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# Isolation and fatty acid analysis of neutral and polar lipids of the food bacterium *Listeria monocytogenes*

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*Listeria monocytogenes* is a Gram-positive bacterium that causes meningitis, septicemia and death in humans. Found in low-acid cheeses, vegetables and meat, *L. monocytogenes* is resistant to osmotic and chill stress. Food handling practices that suppress microbial competitors can therefore promote its growth. In response to hyperosmotic or chill stress, *L. monocytogenes* accumulates the potent protectant glycine betaine from the medium, which decreases the lag time and increases the growth rate of the organism. The molecular basis for activation of glycine betaine transport by chill (7°C), despite the expected membrane lipid phase transition, may reside in the lipid composition. The present research identified the lipids of *L. monocytogenes*. Extraction of total lipids yielded $7 \pm 1$ mg ml$^{-1}$ wet cells, with a 5–6% phosphorus content. Polar lipids represented 64% of total lipids. There was a clear difference in the relative complexity of the fatty acids: neutral lipids were more varied and unsaturated fatty acids represented 19% of the total. Polar lipid fatty acids were primarily 15:anteiso (50%) and 17:anteiso (25%). Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

*Listeria monocytogenes*, a Gram-positive, food-borne bacterium, has been recognized as a human pathogen for more than 60 years (Gellin & Broome, 1989). *L. monocytogenes* causes serious infections such as septicemia, meningitis and encephalitis in susceptible individuals. Some of the predisposing conditions for susceptibility to listeriosis include neoplastic disease, immunosuppression therapy, AIDS, pregnancy, extremes of age, diabetes mellitus, alcoholism, renal collagen diseases and hemodialysis failure (Farber & Peterkin, 1991). The fatality rate among infected individuals is about 25%.

*Listeria monocytogenes* can be isolated from many foods, including low-acid cheese, ice cream, raw and ready-to-eat meat, poultry (including eggs), seafood and vegetables (Ryser & Marth, 1991). Major factors in the recent ascent to prominence of *L. monocytogenes* as a food-borne pathogen are undoubtedly its ability to grow vigorously at refrigerator temperatures and to grow in osmotically-stressful environments (Cole *et al.*, 1990; Conner *et al.*, 1986; Seeliger & Jones, 1984; Walker *et al.*, 1990), such as foods with salt concentrations up to 10% NaCl, and dry surfaces (Marth, 1988). On the other hand, *Listeria* does not compete well in mixed cultures and is susceptible to bacteriolysins. Food handling and storage practices, such as refrigeration, that eliminate or suppress competitors may therefore promote the growth of *L. monocytogenes*.

As for many other organisms, to counter-balance osmotic pressure, *L. monocytogenes* accumulates glycine betaine, a ubiquitous and effective osmolyte (compatible solute), intracellularly when grown under osmotic stress. However, it also accumulates glycine betaine when grown under chill stress at refrigerator temperatures (Ko *et al.*, 1994). Exogenously added glycine betaine enhances the growth rate of stressed cells but not of unstressed cells; that is, it confers both osmotolerance and cryotolerance. Both salt-stimulated and cold-stimulated accumulation of glycine betaine occur by transport from the medium rather than by biosynthesis (Ko *et al.*, 1994). A cold-activated transport system is a novel observation and has intriguing implications concerning the physical state of the cell membrane at low temperature, which is determined by its composition.

Studies have been conducted to determine the lipid composition of *L. monocytogenes* (e.g. Raines *et al.*, 1968). The use of membrane fatty acid analysis in

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taxonomy (Drucker, 1976; Lechevalier, 1977; Müller *et al.*, 1990; Welch, 1991) has not been found to be useful in distinguishing among species of *Listeria* (Von Graevenitz *et al.*, 1991), as the fatty acid pattern differs only marginally. Furthermore, the influence of growth temperature on the fatty acid composition of bulk membrane lipids of *Listeria* is striking. Tadayon & Carroll (1971) found that six strains of *L. monocytogenes* which had similar fatty acid profiles when grown at 37°C, with $C_{15:0}$ and $C_{17:0}$ branched-chain fatty acids predominating, showed a marked decrease in the proportion of $C_{17:0}$ branched-chain fatty acids at 4°C. In addition, Püttmann *et al.* (1993) noted that the relative amount of the $C_{15:0}$ fatty acids increased in most of the species at lower temperatures. This kind of adjustment in the fatty acid composition of membrane lipids in response to changes in temperature has been observed in other organisms (Hazel & Williams, 1990; Jones *et al.*, 1993; Russell & Fukunaga, 1990). Adaptation to low temperature may also involve changes in cellular components other than lipids. In any event, a key element in adaptation is the maintenance of metabolic activity and biosynthetic capability under stress during the adaptation process.

It has been shown that intracellular accumulation of glycine betaine facilitates the adaptation of *L. monocytogenes* to low temperature, as evidenced by a shortening of the lag phase and an increase in the rate of growth caused by the addition of betaine to chill-stressed cultures. The active transport required for glycine betaine accumulation is stimulated by a decrease in temperature from 30°C to 7°C (or 4°C), despite the expected lipid phase transition. The nature of a membrane that is adapted for growth at 30°C yet supports cold-stimulated active transport requires investigation. The fact that one of the processes enabled by glycine betaine, although probably indirectly, is the alteration of the fatty acid complement of membrane lipids, suggests a dual role for the membrane in chill adaptation. A first step in understanding these roles is to obtain compositional information for the organism under defined conditions, with and without stress (e.g. Russell, 1989). The present investigation was designed to analyze in greater detail the lipid classes of unstressed *L. monocytogenes* in order to understand the role of the membrane in stress tolerance.

## MATERIALS AND METHODS

### Culture of organism

An avirulent strain of *L. monocytogenes*, DP-L1044 (D. Portnoy, University of Pennsylvania) was maintained on Trypticase–soy agar slants. Inocula were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth at 30°C (approx. 24 h). Ten milliliters of this culture were used to inoculate 1 liter of BHI broth, which was then incubated at 30°C until late log phase. Cell density was determined by turbidity with a Klett–Summerson colorimeter with a green (No. 45) filter (209–212 Klett units). Cells were harvested by centrifugation at 6000 r.p.m. for 10 min and washed in 100 mM phosphate buffer, pH 7.0. Cells were washed again with the same buffer and harvested by centrifugation at 1500 r.p.m. for 1 h.

### Extraction and separation of polar from neutral lipids

Extraction of lipids was performed essentially according to the method of Folch *et al.* (1957). After phase equilibration, the lower chloroform layer (total lipids) was evaporated to dryness, redissolved in chloroform/methanol (2:1) and stored at 0°C. Total lipids were fractionated by solid-phase chromatography using a modification of the method of Berger *et al.* (1992) as follows. Lipid extracts (7 mg solid) were dissolved in 0.5 ml of chloroform/hexane (1:1) and applied to a prewashed 100-mg silica column equipped with stainless steel frits and 10-ml reservoirs (Bond Elut; Varian Sample Preparation Products, Harbor City, CA). The column was mounted on a Visiprep™ solid-phase extraction vacuum manifold (Supelco, Bellefonte, PA). Neutral lipids were eluted three times with 6-ml aliquots of chloroform, and polar lipids were eluted with 7 ml of methanol.

### Analytical methods and quantitation of lipid components

Total phosphorus in the combined lipids was determined by the method of Long & Staples (1961). The polar lipids in bulk were quantitated by weight after elution from the solid-phase extraction columns as described above.

### Chromatography of polar and neutral lipids

High-performance thin-layer chromatography (HPTLC) was carried out on pre-coated Silica gel 60 plates (E. Merck, Darmstad, Germany). For detection of bacterial polar lipids, one-dimensional development was used with the following solvent systems: A (acidic), chloroform/methanol/acetic acid/water (50:25:6:2, v/v/v/v); B (alkaline), chloroform/methanol/28% ammonium hydroxide (65:35:5, v/v/v); C (neutral), chloroform/methanol/water (65:20: 3, v/v/v). For detection of neutral lipids, the following system was used: D, petroleum ether/ether/acetic acid (80:20:1, v/v/v). Visualization of spots was effected by exposure to iodine vapors, followed either by spraying with 50% sulfuric acid and charring for neutral lipids or by spraying with ninhydrin reagent and/or the phosphomolybdenum blue reagent for polar lipids (Dittmer & Lester, 1964). Lipid bands were also visualized using cupric sulfate spray and heating at 100°C for 2–5 min. The cupric sulfate reagent was 10% (w/v) cupric sulfate in 8% (v/v) phosphoric acid (Bhat & Ansari, 1989; Pucsok *et al.*, 1988). An orcinol ferric chloride spray (Christie, 1973) was used for detection of glyco- or sulfolipids (Sigma Chemical Co., St. Louis, MO).

**Lipid standards**

The reference lipid standards cholesterol oleate, cholesterol, bovine heart cardiolipin (sodium salt), L-α-phosphatidyl-DL-glycerol from egg yolk lecithin (sodium salt), L-α-phosphatidylcholine, β-arachidonyl-γ-stearoyl, L-α-phosphatidyl-L-serine from bovine brain and L-α-phosphatidylethanolamine dioleoyl were purchased from Sigma; tripalmitin was from Calbiochem (La Jolla, CA) and free fatty acids were from Aldrich (Milwaukee, WI).

**Methanolysis of lipids and gas chromatography or gas chromatography/mass spectrometry analysis**

The fatty acids from the (bulk) neutral lipids and (bulk) polar lipids obtained from solid-phase extraction were converted to methyl esters with dry acid methanolysis using the method of Vance & Sweely (1967) with the following modification: 500 µl of 3 N methanolic HCl (10%, w/v; Supelco) reagent was added to 1 mg of the lipid sample and methylation was allowed to proceed at 100°C for 5–6 h. Fatty acid methyl esters were extracted four times with 0.8–1-ml aliquots of petroleum ether. The samples were then dried and redissolved in hexane

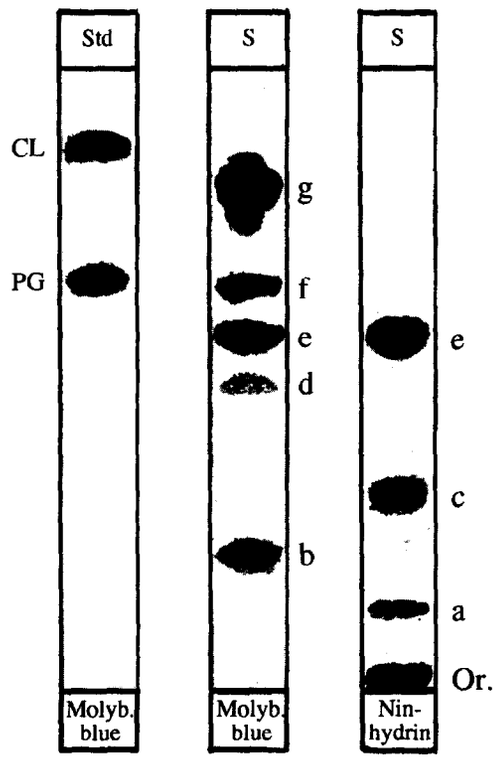


**Fig. 1.** Thin-layer chromatographic separation of the polar lipid fraction of *Listeria monocytogenes* on an HPTLC silica gel 60 plate after separation from neutral lipids by solid-phase extraction. The diagram on the left shows the standards of cardiolipin and phosphatidylglycerol; the center diagram shows the sample after spraying with molybdenum blue reagent; the diagram on the right shows the sample after spraying with ninhydrin reagent. The solvent system was chloroform/methanol/acetic acid/water (50:25:6:2, v/v/v/v). S, sample; Std., standard; CL, cardiolipin; PG, phosphatidylglycerol; or, origin. The letters ***a***, ***b***, ***c***, ***d***, ***e***, ***f***, ***g*** and Or. denote the polar lipid components of *Listeria* as described in the text.

for gas chromatographic analysis. Fatty acid methyl esters were separated using a Varian 3400 gas chromatograph (Varian, Sunnyvale, CA) equipped with a septum-programmable injector, a flame-ionization detector and on-column injector. Methyl esters were separated on a DB®23 30m×0.25 mm capillary column (J&W Scientific, Folsom, CA) with 50% cyanopropyl phase, 0.25 µm film thickness. The make-up gas was nitrogen and the carrier gas was hydrogen. The oven was temperature programmed from 90°C to 130°C at a rate of 50° $min^{-1}$ and 130°C to 210°C at 3° $min^{-1}$. The temperature of the injector was programmed from 90°C to 250°C at 70° $min^{-1}$.

Neutral fatty acid methyl esters were also analyzed using a Hewlett-Packard 5800A (Hewlett-Packard, Palo Alto, CA) gas chromatograph and the same column as described above (split ratio, 50:1). The column temperature was programmed from 130°C to 210°C at a rate of 2° $min^{-1}$, with initial and final column holding times of 1 and 3 min, respectively. The injector temperature was 230°C and the detector temperature was 250°C.

The fatty acid methyl esters were identified by comparison with a standard mixture of bacterial fatty acid methyl esters (CP™ Mix, Catalog No. 1114; Matreya, Pleasant Gap, PA). Gas chromatography/mass spectrometry was performed on the column described above according to the method of Berger *et al.* (1992). For confirmation of the results, additional analyses were performed using a DB®5 30 m×0.25 mm capillary (J&W Scientific, Folsom, CA) 5% phenyl column.

## RESULTS

The total lipids of *L. monocytogenes* grown at 30°C were extracted from harvested cells by the Folch procedure (Folch *et al.*, 1957). Total yield of combined lipids accounted for 7 ± 1 mg $ml^{-1}$ wet harvested cells ($n = 4$). Total phosphorus was determined and the lipid-phosphorus content of the total lipids was about 5–6%.

Solid-phase extraction was used to fractionate 7 mg of total lipids. The polar lipids in bulk were quantified by weight after elution from the columns (4.5 mg). The polar lipids represented 64% of total lipids, and the lipid-phosphorus content of the total polar lipids was about 9%.

Initial large-scale experiments with TLC mobilities of total lipids of *L. monocytogenes* were designed to provide a deeper insight into the composition and nature of its individual lipid classes. Experiments using several developing solvent systems (A, B, C and D) indicated that the most successful solvent for the separation of polar lipids was the A (acidic) system (Fig. 1). In the alkaline solvent (system B), most of the components were concentrated in the same $R_f$ range; in the neutral solvent (system C), the compounds were separated into several spots with similar $R_f$ values, for the most part near the origin of the plate. For neutral lipids, system D was used (Fig. 2).

The mobilities of the components of the polar lipid fraction were compared with those of cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine standards (data not shown). The mobilities of neutral lipids were compared to those of cholesterol ester, cholesterol, triglyceride and free fatty acid standards. The results indicated that the polar lipids of *L. monocytogenes* do not contain phosphatidylcholine or phosphatidylserine. One phosphorus-positive component, which was designated as component *f*, co-chromatographed with the phosphatidylglycerol standard, and the major phospho-compound, which was designated component *g*, had an $R_f$ close to that of cardiolipin.

As determined by TLC ($n = 4$), the polar lipid fraction contained eight components, which were designated as *a*, *b*, *c*, *d*, *e*, *f*, *g* and ***origin*** (Fig. 1). Only *a*, *c* and ***origin*** were free of phosphorus. The major phosphorus-positive compounds were *g* and *e*, followed by *f*. Negligible amounts of lipid-phosphorus were distributed in *b*, *d* and *c*. It is noteworthy that components *e* and the ***origin*** were major amino compounds as shown by strong ninhydrin-positive staining. Several ninhydrin-positive spots migrated between the ***origin*** and *e*, but the most ninhydrin-positive were components *a* and *c*. The orcinol ferric chloride reagent, which is used to detect glycolipids and sulfolipids (Christie, 1973), weakly stained components of the same $R_f$ as component *f* and component *a*, suggesting the presence of such compounds in these spots.

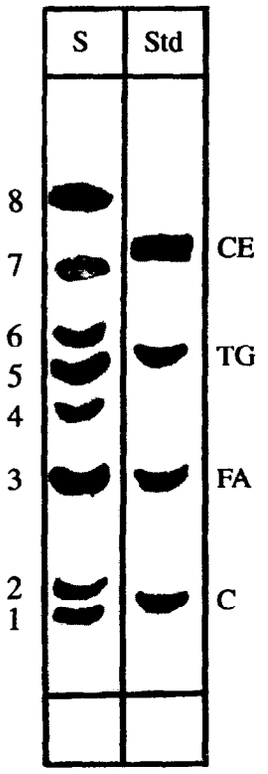


**Fig. 2.** Thin-layer chromatographic separation of the neutral lipid fraction of *Listeria monocytogenes* on HPTLC silica gel 60 after separation from polar lipids by solid-phase extraction. The solvent system was petroleum ether/ether/acetic acid (80:20:1, v/v/v). The plate was visualized by spraying with 50% sulfuric acid and charring. S, sample lane; Std, standard lane; CE, cholesterol esters; TG, triglycerides; FA, free fatty acids; C, cholesterol. The numbers refer to the components of the bacterial neutral lipids as described in the text.

The fatty acid composition of the bulk polar lipid fraction was determined using gas chromatography. They were characterized by the presence of two major fatty acids, $C_{15:anteiso}$ and $C_{17:anteiso}$, 48.95% and 25.70%, respectively, which represented 74.65% of esterified fatty acids of polar lipids (Table 1). The unsaturated fatty acids were present in negligible amounts (1.0%).

Based upon TLC, the neutral lipids consisted of eight components, which are denoted by the numbers 1–8 in Fig. 2. The main components of neutral lipids were free fatty acids (spot 3, Fig. 2), diglycerides (spots 1 and 2, Fig. 2) and another component with an $R_f$ slightly greater than that of cholesterol esters (spot 8, Fig. 2), which is considered to belong possibly to the hydrocarbon class. The remaining were minor components (spots 4–7, Fig. 2).

The fatty acid profile of the neutral lipid fraction was different from that of polar lipids, containing a variety of fatty acids but none in preponderance. It is noteworthy that the fatty acid composition of the neutral lipids was characterized by high percentages of unsaturated fatty acids (18.8%), which were mainly the $C_{18:1}$ and $C_{16:1}$ species (Table 1).

## DISCUSSION

The TLC mobilities of the components of polar lipids of *L. monocytogenes* were determined in alkaline, neutral

**Table 1. Fatty acid composition (weight %) of the polar and neutral lipids of *Listeria monocytogenes* in order of abundance**

| Fatty acid | Polar lipids | Fatty acid | Neutral lipids |
|---|---|---|---|
| $C_{15:anteiso}$[a] | 48.95 | $C_{16:0}$ | 21.73 |
| $C_{17:anteiso}$ | 25.70 | $C_{15:anteiso}$ | 17.12 |
| $C_{15:iso}$ | 10.58 | $C_{18:0}$ | 10.93 |
| $C_{16:0}$ | 6.29 | $C_{18:1(9)}$ | 10.44 |
| $C_{17:iso}$ | 3.38 | $C_{17:anteiso}$ | 9.55 |
| $C_{16:iso}$ | 1.61 | $C_{16:iso}$ | 6.13 |
| C14:0 | 1.00 | $C_{16:1(9)}$ | 6.13 |
| $C_{18:0}$ | 0.68 | $C_{17:0}$ | 5.67 |
| $C_{18:1(9)}$ | 0.66 | $C_{14:0}$ | 4.43 |
| $C_{18:2(9,12)}$ | 0.34 | $C_{15:iso}$ | 3.93 |
| $C_{15:0}$ | 0.09 | $C_{18:2(9,12)}$ | 2.23 |
| $C_{12:0}$ | 0.07 | $C_{15:0}$ | 1.89 |
| | | $C_{17:iso}$ | 1.43 |
| | | $C_{12:0}$ | 1.27 |
| | | 3-OH $C_{14:0}$ | 0.68 |
| | | $C_{11:0}$ | 0.49 |
| | | 2-OH $C_{16:0}$ | 0.02 |
| Total unsaturated | 1.00 | | 18.80 |
| Ratios | | | |
| $C_{15:anteiso/C17:anteiso}$ | 1.90 | | 1.79 |
| $C_{15:anteiso/C15:iso}$ | 4.63 | | 4.35 |
| $C_{17:anteiso/C17:iso}$ | 7.60 | | 6.67 |

[a]Iso and anteiso isomers of branched saturated acids.

and acidic solvent systems (systems B, C and A, respectively). In the acidic solvent (system A), the polar lipid components were resolved into eight distinct spots (Fig. 1). In this system, the major lipid phosphorus was associated with the component designated ***g***. This component had an $R_f$ value lower than that of the cardiolipin standard. The difference was considered to be due to differences in the nature of the constituent fatty acids. This was reported for *Listeria* by Kosaric & Carrol (1971).

The second major phospho-compound of polar lipids, which has been denoted as component ***e***, is also a major amino component, as indicated by the strong ninhydrin-positive staining.

Phosphatidylglycerol is known to be among the lipids of Gram-positive halotolerant bacteria (Russell, 1989). Kosaric & Carrol (1971) reported also the existence of phosphatidylglycerol in *Listeria*. In the present separation, the phosphorus-positive component ***f*** co-chromatographed with the phosphatidylglycerol standard, implying that this component is phosphatidylglycerol.

With regard to the TLC mobilities of acid phospholipids from halobacteria, reports by other researchers (Kates *et al.*, 1993; Stewart *et al.*, 1988; Boggs, 1987) have shown that the relevant $R_f$ is determined not only by the polarity of the phosphate and hydroxyl groups but also may be attributed to the presence of intramolecular hydrogen bonding involving the C–OH group and the P–OH group of the phospho-mono- or diesters.

Additionally, in light of the recent publications by Moreau *et al.* (1995) that hopanoids (a class of pentacyclic triterpene lipids) exist in many species of bacteria, there is the possibility that some of the spots that indicated glyco- or aminolipids in *L. monocytogenes* belong to this class.

Separating total lipids into two classes, polar and neutral, revealed a significant difference in their relative complexity. The polar lipid fatty acid composition is similar to that reported by Von Graevenitz *et al.* (1991) for total lipids, reflecting the relative abundance of polar lipids in the total lipids of *Listeria.* This distinct composition of the neutral lipids implies separate biosynthetic pathways and may thus be important to their function.

Also of notable interest is the high selectivity of the unsaturated fatty acids for neutral lipids and their apparent exclusion from polar lipid classes. This emphasizes the role of branched-chain fatty acids in maintaining membranes in non-crystalline status. In spite of differences in their relative complexity of polar and neutral fatty acids, the ratios of $C_{15:anteiso}/C_{17:anteiso}$, $C_{15:anteiso}/C_{15:iso}$ and $C_{17:anteiso}/C_{17:iso}$ were about the same in both classes, being 1.90 and 1.79, 4.63 and 4.35, and 7.6 and 6.67, respectively.

At the level at which we have analyzed the fatty acid composition of *L. monocytogenes*, two features stand out: the distinctly different profiles of the neutral and polar lipid fractions and the diversity of the neutral lipid fraction. It is possible that it is the diversity of the membrane lipids that leads to the unusual properties of the membrane. Chilling of a membrane adapted to growth at 30°C could lead to local crystallization of the high-melting lipids, leaving the fluid portion of the membrane enriched in low-melting lipids. This sort of fractionation of lipids would alter the properties of the membrane in which the glycine betaine transport protein is embedded. It could also induce distortions in the topography of the membrane. Either event could conceivably activate the transport system. Thus, the membrane itself may be the chill sensor and the osmosensor. This hypothesis lacks precedent in the literature. In fact, local freezing is generally thought to constitute a breech of membrane integrity and to mediate chill-induced damage (Drobnis *et al.*, 1993; Quinn, 1985). In the case of *L. monocytogenes*, however, it is suggested that the composition of the membrane of cells grown at 30°C allows the organism to maintain integrity at the onset of chill stress or osmotic stress, which permits long-term adaptation to occur via the process known as homeoviscous adaptation (e.g. Hazel & Williams, 1990).

*Listeria monocytogenes* survives successfully in unusual environments. This bacterium has obviously evolved a biochemical apparatus that enables it to cope successfully in a medium of high salt concentration and low temperature. Further studies on the range of lipid species are necessary to verify the unique lipid pattern of this bacterium.

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