Coordinated Regulation of Cold-Induced Changes in Fatty Acids with Cardiolipin and Phosphatidylglycerol Composition among Phospholipid Species for the Food Pathogen *Listeria monocytogenes*[†]

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We present here the structural identification of four phospholipid (Phl) classes in *Listeria monocytogenes*, the fatty acid (FA) composition for each individual Phl species, and a description of cold-induced FA changes. Cardiolipin (48.5%) and phosphatidylglycerol (18.1%) are dominated by anteiso-FA, and the previously recognized branched FA chain shortening by cold was observed singularly in these Phls. Phosphoaminolipid (19.9%) and phosphatidylinositol, (9.1%) are significantly different, containing significant amounts of straight-chain FA. These findings are supported by nuclear magnetic resonance analysis.

The bacterium *Listeria monocytogenes*, one of the leading causes of food-borne illness (15), can adapt to cold and salinity (9, 10, 31, 46, 53) and can survive even on dust or flakes of organic material (34). *Listeria* is characterized by >85% branched-chain fatty acids (FA), anteiso-15:0 (a-15:0) and anteiso-17:0 (a-17:0). Cells grown in the cold (5°C) contain significantly less a-17:0 than those grown at higher temperatures (4, 8, 37, 43, 44, 57). A cryotolerance element of *Listeria* is also its ability to use cryoprotectant osmolytes (31), a property shared by other organisms (2, 3, 12, 21, 23). The critical role of odd numbered anteiso-FA in influencing the lower-temperature growth limits has recently been reported (14, 19, 25). We have reported previously (39) that *Listeria* spp. can be cold adapted by an increased content (30%) of neutral lipids (NL) among total lipids (TL) and an increase in the a-15:0/a-17:0 FA ratio (FAr) of TL, polar lipids (PL), and NL (10-, 10-, and 7-fold, respectively), as well as by an increase in FAr (38) for each NL subclasses 1,2-diglyceride, 1,3-diglyceride and free FA (6-, 5-, and 8-fold, respectively).

This study is a continuation (35, 36) of an investigation for the structural characterization of the major phospholipid (Phl) classes of *L. monocytogenes*, in order to (i) elucidate the isolation and quantification of each of class (cells at 30°C), (ii) evaluate the cold temperature (5°C) effect on each individual Phl class FA composition, and (iii) confirm the findings by ¹H-nuclear magnetic resonance (NMR) analysis.

An avirulent strain of *L. monocytogenes*, DP-L1044 (D. Portnoy, University of Pennsylvania), prepared by a transposon insertion (6) in the parent strain (Lm10403S), was grown in brain heart infusion (Difco Laboratories) broth at

30°C (ca. 24 h). A 10-ml portion of this was then inoculated in 1 liter of brain heart infusion broth, followed by incubation until stationary phase (30 or 5°C). Cells pelleted by centrifugation were washed twice in phosphate buffer (pH 7.0). TL were extracted (17) and separated to PL and NL by solid-phase extraction (SPE) as previously described (38). Solvent A (chloroform-methanol-acetic acid-water [50:25:6:2, vol/vol/vol/vol]) was used for one-dimensional thin-layer chromatography (1D-TLC; silica gel G60 aluminum chromatoplate [Merck 1.05554], 0.25-mm thick, 10 by 10 cm). Spots were visualized by using iodine, ninhydrin, phosphomolybdenum blue (13), and copper sulfate (5, 42). Glyco- or sulfolipids were tested by using naphtholsulfuric acid (24). The standards phosphatidic acid (PA), phosphatidylserine (PS), cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), Lyso-PC, and Lyso-PE were purchased from Sigma Chemical Co. Each culture 30°C or 5°C PL sample (85 and 50 µg of phosphorus correspondingly) was fractionated by 1D-TLC (preparative), along with the standards (CL, PG, and PI) and along with another *Listeria* PL sample (30°C) aliquot (3.5 µg of phosphorus) as an alternative standard. Four individual Phl bands were sepa-

TABLE 1. Separation and composition (% of lipid P) of *L. monocytogenes* PL^a from cell cultures at 30°C

Lipid band	Composition (mean % of lipid P ± SD) after separation by HP-TLC ^b
Cardiolipin	48.7 ± 1.0
Phosphatidylglycerol	18.1 ± 0.4
Phosphoaminolipid	19.9 ± 1.6
Phosphatidylinositol	9.1 ± 1.8
Recovery	95.8

^a The lipid sample (10 µg of phosphorus) was separated by high-performance thin-layer chromatography (HP-TLC).

^b The values are means ± the standard deviations obtained from phosphorus determinations performed on each HP-TLC plate (*n* = 3).

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TABLE 2. Comparison of ¹H chemical shifts for different lipid glycerol backbones^a

Compound	Lipid glycerol backbone (ppm)					
	CL		PG, 30°C	PI, 30°C	PhAL	
	30°C	5°C			30°C	5°C
C ₁ H ₂ OC	4.109 (4.08–4.17)	4.064	4.114 (4.02–4.07)	–(4.355)	4.020–4.180	4.010–4.180
	4.355 (4.323–4.340)	4.358	4.325–4.321	4.341 (4.396)	4.338	4.336
C ₂ HOC	5.148 (5.1–5.2)	5.150	5.145 ( )	5.140 (5.147)	5.147	
C ₃ H ₂ OP	3.915 (3.893–3.930)	3.915	3.909 (3.910)	3.90–4.00 (3.978)		
				4.01–4.10 (4.104)		

^a Values in parentheses indicate the ppm values from each lipid reference standard ¹H-NMR spectra.

rated, scraped off, and eluted from silica gel as previously described (35, 39). Each extracted band of residual lipids was used for analytical methods and ¹H-NMR spectroscopy.

Total phosphorus (33), esters (47), nitrogen (20), and glycerol (30) analysis was performed as described previously. Deacylation (27, 54) was also performed in 0.1 ml of NaOH (1.2 N) in 50% (vol/vol) methanol at 45°C for 20 min. The PL in bulk were quantitated by weight after SPE column elution. Each individual Phl band (cells 30°C) was quantified after 1D-TLC fractionation of the PL sample (~10 µg of phosphorus) with solvent A (*n* = 10). Each iodine-visualized band was scraped into a digestion tube, and the total phosphorus content was determined (27). A 25-µg phosphorus sample of the TLC-extracted CL band (cells of 30°C) was dry acid methanolysed (51) by 400 µl of 3 N methanolic HCl, and the molecular ratios (structural data) were determined in hydrolysis aliquots as described previously (28).

For each culture CL, PG, phosphoaminolipids (PhAL), and PI FA were methylated by scraping the 1D-TLC bands into digestion tubes, and a 1-mg PL sample was also methylated as previously described (28, 35). FA methyl esters were separated by using a Hewlett-Packard 6890 gas chromatograph with split/splitless injector, flame ionization detector (30 m by 0.251 mm, capillary column DB-5ms; J&W Scientific). The carrier gas was He. The injector and detector were maintained at 250 and 280°C, respectively. The oven temperature was ramped from 90 to 170°C (70°C/min) and from 170 to 210°C (5°C/min). The

initial and final holding times were 1 and 2 min, respectively. Two standards were used: bacterial FA methyl esters (CP Matreya, Inc.) and marine oil fame (Restek Corp.). Each lipid sample was analyzed by gas chromatography-mass spectrometry (electron impact or chemical ionization with acetonitrile) using a Varian Saturn 2000 with a 30-m-by-0.25-mm DB5-ms column. To each sample of CL (750 µg), PG (270 µg), PhAL (270 µg), and PI (150 µg) 0.5 ml of CDCl₃/CD₃OD (1:2 [vol/vol]) was added, and ¹H-NMR spectra were acquired by using a Varian Unity Plus spectrometer (300 MHz). As standards, CL (1,000 µg), PG (540 µg), and PI (920 µg) were also analyzed.

L. monocytogenes TL extraction (at 30 or 5°C) yielded 5.2 ± 1.2 mg g⁻¹ and 6.5 ± 1.4 mg g⁻¹ wet cells. The TL lipid phosphorus and nitrogen contents were 3.4% ± 0.2% (30°C) and 2.5% ± 0.1% (5°C) and 0.96% ± 0.02% and 0.86% ± 0.02%, respectively. The PL for each culture represented 62.5% ± 2.3% and 53.3% ± 1.1%, respectively, of the TL. PL were separated by 1D-TLC into four phosphocomponents (see Fig. S1 in the supplemental material) designated g, f, e, and b, as we have previously described (36) for *Listeria* (30°C). Two bands, f and b, were cochromatographed with PG and PI standards, respectively. The component f includes (after 2D-TLC separation) two phosphocomponents, f₂ and f₃, but not the glycomponent f₁ (35). The component g was cochromatographed close to the CL standard. The ninhydrin-positive phosphocomponent e (migrated just below the PG position) is the single PhAL of *Listeria* (35, 36), as others (16) have also

TABLE 3. Comparison of ¹H chemical shifts for different lipid head groups^a

Compound	Lipid head group			
	Central glycerol of PG, 30°C	Glycerol of CL, 30°C	Inositol of PI, 30°C	Amino moiety of PhAL
				30°C 5°C
POCH ₂ CH(OH)CH ₂ OP	3.790–3.915 (3.839–3.930)			
POC ₄ H ₂		3.828		
C ₅ HOH		3.645		
		3.703		
C ₆ H ₂ OH		3.529		
		3.564		
CH ₂ NH ₂				3.001 3.001
Ring H				
POC ₄ H			3.900 (3.804)	
C ₅ HOH			4.341 (4.353)	
C ₆ HOH			3.466 (3.327)	
C ₇ HOH			3.561 (3.53–3.57)	
C ₈ HOH			3.2 (3.142)	
C ₉ HOH			3.77–3.87 (3.7)	

^a Values in parentheses indicate the ppm values from each lipid reference standard ¹H-NMR spectra.

FIG. 1. ^1H -NMR spectra of *L. monocytogenes* cardiolipin class, extracted from preparative TLC, from cultures at 30°C (down) and 5°C (up). For samples, the solvent $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1:2 [vol/vol]) was used.

reported, and a lysylcardiolipin structure is suggested. This molecule can serve as a genus-specific chemotaxonomic marker for the genus *Listeria*. Table 1 shows the lipid phosphorus distribution of each lipid component (cells at 30°C). Of note is that the ratio of relative amounts at phosphorus per-

centages for CL/PhAL (~2.4) are similar to that (~2.8) reported by Fischer and Leopold (16). The existence of CL and PG has been reported by others (16, 32). Until now there has been no precedent in the literature for the existence of PI in *Listeria* PL.

FIG. 2. ^1H -NMR spectra of *L. monocytogenes* phosphatidylglycerol class, extracted from preparative TLC, from a culture at 30°C . For samples, the solvent $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1:2 [vol/vol]) was used.

Based on their TLC behavior before and after deacylation, the four phosphocomponents of each culture PL sample were completely hydrolyzed, and the bulk of the lipid phosphorus was distributed in the water-soluble products. This suggests a

basic glycerolipid structure for each phosphocomponent. The hydrolysis products after dry acid methanolysis of the phosphocomponent g, contained total phosphorus, (methyl) esters, and glycerol at a molar ratio of 2.00:4.00:3.12, respectively, and

TABLE 4. Cold temperature dependence of FA composition (wt %) of CL, PG, PhAL, and PI from two sets of *L. monocytogenes* cultures at 30 and 5°C ^a

FA or parameter ^b	FA composition (mean wt % $\pm$ SD)						
	CL		PG		PhAL		PI, 30°C
	30°C	5°C	30°C	5°C	30°C	5°C	
14:0	0.8 ± 0.7	0.8 ± 0.8	0.4 ± 0.4	1.3 ± 0.1	4.4 ± 1.3	0.9 ± 0.9	1.9 ± 0.5
iso-15:0	8.0 ± 2.9	7.3 ± 0.7	5.7 ± 0.6	6.3 ± 0.2	2.5 ± 0.5	4.5 ± 0.5	1.7 ± 1.7
Anteiso-15:0	29.7 ± 7.4	78.7 ± 3.3	28.6 ± 6.0	76.7 ± 0.9	11.0 ± 2.2	40.9 ± 7.0	12.8 ± 3.7
15:0	2.5 ± 2.5	0.2 ± 0.2	0.4 ± 0.4	0.8 ± 0.6	0.3 ± 0.3	0.7 ± 0.7	3.7 ± 0.6
Iso-16:0	9.2 ± 3.1	1.9 ± 0.6	7.1 ± 1.7	1.2 ± 0.3	2.0 ± 1.1	1.6 ± 1.6	4.0 ± 1.0
16:0	13.8 ± 3.6	2.4 ± 0.7	14.7 ± 3.5	3.6 ± 1.0	20.7 ± 5.0	28.2 ± 4.9	14.9 ± 1.0
Iso-17:0	4.4 ± 1.5	0.3 ± 0.3	2.5 ± 2.5	0.5 ± 0.4	2.2 ± 0.5	0.9 ± 0.9	5.0 ± 1.7
Anteiso-17:0	26.9 ± 5.3	7.1 ± 0.7	24.9 ± 5.6	4.0 ± 0.4	6.5 ± 0.3	8.7 ± 0.5	8.0 ± 2.5
17:0	1.5 ± 1.5	0.2 ± 0.2	0.3 ± 0.3	0.4 ± 0.3	-	1.5 ± 1.5	4.9 ± 2.0
18:0	0.4 ± 0.4	0.3 ± 0.3	9.1 ± 0.4	1.3 ± 0.2	15.2 ± 3.8	6.0 ± 0.3	14.0 ± 0.4
$S_{\text{unsaturated}}^b$	1.5 ± 1.5	1.0 ± 1.0	6.4 ± 3.2	4.0 ± 1.5	35.4 ± 13.9	6.1 ± 3.6	29.1 ± 6.7
Anteiso-15:0/anteiso-17:0 ratio	1.2	11.1	1.2	19.2	1.7	4.7	1.6
Branched-15:0/branched-17:0 ratio	1.2	11.6	1.3	18.4	1.6	4.7	1.1
$S_{\text{branched}}/S_{\text{normal}}$ ratio	3.8	19.5	2.2	8.0	0.3	1.3	0.5

^a Mass spectrometry was used to confirm FA structures. Values are means $\pm$ SD except as noted in column 1.

^b $S_{\text{branched}}/S_{\text{normal}}$ is the ratio of the sum of branched-chain to the sum of normal-chain FA. The $S_{\text{unsaturated}}$ value includes 16:1n-9 cis, 18:1n-9, cis, 18:1n-11 cis, and 18:2n-9,12 cis.

TABLE 5. Temperature dependence of the $-\text{CH}_2/-\text{CH}_3$ ratio of CL, PG, and PhAL FA chains from cells of *L. monocytogenes* grown at 30 and 5°C^a

Parameter	CL		PG		PhAL	
	30°C	5°C	30°C	5°C	30°C	5°C
Integral of $\omega\text{-CH}_3$, br- CH_3	19.30 (0.72–0.82)	20.99 (0.72–0.82)	21.40 (0.72–0.82)	23.68 (0.72–0.82)	20.77 (0.72–0.82)	21.96 (0.72–0.82)
Integral of $-\text{CH}_2-$	80.70 (1.12–1.32)	79.01 (1.12–1.32)	78.60 (1.12–1.32)	76.32 (1.12–1.32)	79.23 (1.12–1.32)	78.04 (1.12–1.32)
Ratio variation ($-\text{CH}_2/-\text{CH}_3$)	4.18	3.76	3.67	3.22	3.81	3.55

^a The phospholipids were dissolved in C/M 1/2 (vol/vol). The percent reductions of ratio variation ($-\text{CH}_2/-\text{CH}_3$) were 10.05, 12.26, and 6.82 for CL, PG, and phosphoaminolipid, respectively. The ppm values of the peaks from each ¹H-NMR spectra are indicated in parentheses.

this suggests a diphosphatidylglycerol (cardiolipin) molecular structure.

The four isolated glycerolipid components were also identified by ¹H-NMR analysis (Tables 2 and 3). For each lipid class, the spectral assignments were compared to standard spectra of CL, PI, and PG (1, 7, 48). Diagnostic resonances may be considered to be the CL resonances of the central glycerol head group, sandwiched between two phosphate groups (OPCH₂CHOHCH₂OP) (Table 3 and Fig. 1) and, in principle, the PG resonances of its glycerol head group C₄H₂C₅HC₆H₂ (Table 3 and Fig. 2). The PI head group is complicated because it represents a network of six interacting spins corresponding to the six inositol CH protons (see Fig. S3 in the supplemental material). The three features distinguishing PI are the C-4(POCH), C-6(CHOH), and C-7(CHOH) proton resonances (Table 3). The C-8(CHOH) signal at 3.200 ppm is overlapped by the methanol resonance. C-5 has the same peak (4.341ppm) as glycerol backbone C-1 (1, 7). The PhAL head

group is suggested to be the same as the CL headgroup, with an esterified lysine moiety at the central free OH group. The methylene protons neighboring the ε-amino group of lysine (CH₂NH₂) occur at 3.001 ppm. The central glycerol sandwiched between two phosphate groups could also be used as a diagnostic for this lipid (see Fig. S2 in the supplemental material) as for the CL structure described above.

This study represents the individual isolated Phl species (CL, PG, PhAL, and PI) FA composition of *Listeria* (30°C). FA profiles of CL and PG are very similar and dominated by anteiso-FA. The PhAL and PI FA compositions are significantly different, containing significant amounts of straight-chain FA (16:0 and 18:0) and unsaturated FA (Table 4).

Upon cold stress the a-15:0 FA proportion in their CL and PG of *Listeria* increased dramatically (Table 4). The main cold change for PhAL FA was, apart from the increase of anteiso-15:0, the decrease of the sum of unsaturated FA. Cold differences between the FA composition of PI species (unpublished

FIG. 3. ¹H-NMR spectra (acyl chains only) of *L. monocytogenes* cardiolipin (A) and phosphatidylglycerol (B) extracted from preparative TLC, from cultures at 30°C (down) and 5°C (up). For samples, the solvent CDCl₃/CD₃OD (1:2 [vol/vol]) was used.

data) were mainly independent of the growth temperature (the differences varied between ± 4.0 percentage points, with the exception of a 15:0 FA, which showed a difference of +6.2). Conclusively, the cold metabolic change in CL and PG is different from the rest of the PL subclasses (PI and PhAL). The cold decrease of the $-\text{CH}_2/-\text{CH}_3$ ratio of the acyl chains by NMR analysis (Table 5 and Fig. 3) reflects the cold increase of the branched-15:0/branched-17:0 FA ratio (as determined by gas chromatography analysis) for CL and PG, as well as for PhAL (Table 4). The determination of branched FA by ^1H -NMR analysis can be a useful interpretation tool of FA microorganism metabolism at cold temperature.

The potential impacts of studying *L. monocytogenes* membrane Phl composition are that (i) molecules can serve as a chemotaxonomic marker, (ii) resistance to antimicrobials and nisin (11, 40, 41, 52, 56) or other antimicrobial peptides (49, 50) is correlated with both altered FA composition (26) and Phl compositional changes, (iii) antibacterials encapsulated in liposomes produced with PhL of similar structure as that included in *Listeria* membrane are more effective (improved permeation) (55), (iv) an understanding of the cold stress response is helpful in the design of effective food preservation strategies (such as membrane-disrupting techniques like pulsed electric fields, ultrahigh pressure, etc.) applicable to hurdle technology, etc. (45), and (v) FA biosynthesis is coordinately regulated with Phl synthesis and growth as part of the bacteria response to the changing environment and offers unique sites for selective inhibition (22) to combat microbial infection (chemotherapeutic agents). Phl FA fingerprints help us to monitor the impact on bacterial growth conditions (18).

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