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The lack of *dam* gene induces modification on the lipids membrane composition of *Salmonella* typhimurium exposed to the Ox bile extract

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ABSTRACT

The effect of methylation on the bacterial growth, the phospholipids (PL) and the membrane fatty acid (FA) composition in *S.*Typhimurium wild type (WT) and *dam*- mutant strains exposed to the ox bile extract (OBE) stress was examined. Phosphatidylethanolamine (PE) Cardiolipin (CL), and Phosphatidylglycerol (PG) are the major PL present in all the strains and accounted for greater than 95% of the total lipid (TL) phosphorus. Phosphatidic acid and phosphatidylserine are the minor ones.

The OBE stress highly affected the growth rate of the *dam*- mutant and its PL contents. We noticed a great increase in the acidic PL proportion (CL and PG) and an unusual decrease in PE compared with the untreated *dam*- and the isogenic WT strains. FA composition of the TL and the different fractions containing PL were altered. Moreover, OBE stress combined with the *dam* mutation caused an increase in the ration of unsaturated to saturated fatty acids (UFA/SFA) and there was an increase in the content of palmitoleic acid ($C_{16:1w7}$) and oleic or vaccenic acid ($C_{18:1w9}$). This increase in UFA content was compensated by a decrease in the corresponding cyc17 and cyc19 cyclic FA (CFA). So these CFA were converted to UFA, which resulted in a high UFA/SFA ratio.

Based on these observations, we suggest that the Dam protein might regulate biosynthesis of membrane lipids of *S.*Typhimurium and it is required for membrane protection from bile salts during bacterial growth and infection process. ! 2014 Trade Science Inc. - INDIA

KEYWORDS

*S.*Typhimurium;
Fatty acids;
Phospholipids;
OBE stress;
Dam enzyme;
Methylation.

INTRODUCTION

Salmonella enterica comprise a family of Gram-negative enteropathogenic bacteria that successfully colonize a wide range of animal hosts including humans[1]. They are an etiological agent that causes a variety of food and water-borne diseases ranging from gastroenteritis, food and blood poisoning to typhoid fever[2]. The serovar *S.*Typhimurium represents a concern regarding food safety and its ability to grow in a wide range of adverse environmental conditions. Ingested bacteria colonize the intestinal epithelium by triggering their own phagocytosis, using a sophisticated array of effector proteins that are injected into the host cell cytoplasm. Moreover, *S.*Typhimurium which contaminates foods may be derived from environments in

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which its previous growth occurs at moderate acidic conditions and, consequently, it may develop adaptive responses which enhance its resistance to other stress conditions occurring during food processing[3-5]. One of the most important and intensively studied stress responses in *S.*Typhimurium is the acid tolerance response which increases its subsequent ability to survive in high acid foods, as well as in the extreme acid conditions of the gastrointestinal tract, increasing the risk of illness[6-9].

The initiation of replication of chromosomal DNA is coordinated with cell division. It has been demonstrated that DNA replication in bacterial cells is initiated on membranes and the activities of replication proteins are regulated by membrane components[10]. Indeed, initiation of DNA replication is precisely regulated in the cell cycles[11]. The DNA adenine methyltransferase (Dam) protein seems to be one of the key proteins in the control of this process.

The Dam enzym of *S.*Typhimurium catalyzes the methylation of adenosine moieties in the sequence 5'-GATC-3' using *S*-adenosyl-methionine as a methyl donor. This methylation of DNA by Dam protein provides a biological and an epigenetic signal that influences and regulates pathogenesis[12] and numerous physiological processes in the bacterial cell including chromosome replication and nucleoid segregation, mismatch repair, transposition, and transcription of certain genes[2,13]. A growing number of reports describe a role for DNA adenine methylation in regulating the expression of various bacterial genes related to virulence in diverse pathogens, suggesting that DNA methylation may be a widespread and versatile regulator of virulence gene expression[14-17]. Aside from these roles, Dam protein is known to regulate the metabolism of membrane lipids (Phospholipids and fatty acid synthesis). Disruption of the *dam* gene of *S.*Typhimurium causes filament formation, aberrant nucleoid segregation, induction of the SOS response, envelope instability, and increased sensitivity to membrane-damaging agents like bile salt.

The purpose of this study was to investigate the changes brought about in the growth and in the membrane PL and FA composition of *S.*Typhimurium grown in the presence of OBE in an attempt to clarify the role of membrane composition and fluidity in *Salmonella* stress responses and to observe any sensitivity in the extent of bile salts injury induced by such a change in membrane lipid composition.

MATERIALS AND METHODS

Bacterial strains

The strains of *Salmonella enterica* used throughout these experiments belong to serovar Typhimurium and are derived from the mouse-virulent strain ATCC 14028. Two isogenic strains were used and their relevant genotypes are: 14028: (WT: *dam*$^+$); SV4698: (*dam-225*::Mud*J* Km^r: *dam*$^-$).

The strains were kindly provided by Dr. Fransisco Ramos-Morales (Departamento de Genética Facultad de Biología Universidad de Sevilla Spain)[18].

Media and chemicals

Luria-bertani broth (LB) was used as a rich medium. Solid media contained agar at 1.5% final concentration. Sodium choleate (OB extract) was from Sigma Chemical Co., St. Louis, MO.

Bacterial growth with OBE

The *S.*Thyphimurium *dam*$^+$ and its isogenic mutant strain *dam*$^-$ were stored at -80°C. LB broth and nutrient agar (15 g.L^{-1}) were used for cultivation of the bacteria. The media was prepared as described by Miller[19] and was autoclaved at 120 °C for 30 min. Cultures were prepared by inoculating liquid media with a single colony from nutrient agar. The medium was supplemented, for the *dam*$^-$ strain, with kanamycin to a final concentration of 50 µg.mL^{-1}. Pre-cultures were performed overnight at 37°C in 10 mL of culture medium. They were then diluted in 50 mL of sterile LB broth to an initial concentration of 0.1 at an optical density (OD) of 600 in an Erlenmeyer flasks (250 ml) containing OBE (1%).

Bacterial growth were done aerobically in a shaker water bath at 37 °C and aerated by shaking during the experimental procedure. *dam*$^+$ and *dam*$^-$ were grown exponentially, the doubling time was calculated for each experiment and the speed of growth of a bacterial culture was appreciated by measuring its absorbance at 600 nm using a spectrophotometer: samples are taken every 2 hours and OD was measured. Spectrophotometric measurements was expressed as the log10 of the surviving fraction (number of the colony forming units [CFU] per milliliter [N] divided by the initial number of

CFU per milliliter [N_0]). The logarithms (N/N_0) were plotted against time. The number of CFU was determined 2, 4, 6, 8 and 10 hours after the start of the cultivation. CFU measurements were done for three different culture experiments.

Lipids manipulation

Culture conditions

Pure cultures of bacterial strains were maintained on LB agar plates at 4°C. Precultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 ml of sterile LB followed by aerobic incubation in a shaker water bath at 37°C for 24 h. Erlenmeyer flasks containing 50 ml of sterile LB were inoculated with the subculture to a final concentration of 10^3 cells/ml. The OD of the cultures and the dry-weight contents were assayed as described by Ames[20].

OBE sensitivity

An exponential bacterial culture in LB was washed and resuspended in phosphate-buffered saline solution (PBS) containing OBE at a concentration of one percent. In parallel, control samples were prepared in PBS. Treatments were carried out during 12 h of incubation at 37°C and growth was visually monitored. Assays were carried out in triplicate.

Extraction of total lipids

The lipid extraction procedure was previously described[21]. The method consists of an extraction of the lipids in a monophasic system in which methanol, chloroform, and water were in the proportions 2:1:0.8, by vol. The lipids were separated from the water-soluble material by diluting the extraction mixture with 1 volume of chloroform followed by 1 volume of water. After centrifugation, the chloroform layer was removed completely by gently inserting a pipette through the water-methanol phase and through the pellet which was formed at the interphase. The extractions were performed at 0°C. Chloroform layers so obtained were evaporated to dryness in a rotary evaporator at 40°C or less. The lipid residue was immediately dissolved in a small volume of hexane and stored at -18°C under a nitrogen atmosphere until chromatographic analyses were carried out.

Analysis of FA in total lipids and phospholipids

These analyses were carried out from 1L of each bacterial culture obtained as previously described. All reagents for saponification, methylation, extraction, and washing were dispensed with autopipets into this same tube, making the hands-on time minimal. Next, the final extracts were analyzed by a GC system (Agilent Technologies 6890N^R model [Network GC System]) equipped with a flame ionization detector (FID), an electronic pressure control (EPC) injector. A polyethylene glycol fused silica capillary column (Innowax, 30 m × 0.25 mm × 0.25 µm film thickness) purchased from Agilent (Wilmington, Delaware, USA) was used. The column was operated at 150°C for 1 min, the temperature was raised by 15°C/min^{-1} to 210°C for 5 min and then raised by 5°C/min^{-1} to 250°C and maintained until the end of analysis (25 min). N_2 was used as the carrier gas at a flow rate of 150 KPascal, H_2 at a flow rate of 25ml.min^{-1} and the split ratio used was 60:1. Peak areas were quantified using chromatography software (Agilent Technologies ChemStation Family^R data analysis). The identification of FA methyl esters (FAME) was performed by external standards (all purchased from Sigma Chemical Co.) submitted to the same processes of manipulation as the analyzed biological samples. A known quantity of heneicosanoic FAME [$C_{21:0}$] was used as an internal injection standard. The values of FA are presented as area percentage of total FA. Total SFA, total UFA and total CFA were used to determine the differences among membrane FA of *S.*Typhimurium cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane fluidity.

Incorporation of *cfa* plasmid or exogenous CFA to *dam*$^-$ mutant cells

Transformation by the calcium chloride method was used. Routinely 40 ng of pAYW19 plasmid (Carrying *cfa* gene on pGEM5 vector) was used per 200 µl of competent cells. Competent *dam*$^-$ cells were grown in LB sterile liquid medium, and transformants were selected on kanamycin plates. The concentration of antibiotic used in media was 50 mg.L^{-1} kanamycin sulphate.

To test the effects of CFA-supplemented medium on the OBE sensitivity, *dam*$^-$ strain was grown in LB medium containing the OBE and the proper CFA supplements for 16 h at 37°C. The cyc-17 CFA, (cis-9,10-methylenehexadecanoic acid) and the cyc-19 CFA, (cis-9,10-methyleneoctadecanoic acid:

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dihydrosterculic acid) was obtained from the Sigma Chemical.

Statistical analysis

Statistical analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. Both differences in the CFA and UFA/SFA were examined by the Friedman test, followed by the Wilcoxon signed ranks test. p-values of < 0.05 were considered as significant.

RESULTS

The overall purpose of this work was to study the effect of OBE on bacterial growth and the modifications of the cell membrane compounds of *S.*Typhimurium grown in the presence of OBE doses. The results obtained proved that the tested substances induced a significant growth alteration and noticeable modifications of the PL and FA composition of cell membrane of *dam*⁻ mutant cells, during bacterial growth.

Effects of OBE on *S.*Typhimurium WT and *dam*⁻ growth abilities

In order to check the effect of OBE on the growth of our bacterial strains, we carried out an analysis of the biomass in a liquid culture media. The ability of *S.*Typhimurium *dam*⁺ and *dam*⁻ to grow in the presence of bile salts was examined over 10 hours and the results are represented on Figure 1: A and B (the average results of three experiments are presented on these figures).

The growth data are plotted as the $\log_{10}$ of the ratio of the number of surviving colony-forming units (CFU) per milliliter divided by the initial number of CFU per milliliter at time zero. The logarithms are plotted against time. The values are the means ± SD of three different CFU measurements. * $p < 0.05$ vs. control samples.

The follow-up of the evolution of the biomass and the establishment of the curve showed a significant altered growth ($p < 0.05$) of the mutant strain cultured with OBE compared to the non exposed one. This alteration starts at the beginning of the exponential phase and continues in stationary phase. However, for *S.*Typhimurium *dam*⁺ grown with bile salts, no significant growth rates differences were found with the non exposed WT strain. Also, the establishment of a competitive index (CI) confirms the growth advantage. Moreover, the knowledge of the microbial speed growth

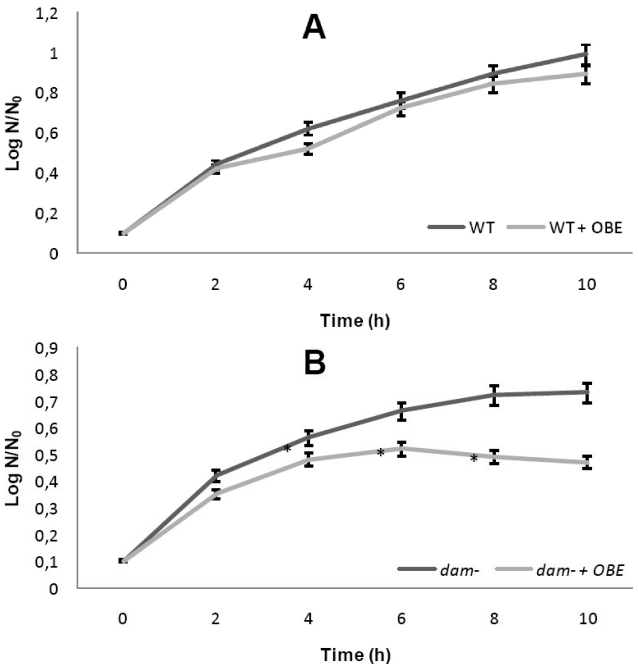

Figure 1 : Changes in the viable cell number of *S.*Typhimurium (A) WT strain (*dam*⁺) and (B) mutant strain (*dam*⁻) during bile salts exposure.

during the cell cycle (exponential phase) would be especially useful and essential in elucidating this difference and the influence of the acidic stress on the growth of our bacterial strains. For that, we determined the generation times of WT and mutant strains grown with and without bile salts and we obtained the values marked in TABLE 1.

The analysis of these results shows that the doubling times of the WT strain (exposed and none exposed) are the same and no significant differences were found. For *dam*⁻ the doubling time of the exposed strain was higher compared to that of the WT (exposed and none exposed) and the *dam*⁻ strains, it makes the double time of the parental strain (*dam*⁻ none exposed). However, the *dam*⁻ mutant cultured with bile salts, gave smaller colonies on LB plates (at 37°C), reflecting a slight growth disadvantage compared to the control strains (WT exposed and none exposed / *dam*⁻ none exposed) and the unique explanation for these differences must be the OBE stress added to the lack of methylation.

Effect of OBE on membrane PL composition of *S.*Typhimurium WT strains

The major PL present in *S.*Typhimurium WT strain membrane was the acidic PL (PG and CL) and the zwitterionic PE. These PL distributions were in accor-

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TABLE 1 : Generation time of bacterial strains

Strains	dam^+		dam^-	
Medium culture	LB	LB + OB	LB	LB + OB
Generation time	0,4 h^{-1} (24 min)/gen	0,43 h^{-1} (26 min)/gen	1,0 h^{-1} (60min)/gen	1,83 h^{-1} (110min)/gen

h: hour; min: minute; gen: generation. The generation times of WT and mutants cells growing exponentially with and without bile salts at 37°C in LB media were 24/26 min (dam^+) and 60/110 min (dam^-), respectively

dance with those reported in the literature for *S.*Typhimurium[20].

Compared with the non treated cells, our results indicated that the PL composition of the bile treated WT strain was in the majority similar to the non treated WT strain (PE: 75.2%, PG: 19.4% and CL: 5.3%). So it appeared to be unaffected by the OBE stress. The acidic PL fractions (PG and CL) amounted to 25.4% of total PL distributed in 20.7% of PG and 4.7% of CL. A non significant decrease in the PE fraction (74.6%) was observed (Figure 2).

Exponentially growing WT strain was incubated at 37°C with Sodium choleate (1%). Their contents were also calculated from the FA contents. Average values of triplicates were given, and the deviation was less than 5% of each value.

Effect of OBE on membrane PL composition of *S.*Typhimurium *dam*⁻ mutants

The PL composition of the *dam*⁻ mutant membrane was determined. It shows that the PE, PG, and CL proportions were affected by the *dam* mutation while comparing them with the WT strain. In the *dam* mutant, the zwitterionic PE fraction decreased to 22.25%. However, the acidic PL fraction (PG and CL) becomes a majority of total PL with 77.75%, distributed in 30.05% of PG and 47.7% of CL. To evaluate the combined effects of the *dam* mutation and the OBE stress on the bacterial membrane integrity, we compared the PL composition of the exposed with the non exposed *dam*⁻ mutant strains. The results showed that the acidic PL (PG and CL) showed a great increase with 42.4% and 50.1%, respectively. However, the PE proportion decreased dramatically to 7.5%. The analysis of PL levels in *S.*Typhimurium *dam*⁻ non exposed (A) and exposed

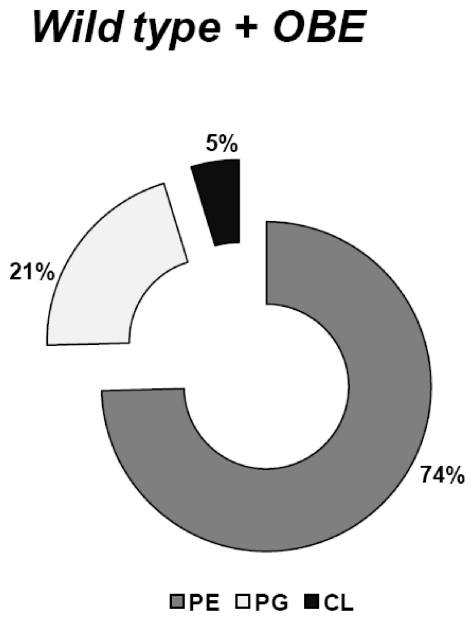

Figure 2 : Analysis of PL levels in *S.* Typhimurium wild type exposed to OB

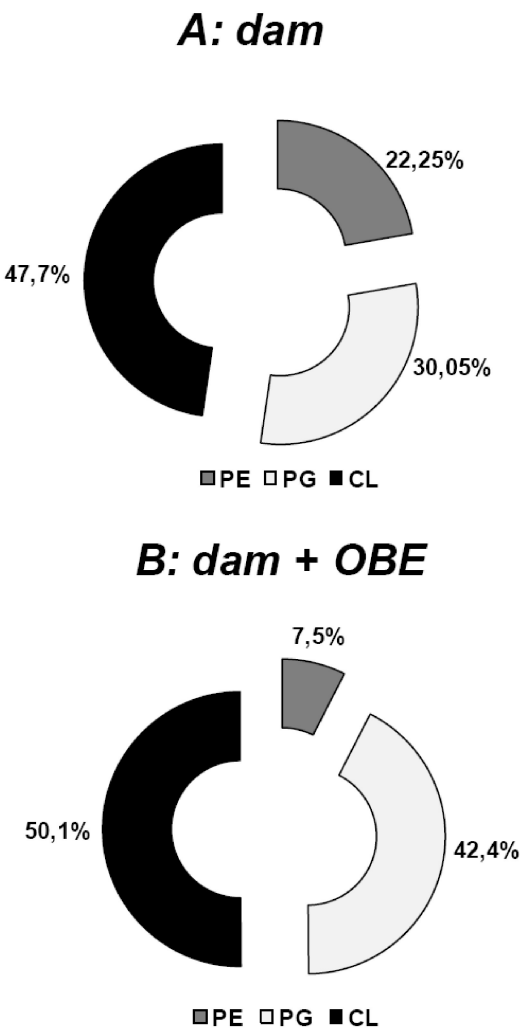

Figure 3 : Analysis of PL levels in *S.*Typhimurium *dam* none exposed (A) and exposed (B) to OB

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to OBE (B) are shown in Figure 3.

Effect of OBE combined to the *dam* mutation on membrane FA composition of *S.*Typhimurium

The membrane FA composition (molar percent) of the WT strain was determined. Many FA were found and seven main peaks were identified by comparing their retention times with those of known standards. Three SFA were myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), and stearic acid ($C_{18:0}$), two UFA were palmitoleic acid ($C_{16:1w7}$) and oleic (or vaccenic) acid ($C_{18:1w9}$), and two CFA were the cis-9,10-methylenehexadecanoic acid (cyc17) and the cis-9,10-methyleneoctadecanoic (lactobacillic) acid (cyc19). Their relative percentages were between 2% and 46% corresponding to more than 96% of all FA observed. Some other minor FA were also detected at lower relative concentrations: $C_{17:0}$, $C_{18:2w6}$, $C_{18:3w6}$, $C_{18:3w3}$, $C_{19:0}$, and $C_{20:0}$.

Membrane FA composition of S.Typhimurium WT strains exposed to the OBE

In the WT strain palmitic acid, palmitoleic acid, and stearic acid were the main constituents, representing about 60% of total FA. The proportion of TL CFA obtained was about 25.79%. However, minimum CFA levels were observed for PE and CL (6.43% and 5.08%, respectively) and higher one for PG (38.35%). The UFA/SFA ratio was mostly similar between the PL fractions. For the WT strain cultured with the OBE, no significant changes were observed in both TL and PL. The FA composition appeared to be unaffected by the OBE stress with an UFA/SFA ratio mostly similar to that of WT control strain (TABLE 2).

Exponentially growing *S.*Typhimurium strains were incubated at 37°C, centrifuged when the OD at 600 nm reached 0.5. Extraction of the total PL and purification of each PL were performed as described in the experimental procedures. The contents of FA in TL, PE, PG, and CL were determined as described in the experimental procedures. The composition of each FA was shown as the relative value to the total content of FA. Average values of triplicates were given, and the deviation was less than 5 % of each value (the mean value ± the standard deviation of replicate value). Significance was assessed as described in experimental procedures. (MFA: Minor Fatty Acids; SFA: Saturated Fatty Acids; UFA: Unsaturated Fatty Acids; CFA: Cyclic Fatty Acids; UFA/SFA: Unsaturated to Saturated Ratio).

TABLE 2 : Membrane FA composition (Molar percent) in TL and different PL classes in the *S.*Typhimurium WT strain exposed to OB

Fatty acids	*Total lipids*	*Phosphatidylethanolamine*	*Phosphatidylglycerol*	*Cardiolipin*
$C_{14:0}$	6.78 ± 0.29	6.95 ± 0.5	4.54 ± 0.45	13.74 ± 0.13
$C_{16:0}$	47.48 ± 0.32	51.22 ± 0.09	37.68 ± 0.33	61.2 ± 0.54
$C_{18:0}$	9.43 ± 0.09	2.51 ± 0.34	10.02 ± 0.35	6.70 ± 0.33
ΣSFA	*63.69*	*60.68*	*52.24*	*81.64*
$C_{16:1\ w7}$	6.56 ± 0.32	9.74 ± 0.91	4.43 ± 0.72	9.04 ± 0.86
$C_{18:1\ w9}$	1.57 ± 0.11	1.45 ± 0.21	2.58 ± 0.61	1.24 ± 0.74
ΣUFA	*8.13*	*11.19*	*7.01*	*10.28*
UFA/SFA	*0.120*	*0.184*	*0.134*	*0.125*
cyc_{17}	8.41 ± 0.06	7.96 ± 0.84	11.70 ± 0.21	4 ± 0.01
cyc_{19}	19.77 ± 0.18	20.17 ± 0.03	29.05 ± 0.02	4.08 ± 0.12
ΣCFA	*28.18*	*28.13*	*40.75*	*8.08*
MFA	*6.04*	*5.35*	*5.96*	*4.65*

Membrane FA composition of S.Typhimurium dam⁻ mutant strains exposed to the OBE

To determine whether the mutation in the *dam* gene added to the OBE stress affected membrane lipid components, FA composition was quantified. It was shown that the *dam* mutation affected highly the FA composition of the TL. The FA were characterized by high level of CFA (about 40% of total content), and low level of UFA (about 2%). These changes were due to a concomitant decrease in $C_{16:1w7}$ and $C_{18:1w9}$ and an increase in their cyclopropane derivatives cyc17 and cyc19 CFA and resulted in a low UFA/SFA ratio. The accumulation of the CL fraction was accompanied with a relative stability in the FA composition, which appeared to be unaffected by the *dam* mutation. In the PG fraction, data confirmed that SFA are the most prominent spe-

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cies accounting for about 61%. Finally, we noticed that the PE was characterized by a reduction in UFA to the profile of their CFA derivatives. These PL changes resulted in a low UFA/SFA ratio.

The membrane FA composition of the TL was highly affected by the OBE stress (TABLE 3). Our data showed that the OBE added to the *dam* mutation decreased the CFA levels from 40.16% to 11.28% (About four times lower than control *dam*$^-$ cells), but increased those of UFA from 2.46% to 19.62%. These changes resulted in very high level of acyl chain unsaturation of FA (UFA/SFA). For the other FA, minor changes were observed (the SFA and MFA levels increased to 65.06% and 4.04% respectively). In CL and PE, FA were characterized by low levels of CFA and high levels of UFA. Other FA species such as myristic ($C_{14:0}$) and palmitic ($C_{16:0}$) were more abundant. The PG FA composition appeared to be unaffected by the OBE with an UFA/SFA ratio, in the majority, with respect to that of *dam*$^-$ non exposed strain (TABLE 3).

Incorporation of cfa plasmid or exogenous CFA renders dam$^-$ more bile resistant

We tested whether the increased sensitivity to the OBE in *dam*$^-$ mutant strain was directly due to the loss of CFA, so we introduced the *cfa* plasmid pAYW19 into this strain. The transformed *dam*$^-$ strain became OBE resistant as its WT parental strains, indicating that the OBE sensitivity was specific to the loss of the *cfa* gene product after their conversion to UFA. To test this possibility, we bypassed the loss of CFA by providing chemically synthesized CFA (cyc-17 and cyc-19) in the growth medium. We grew the *dam*$^-$ strain in LB medium supplemented with CFA and OBE for many generations of growth to ensure that the added FA was the sole CFA component of the membrane lipids. *dam*$^-$ strain had shown a decreased sensitivity when grown with CFA supplement than when the strain was grown without the corresponding CFA. These data therefore directly demonstrate that cells with membranes containing CFA resist OBE stress better than the corresponding mutant cells.

TABLE 3 : Membrane FA composition (Molar percent) in TL and different PL classes in the *S.*Typhimurium *dam*$^-$ mutant strain exposed to OB

Fatty acids	*Total lipids*	*Phosphatidylethanolamine*	*Cardiolipin*	*Phosphatidylglycerol*
$C_{14:0}$	6.43 ± 0.29	8.95 ± 0.5	12.01 ± 0.13	3.24 ± 0.48
$C_{16:0}$	44.08 ± 0.28	54.22 ± 0.09	65.2 ± 0.54	42.18 ± 0.35
$C_{18:0}$	14.55 ± 0.11	4.51 ± 0.34	7.1 ± 0.33	21.02 ± 0.23
ΣSFA	*65.06*	*67.68*	*84.31*	*66.44*
$C_{16:1\ w7}$	13.56 ± 0.32	9.74 ± 0.31	6.04 ± 0.86	2.43 ± 0.72
$C_{18:1\ w9}$	9.6 ± 0.11	4.45 ± 0.16	3.73 ± 0.74	2.08 ± 0.61
ΣUFA	*19.62*	*14.19*	*9.77*	*4.51*
UFA/SFA	*0.301*	*0.209*	*0.115*	*0.067*
cyc_{17}	2.51 ± 0.06	2.96 ± 0.84	0.79 ± 0.01	8.70 ± 0.21
cyc_{19}	8.77 ± 0.18	9.82 ± 0.03	1.08 ± 0.12	17.19 ± 0.02
ΣCFA	*11.28*	*12.78*	*1.87*	*25.89*
MFA	*4.04*	*5.35*	*4.05*	*3.16*

DISCUSSION

Previous works had shown that the *dam* mutation renders *Salmonella enterica* sensitive to agents known to be antimicrobially active in the host like OBE. In the present study we tried to investigate a possible connection between both the lack of *dam* gene (coding for the Dam protein) and some membrane components, and the sensitivity to the OBE observed in *S.*Typhimurium. The PL and FA were the object of attention because the membrane delimiting the cell is supposed to be constituted largely of lipids. These lipids presumably confer to the membrane its characteristic impermeability and resistance to stressing agents.

To better observe the influence of OBE on the dynamics of growth, the follow-up of the biomass was used to quantify the biological effects of this stress. The inhibitory effect of acidic stress on the growth of bacteria has been also highlighted by several studies[22]. However, in this study we found that the OBE did not affect the growth of *S.*Typhimurium *dam*$^+$, spread on liquid medium and agar plate. In addition, our results showed

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that *S.*Typhimurium WT and *dam*⁻ mutant reacted differently to bile stress in terms of growth. The WT strain cultured with OBE recovered more rapidly its growth than *dam*⁻ strain since an increase was observed for the first one from 4 to 10 hours with comparable values between stressed and control cells. For the *dam*⁻ mutant, no growth recovery was observed until 10 hours.

These results provide an additional element confirming the existence of a variation of the OBE effects on cell growth depending on the microorganism. Furthermore, the analysis of the bacterial behavior allowed us to suppose that the OBE exerted a possible bactericidal effect on a certain number of cells. However, we do not exclude that the increased sensitivity of *S.*Typhimurium *dam*⁻ to OBE is probably due to changes in the permeability of ionic channels in the membrane or membrane lipid composition, suggesting that the OBE can lead to metabolic and morphological changes, which can weaken the resistance of *S.*Typhimurium *dam*⁻ to external aggression. So we propose that under the influence of the OBE, *S.*Typhimurium *dam*⁻ cells would require the implementation of mechanisms of defense in order to overcome the effects of this substance.

It has been well established that Dam methyltransferase is important in global gene regulation, which can up- and down-regulate molecules involved in energy, nucleotide metabolism and cellular processes, as well as SOS and stress response genes, like *barA, katG, katF, oxyR, dnaK, dnaJ, uvrA, uvrB, ompR, copR*[23]. Regulation is done most commonly by a competition of regulatory proteins in the expression of target genes with the Dam methyltransferase in GATC sites, as is the case of several virulence genes in *Salmonella*[17,24,23]. In addition, it is well known that *dam*⁻ mutants are generally more sensitive to several components like bile salts and hydrogen peroxide than the WT strain[22]. So, we suggest that these data could in part explain the differential behavior of OBE stressed WT and *dam*⁻ mutant strains. Here, the question to be addressed concerns the adaptive mechanisms of membrane lipid composition to overcome acidic stress in *S.*Typhimurium and the implication of the Dam enzyme in their regulation.

It is generally admitted that cells regulate their lipid composition to achieve a degree of fluidity compatible with life[25]. In addition, previous results showed that synthesis of different FA, which are the major components of the PL[26], plays a key role in membrane integrity against both many external and internal biological challenges. These FA has significant implications for the survival of pathogenic bacteria. This hypothesis agreed very closely with the results of[27], who showed that during acid habituation, *E.coli* can changes the membrane lipid composition. Moreover, they showed that the acid tolerance of individual strains of *E.coli* appeared to be correlated with membrane FA, and thus, they postulated that modified levels of FA may enhance the survival of microbial cells exposed to low pH.

*S.*Typhimurium cells have developed efficient protection systems to cope with a variety of physicochemical unfavorable conditions and to adapt to the environmental stresses. In particular, fundamental for the microbial cells is to maintain membrane integrity and functionality in response to environmental stresses encountered during infection. In response to stresses, the PL can alter their acyl chain structure by changing the ratio of saturation to unsaturation, cis to trans unsaturation, branched to unbranched structure and type of branching and acyl chain length[28]. Different modulation mechanisms can be used in relation to the physiological state of the cells[29].

In this report, we have shown that zwitterionic (PE) and acidic PL (PG and CL) proportions as well as their FA and their acyl chain unsaturation levels were strongly affected in the *dam*⁻ mutant exposed to the OBE. We demonstrated recently that in *S.*Typhimurium, the deficiency of Dam protein causes marked envelope defects and abnormal PL and FA composition. We showed an increased sensitivity to the OBE. Hence, a tentative explanation for this OBE sensitivity in *dam*⁻ mutants may be the alteration of their PL and FA components. Besides the increase in CL and PG synthesis, the *dam*⁻ mutant exposed to the OBE showed other differences in the PE content compared with the none exposed and the WT strains, suggesting a cross-regulation of Dam protein in membrane lipid pathways, crucial for the maintenance of membrane functionality and integrity. We showed here that in the absence of the Dam protein, the bacterial membrane decreased the zwitterionic PE synthesis, which was accompanied with compensatory increase in the acidic PL (CL and PG) content (77.75%, distributed in 30.05% of PG and 47.7% of CL). Thus, we might assume that the expression of genes coding for components of the membranes (like PE, PG, and CL synthase) or for proteins involved in the synthesis of

such components might be impaired in the *dam*- mutant. In addition, dramatic changes in FA composition occurred with the OBE stress. A membrane response to this agent resulted in a relative stability in SFA levels, an increase in the MFA and UFA levels, and a decrease in CFA levels. All these changes might be the origin of OBE sensitivity as far as the membrane fluidity is concerned.

During passage through the small intestine, *Salmonella* faces periodic release of bile, and the extreme sensitivity of *dam*- mutants to bile salts may be due to the reduction of CFA contents and the concomitant increase in the UFA levels probably due to the fact that a significant proportion of the CFA (cyc17, cyc19) was converted to UFA (C16:1w7, C18:1w9, respectively) during responses to the OBE stress. This last observation resulted in a progressive increase in the UFA/SFA ratio and membrane fluidity. It has been previously reported that membranes with high UFA/SFA ratio show high fluidity[30] and a low UFA/SFA ratio, has been linked to less membrane fluidity[30,31] and it is generally admitted that cells regulate their lipid composition to achieve a degree of fluidity compatible with life[25]. In addition, Makise et al.,[32] have reported that UFA increase membrane fluidity which could render the bacterial cell more sensitive to the OBE.

Our results show that CFA, which are the major components of the PL[26] and a postsynthetic modification of the lipid bilayer that occurs as cultures of many other bacteria enter stationary phase[33], play a key role in membrane integrity and for the survival of pathogenic bacteria and the loss of these CFA resulted in a great sensitivity to OBE and perhaps to many external and internal biological challenges. In addition, there are other membrane FA components that are altered in the *dam*- strain treated with bile. That is why, and in additional experiments, we showed that only the addition of CFA to the culture medium with OBE or the restoration of their production by the introduction of a plasmid with the *cfa* gene restored bile resistant of the *dam*- mutant. These data therefore directly demonstrate that cells with membranes containing CFA resist OBE stress better than the corresponding mutant cells. So we can admit that the only cause for bile sensitivity in the *dam*- mutant is the lack of CFA. Brown et al.,[27], showed that the acid tolerance of individual strains of *E.coli* appeared to be correlated with high membrane CFA, and thus, they postulated that increased levels of CFA may enhance the survival of microbial cells exposed to low pH. Other results that confirmed the key role of CFA in membrane integrity was also postulated by Chang and Cronan[33]. They showed that cultures of strains lacking CFA (as a result of a null mutation in the *cfa* gene) are abnormally sensitive to killing by a rapid shift from neutral pH to pH 3. This sensitivity is dependent on CFA itself because resistance to acid shock is restored to *cfa* mutant strains by incorporation of CFA to the growth medium or by introduction of a functional *cfa* gene on a plasmid[33]. So the new phenotype of the *dam*- sensitive strain was the direct consequence of abnormalities in membrane PL and FA composition. It has been proposed that intracellular pathogens like *Salmonella* are exposed to several stressing agents such as bile during the infection process. Stress conditions which pathogenic bacteria encounter during infection course can affect membrane components. The environmental control of regulatory mechanisms is mediated by complex processes. *Salmonella* comes in contact with bile salts in the intestine and is able to resist the action of bile and respond to escalating bile concentrations by increasing mechanisms of resistance.

Previous studies showed that bacteria with enhanced tolerance to acid, bile and blood serum survive[34,35] and cause disease[36,3] better than sensitive bacteria and showed that *in vitro* acid adapted *Salmonella* were more resistant towards bile[37] and acids[3] in comparison to non-adapted cells. Results obtained in this study show that the OBE stress added to the *dam* mutation is an important factor affecting *S.*Typhimurium resistance and could contribute to find new strategies based on intelligent combinations of hurdles, which could prevent the development or survival of *Salmonella spp.* in gastrointestinal tract. In *E.coli*, *dam*- mutants show altered membrane permeability and abnormal PL composition[38], which may explain their increased sensitivity to a number of dyes. The observation that the envelope of *S.enterica dam*- mutants is likewise unstable can be tentatively correlated with bile sensitivity, because unconjugated bile salts can enter the cell by diffusion[39]. Thus, a structural role of Dam protein in envelope stability cannot be discounted. An alternative explanation is that Dam might regulate the expression of genes involved in the stability and the integrity of the cell membrane against bile salts during infection process, a possibility also considered in *E.coli*[40].

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As future perspectives, additional experiments would be necessary to investigate the genes involved in the bacterial growth and in the synthesis of components of the membranes that are impaired in the *dam*$^-$ mutant under bile salts stress. Indeed, further experiments have also to be done to determine if there is an expression of genes coding for chaperone proteins like *dnaK* (chaperone Hsp 70) or catalase like *katN* under this stressing status and to answer the question of how these genes are regulated in response to bile salts stress in WT and *dam*$^-$ mutant strains.

ABBREVIATIONS

PL: phospholipids; FA: fatty acid; *S.*Typhimurium: *Salmonella enterica* serovar Typhimurium; WT: wild type; OBE: ox bile extract; PE: phosphatidylethanolamine; CL: cardiolipin; PG: phosphatidylglycerol; TL: total lipid; UFA: unsaturated fatty acid; SFA: saturated fatty acid; MFA: minor fatty acid; CFA: cyclic fatty acid; Dam: DNA adenine methyltransferase; SeqA: sequestration protein; LB: Luria-bertani broth; OD: optical density; CFU: colony forming units; *dam*$^+$: strain with *dam* gene; *dam*$^-$: strain without *dam* gene

CONCLUSION

In summary, *S.*Typhimurium Dam protein is required for maintenance of membrane integrity against the OBE. Mutation in the *dam* gene causes growth and envelope defects and enhances sensitivity to the OBE which together may contribute to the attenuation of virulence, may induce strong immune responses in infected animals and effectively may be applied to the design of a live vaccines. In conclusion, our results demonstrate that *dam*$^-$ mutant strains have an altered membrane composition of PL and FA and this alteration could be the main factor in the defects of growth rate and sensitivity for the OBE that we have shown for *dam*$^-$ mutant. Finally, the way in which Dam participates in the membrane integrity is a critical question that deserves further investigation in the near future, and may be research studies will have to identify explanations.

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DISCLOSURE STATEMENT

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