphosphatidylserine; OD, optical density; OM, outer membrane; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGpg, phosphatidylglycerolphosphoglycerol; PLPG, phosphatidyllysophosphatidylglycerol; PLPTris, phosphatidyllysophosphatidyltris(hydroxymethyl)aminomethane; PTris, phosphatidyltris(hydroxymethyl)aminomethane; PTris-pg, phosphatidyltris(hydro xymethyl)aminomethanephosphoglycerol; Tris, tris(hydroxymethyl)aminomethane.

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(LPE), 2%; PG, 12%; dPG, 1%; phosphatidic acid (PA), 2%; phosphatidylcholine (PC), 8%; lysophosphatidylcholine (LPC) 4% [9]. Such production of PCs and LPCs by *P. aeruginosa* is a common feature of bacteria interacting with eukaryotes [10,11].

In the present paper, we characterized the lipid composition of the IM of *P. aeruginosa* when grown in medium buffered with tris(hydroxymethyl)aminomethane (Tris). We show that this bacterial species may produce Tris-containing glycerophospholipids as well as unexpected PG derivatives. We also report that the substitution of phosphate by Tris in the culture medium induces an increase of the bacterial sensitivity to some antibiotics.

2. Material and methods

2.1. Bacterial strain and planktonic culture

P. aeruginosa PA14 strain was stocked in 30% (v/v) glycerol. Preculture was performed in a 50 mL flask containing 1 mL of bacteria stock suspensions and 10 mL of Muller-Hinton broth (MHB, Difco). The flask was incubated at 37 °C on a rotary shaker (140 rpm) for 18 h. The preculture was then used to inoculate 100 mL of culture broth at 10^7 Colony Forming Units (CFU)/mL. Three culture broths with a pH of 7.4 were used: MHB and minimum glucose medium (MGM: 0.5 g/L NH_4Cl; 2 g/L yeast extract; 0.05 g/L $CaCl_2$; 0.05 g/L $MgSO_4$; 0.005 g/L $FeSO_4$; 0.005 g/L $MnSO_4$; 15 g/L glucose) with either 100 mM Tris buffer (15 g/L TrisHCl; 0.6 g/L TrisBase), either 100 mM phosphate buffer (5.44 g/L KH_2PO_4; 5.44 g/L K_2HPO_4). Cultures were incubated on a rotary shaker (140 rpm) at 37 °C for 24 h to reach the stationary phase of growth. After 24 h of incubation, planktonic organisms were recovered by centrifugation (10 min at 2600 ×*g* at 4 °C).

Generation times of 39 ± 9 min and 40 ± 5 min were measured in phosphate- and Tris-buffered media, respectively.

2.2. Inner membrane lipid extraction

IM extraction was carried out following the spheroplast protocol described by Mizuno and Kageyama [12]. Rapidly, bacterial pellets were washed in 10 mL of 20% (w/v) sucrose and centrifuged. Pellets were weighted and resuspended in a digestion solution of the following composition (for 1.5 g bacteria wet weight): 18 mL of 20% sucrose; 9 mL of 2 M saccharose; 10 mL of 0.1 M Tris–HCl; 0.8 mL of 1% EDTA; 1.8 mL of 1% lysozyme; 1 μL of 1 mg/mL Rnase; 5 μL of 20 mg/mL DNase. Spheroplast formation at 37 °C was monitored by optical microscopy. When only ovoid forms were observed, the suspension was centrifuged at 30 °C for 15 min at 5200 ×*g* to recover spheroplasts. The pellet was resuspended in 5 mL of 0.01 M phosphate buffer saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) and was subjected to sonication (cycles of 30 s for 2 min, Transsonic 950, Prolabo, France). The suspension was then centrifuged at 30 °C, for 20 min at 5200 ×*g* (Sigma 3-16KL). The supernatant was diluted in 100 mM sodium carbonate, stirred at 4 °C for 1 h to separate soluble and insoluble phases and was ultracentrifuged (60,000 ×*g* for 1 h at 4 °C, Beckman Coulter TL100) to harvest bacterial membrane. Pellets were washed twice with 40 mM Tris buffer (pH 7) and frozen at −20 °C. This protocol allowed us to obtain IM enriched lipid extracts [13].

Lipid extraction was carried out according to Bligh and Dyer protocol [14]. For 1 mL of IM extract, 3.75 mL of chloroform: methanol (1:2 v/v) solution was added. The mixture was sonicated for 5 min and vortexed for 15 min until obtaining a milky-mixture. After adding 1.25 mL of chloroform, the mixture was vortexed for 1 min. A volume of 1.25 mL of 1 M NaCl aqueous solution was added and the mixture was vortexed again for 15 min. Finally, the mixture was centrifuged (670 ×*g* for 10 min at 30 °C) to separate organic and aqueous phases. The organic phase was recovered with a Pasteur pipette and 1.88 mL of chloroform was added to the aqueous phase. After stirring for 15 min, the mixture was centrifuged (670 ×*g* for 10 min at 30 °C) to separate the two phases. The aqueous phase was removed and organic phases were mixed and evaporated under argon.

To degrade and eliminate lipoproteins, 1 mL of methanol was added to lipid extracts. The mixture was vortexed for 5 min. Methanol was then evaporated. A chloroform: methanol (1:2, v/v) solution was added and the mixture was centrifuged (670 ×*g* for 10 min at 30 °C), to sediment proteins. The organic phase was recovered and evaporated under argon. The lipid extracts were conserved at −20 °C. The lipid concentration was evaluated by measuring the surface pressure of Langmuir film for each lipid extract using a Wilhelmy balance (Nima, UK) [15]. Lipid extracts were diluted in a chloroform:methanol (80:20, v/v) solution. The volume of solvent was adjusted in order to obtain a 1 mM solution for thin layer chromatography (TLC) analysis and a 30 μM solution for electrospray-mass spectrometry (ESI-MS) analysis.

2.3. Thin layer chromatography

TLC plates AdsorbiolTM Plus 1 SL 20 × 20 (Alltech, France) were heated at 100 °C for 30 min. The developer container for TLC was conditioned for 1 h with the mobile phase in order to saturate the TLC chamber with solvent vapor. Mobile phase was chloroform:methanol:acetic acid:water (85:22.5:10:4, v/v). One hundred μL of lipid samples was deposited at the bottom of the plate. The plate was then placed in the developing container for migration for about 1 h. After evaporation of the developing solvent mixture, lipid spots were revealed by using iodine vapor. For phospholipid identification by mass spectrometry, each spot was extracted from the scraped silica gel by two elutions with 1 mL of chloroform:methanol (2:1, v/v). Lipid-containing supernatant, collected after centrifugation (670 ×*g* for 10 min), was evaporated and stored at −20 °C. Comparing the spot intensities was carried out on five TLC plates for each growth culture media: three independent biological replicates for phosphate-buffered lipid extracts (two being technically duplicated); and four independent biological replicates for Tris-buffered lipid extracts (one being technically triplicated).

2.4. Mass spectrometry analyses of phospholipid extracts

Lipid extracts were analyzed by ESI-MS using two mass spectrometers. First, a Linear Quadruple Ion Trap (QTRAP®, AB Sciex Instruments™) mass spectrometer, equipped with a turbo spray ionization source heated to 300 °C, was used, as previously described [13,15]. The potential applied during the acquisition was −4500 V. Samples were infused at a flow rate of 10 μL/min in negative ionization mode. Spectra were acquired at 1000 amu·s^{-1} over the 50–1700 m/z range. In order to identify main ions of MS spectra, the mass spectrometer was set in the enhanced production ion (EPI) scan mode. Selected parent ions were trapped in the LINAC collision cell during 200 ms with Q1 unit resolution. Pure nitrogen was used as collision gas. Gas pressure was set to "low" value and collision energy ranged step by step (5 V) between −120 and −10 V. The mass spectrum of the fragment ions was acquired at 1000 amu·s^{-1} over the 50–1700 m/z range. MS/MS spectra were the average of 35 consecutive spectra recorded for 2.5 min. The chosen acquisition conditions allowed to point out a large variety of daughter ions and the pseudo-molecular ion.

Second, a high resolution mass spectrometer (HRMS, LTQ Orbitrap Elite, Thermo Scientific) was used. The capillary voltage and the source temperature were set at −4 kV and 300 °C, respectively, in negative mode. Samples were infused at a flow rate of 5 μL/min. The mass range was m/z 200–2000 and the resolution was set at 120,000. MS/MS spectra were recorded both in the orbitrap (in CID or HCD mode) and in the ion trap analyzers. MS/MS was achieved at a collision energy varied from 35 to 50, according to the parent ions.

To confirm the impact of Tris on the *P. aeruginosa* lipidome, mass spectra were done on six independent biological lipid extracts obtained with Tris-buffered medium. In the case of the reference lipid extracts