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Polar lipids of four listeria species containing L-lysylcardiolipin, a novel lipid structure, and other unique phospholipids

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The membrane lipids of *Listeria innocua*, *Listeria monocytogenes*, *Listeria seeligeri* and *Listeria welshimeri* were fractionated on DEAE-cellulose and purified by chromatography on silica gel and/or preparative TLC. The lipid structures were elucidated by chemical and chromatographic means. The polar lipid composition of the four listeria species was similar. Phospholipids predominated. They consisted of phosphatidylglycerol, L-lysylphosphatidylglycerol, cardiolipin [bis(phosphatidyl)glycerol] and L-lysylcardiolipin. A phospholipid more polar than cardiolipin, possibly two L-lysyl derivatives of it, *sn*-glycero-1-phosphoglycolipid, its D-alanyl derivative, and polyprenol phosphate were also detected. Towards the end of exponential growth, the relative amounts of cardiolipin and L-lysylcardiolipin increased, approaching 47–78% lipid phosphorus with a ratio of L-lysylcardiolipin to cardiolipin of 0·25–1·6. As shown by fast atom bombardment-mass spectrometry, cardiolipin and L-lysylcardiolipin consisted of five molecular species due to various fatty acid combinations. L-Lysylcardiolipin has so far not been found in nature. It belongs to the recently discovered class of substituted cardiolipins. Its occurrence in the four listeria species tested shows that it is a characteristic lipid component of the *L. monocytogenes* line of descent. Further studies on the lipid pattern of members of the other descent line are required to decide whether lysylcardiolipin can serve as a genus-specific chemotaxonomic marker for listeriae.

Keywords: aminoacylphospholipids, glycerophosphoglycolipids, listeriae, L-lysylcardiolipin, L-lysylphosphatidylglycerol

INTRODUCTION

The polar lipid composition of listeriae has not been studied in detail. In one report, the major phospholipids of *Listeria monocytogenes* were identified as bis(phosphatidyl)glycerol (cardiolipin; Ptd_2Gro), phosphatidylglycerol (PtdGro) and a phospholipid, that was described as bis(phosphatidylglycerol) phosphate [$(PtdGro)_2P$; Kosaric & Carroll, 1971]. In a review (Shaw & Stead, 1972), the occurrence of a glycerophosphogalactosyl-glucosyldiacylglycerol in *L. monocytogenes* was mentioned. The glyceroglycolipid of *L. monocytogenes* was shown to possess the structure Gal(α1-2)Glc(α1-3)$acyl_2$Gro (Deroo, 1969), and this glycolipid was also identified as one of the two lipid anchors of lipoteichoic acid (LTA); the other is Gal(α1-2)Ptd-6Glc(α1-3)$acyl_2$Gro, a phosphatidyl derivative of the glyceroglycolipid (Hether & Jackson, 1983, Uchikawa *et al*., 1986; Ruhland & Fiedler, 1987; Fischer *et al*., 1990). All species of the genus *Listeria* possess poly(glycerophosphate)-containing LTA (Ruhland & Fiedler, 1987). The glycerophosphate (Gro*P*) units of the hydrophilic chains in part are substituted at *O*-2 with D-alanyl esters and, in most species, D-alanyl esters alternate with α-D-galactopyranosyl residues.

In the present study, the membrane lipids from one strain of each of *L. monocytogenes*, *Listeria innocua*, *Listeria seeligeri* and *Listeria welshimeri* were

Abbreviations: $acyl_2Gro$, diacylglycerol; APL, aminophospholipid; FAB-MS, fast atom bombardment-mass spectrometry; Gro*P*, glycerophosphate; LTA, lipoteichoic acid; $LysPL_X$, lysine-containing phospholipids; LysPtdGro, L-lysylphosphatidylglycerol; $LysPtd_2Gro$, L-lysylcardiolipin; PL_U, unidentified ninhydrin-negative phospholipid; PtdGro, phosphatidylglycerol; Ptd_2Gro, bis(phosphatidyl)glycerol (cardiolipin); $(PtdGro)_2P$, bis(phosphatidylglycerol) phosphate.

extracted. The extraction and purification of lipids were performed under conditions that avoid hydrolysis of the labile amino ester bond of aminoacyl phospholipids (Houtsmüller & van Deenen, 1964, 1965; Gould & Lennarz, 1970). In this way, four L-lysyl-containing phospholipids and a D-alanyl-substituted glycerophosphoglycolipid could be detected.

METHODS

Materials. DEAE-Cellulose and TLC plates were purchased from Merck, enzymes and co-substrates were from Boehringer Mannheim. Reference straight-chain and anteiso- and iso-branched fatty acids (a-, i-14:0; a-, i-15:0; a-, i-16:0; a-, i-17:0), decaprenol phosphate and octadecaprenol phosphate were from Sigma-Aldrich. Iatrobeads 6 RS-8060 were obtained from Iatron Laboratories. Reference glyceroglycolipids, phosphoglycerolipids and glycerophosphoglycolipids were from previous work (Fischer, 1977b; Nakano & Fischer, 1977; Landgraf, 1976).

Bacteria and growth. *L. innocua* NCTC 11288^T (= DSM 20649^T), *L. monocytogenes* NCTC 7973, *L. seeligeri* SLCC 3954^T (= DSM 20751^T) and *L. welshimeri* SLCC 5334^T (= DSM 20650^T) were kindly provided by H. Hof (Institute of Microbiology & Hygiene, Mannheim, Germany). Bacteria were stored in growth medium containing 10% (w/w) glycerol at −80 °C. The growth medium (pH 7·0) contained (l^{-1}): 12·5 g casein peptone; 7·5 g yeast extract; 5 g trisodium citrate; 5 g NaCl; 5 g K_2HPO_4; 14 mg $MgCl_2.6H_2O$; 40 mg $FeSO_4.7H_2O$; 0·8 g $MgSO_4.7H_2O$; and 15 g glucose. The medium was sterilized by filtration. For inoculation of batch cultures (20 l), overnight cultures were diluted 50-fold. The bacteria were grown at 37 °C under moderate stirring without aeration until OD_{578} of the culture approached values of $3 \pm 0{\cdot}5$ and the pH had dropped to $5{\cdot}0 \pm 0{\cdot}5$ (post-exponential growth phase). The bacteria were harvested with a refrigerated continuous-flow centrifuge. The yield was 1·3–3·1 g wet cells (l medium)$^{-1}$.

Extraction and purification of lipids. Immediately after harvesting, the bacteria were suspended [4 g wet cells (10 ml^{-1})] in ice-cold 0·1 M sodium acetate, pH 4·7 (buffer A) and disintegrated with glass beads in a Braun disintegrator as described previously (Fischer *et al.*, 1983). Lipids were extracted from the disintegrated bacteria by a Bligh–Dyer procedure (Kates, 1986) in which water was replaced by buffer A. The crude lipid extracts were dissolved in $CHCl_3$ and fractionated by column chromatography on DEAE-cellulose as described in Results. Fractions from the column, containing ammonium acetate in $CHCl_3$/methanol (2:1, v/v) were de-salted by twofold extraction, each with one-quarter vol. water, containing 0·9% (w/v) NaCl, 0·2% (w/v) $CaCl_2$ and 10 mM acetic acid. Purified lipids were taken to dryness with several additions of methanol/benzene (1:1, v/v), then dissolved in $CHCl_3$/methanol (2:1, v/v; slightly acidified with acetic acid) and stored at −20 °C.

Analytical procedures. Hydrolyses and measurements of the released components were essentially performed as described previously (Fischer & Arneth-Seifert, 1998). D-Alanine was identified and measured by an enzymic procedure (Grassl & Supp, 1985). L-Lysine was differentiated from the D-enantiomer by a stereoselective HPLC procedure (Brückner *et al.*, 1991) and quantified, using taurine as an internal standard. The separation of L- and D-lysine is shown in Fig. 1.

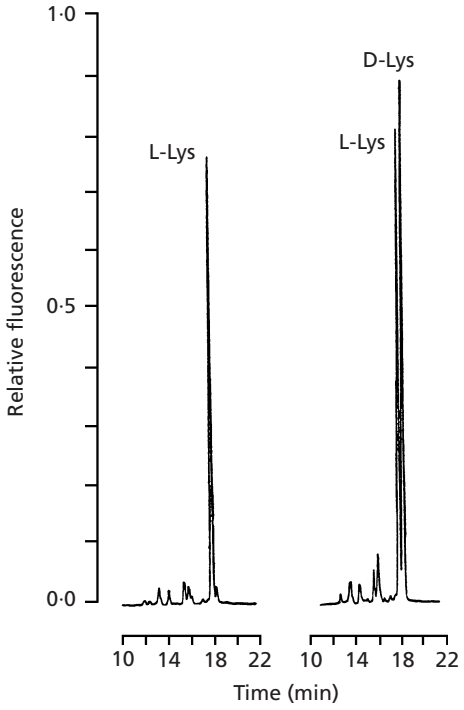

Fig. 1. Separation of L- and D-lysine by HPLC (see text). Left-hand plot, lysine released from phospholipids by HCl; right-hand plot, lysine released from phospholipids after addition of D-lysine to the hydrolysate. The fluorescent derivatives (Brückner *et al.*, 1991) were analysed on a LiChrospher RP-18e column (5 μm, 4 × 25 mm; Merck) by elution at a flow rate of 1 ml min^{-1} with a linear gradient (5 min) from buffer A (23 mM sodium acetate adjusted with 10% acetic acid to pH 6·0) to 50% buffer B (50 ml acetonitrile in 600 ml methanol) and then from 50 to 75% buffer B (17 min).

Fatty acids were released by HCl hydrolysis and converted to methyl esters as described previously (Fischer & Arneth-Seifert, 1998). For quantification, pentadecanoic acid was added before acid hydrolysis. Fatty acid methyl esters were analysed by GLC on a Hewlett Packard gas chromatograph 5890 Series II, equipped with a flame-ionization detector. An HP-5 fused silica capillary column (5% diphenyl-polysiloxane/95% dimethylpolysiloxane; 30 m; i.d., 0·24 mm; film thickness, 0·25 μm) was used at oven temperatures of 150–250 °C with a programmed temperature rise of 5 °C min^{-1}.

TLC. Lipids and the deacylation products of glycolipids were separated on silica gel plates (Merck 60). The following solvents were used: A, chloroform/methanol/water (65:25:4, by vol.); B, chloroform/acetone/methanol/acetic acid/water (50:20:10:10:4, by vol.); C, chloroform/methanol/acetic acid/water (80:18:12:5 by vol.); D, propanol/pyridine/water (7:4:2, by vol.); and E, propanol/25% (w/v) ammonia/water (6:3:1, by vol.). Lipids were visualized with iodine vapour or phosphomolybdic acid and charring, glycolipids were stained with 1-naphthol/H_2SO_4, aminolipids with ninhydrin (Merck reagent), and phospholipids with the Dittmer–Lester reagent. Deacylated phospholipids were separated on cellulose plates (Merck) using solvent E and the Hanes-Isherwood reagent for visualization. The references to the staining procedures can be found in previous work (Fischer *et al.*, 1973a, 1994).

Deacylation. Deacylation was performed as described by Kates (1986).

Selective amino acid ester cleavage. The procedure is based on previous work (Fischer & Arneth-Seifert, 1998). Lipid extracts or individual aminoacylphospholipids were dissolved in 200 µl chloroform/methanol (2:1, v/v) containing 0·05% (w/v) Triton X-100. After drying (rotavapor), the sample was dispersed in 0·1 M ammonium borate, pH 9 (250 µl) and incubated for 10–24 h at 37 °C. Water (200 µl), methanol (500 µl) and chloroform (500 µl) were then added and, after mixing, the phases were separated by centrifugation (3000 ***g***). The lower phase was washed with water/methanol (1:1, v/v) and taken to dryness with several additions of benzene/methanol (1:1, v/v) under a stream of N_2.

Stereochemical analysis of the Gro*P* residues. Since in alkali, phosphate migration between adjacent hydroxyls does not occur (Baer & Kates, 1950), the α-Gro*P* released from phosphoglycerolipids by alkali hydrolysis retains the original stereochemical configuration (Fischer *et al*., 1973a, b; Brotherus *et al*., 1974; Fischer & Landgraf, 1975; Joutti *et al*., 1976). Hydrolysis, neutralization (Fischer & Arneth-Seifert, 1998), separation of the products on DEAE-cellulose, HCO_3^- (Fischer, 1977b), and subsequent quantification of the products (Fischer & Arneth-Seifert, 1998) were essentially as described in the references.

RESULTS

Polar lipid composition

Fig. 2 shows a chromatogram of the polar lipids from late exponential growth phase cells of *L. welshimeri*, Fig. 3(a) shows a chromatogram of the polar lipids from stationary phase cells of *L. innocua*. In both extracts, phosphoglycerolipids predominated. The phosphoglycerolipid composition of the four *Listeria* species is summarized in Table 1. In all species, Ptd_2Gro, L-lysylcardiolipin ($LysPtd_2Gro$), PtdGro, L-lysylphosphatidylglycerol (LysPtdGro) and a glycerophosphoglycolipid were found. Common constituents were also a ninhydrin-negative phospholipid (PL_U) and two L-lysine-containing phospholipids ($LysPL_X$). In stationary phase cells of *L. welshimeri* and *L. innocua*, Ptd_2Gro and $LysPtd_2Gro$ together amounted to 70–80% of the lipid phosphorus and the ratio of $LysPtd_2Gro$ to Ptd_2Gro varied between 1·1 and 1·6. In *L. monocytogenes* and *L. seeligeri*, Ptd_2Gro and $LysPtd_2Gro$ together contributed 47–63% to the lipid phosphorus. In these species, Ptd_2Gro was prevailing. The values in Table 1 are tentative because Ptd_2Gro and $LysPtd_2Gro$ increased drastically towards the end of exponential growth (see below). PL_U was more polar than Ptd_2Gro and PtdGro (Figs 2 and 3; lipid 8). As shown in Table 1, in *L. monocytogenes* and *L.*

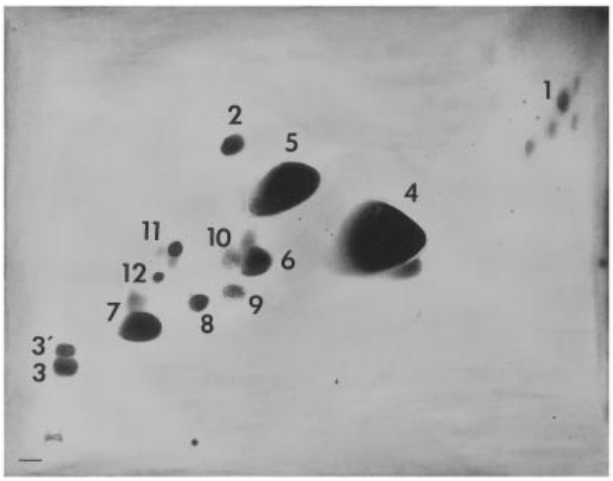

Fig. 2. Polar lipids of *L. welshimeri*. Two-dimensional TLC on silica gel plates. The first and second dimensions were developed with solvents A and C, respectively. The lipids were stained with a phosphomolybdic acid spray and charring (Kritchevski & Kirk, 1952). The sample was applied on the line at the lower left. 1, Glc-acyl$_2$Gro; 2, Gal-Glc-acyl$_2$Gro; 3′, AlaGro*P*-Gal-Glc-acyl$_2$Gro; 3, Gro*P*-Gal-Glc-acyl$_2$Gro; 4, Ptd_2Gro; 5, $LysPtd_2Gro$; 6, PtdGro; 7, LysPtdGro; 8–10, ninhydrin-negative phospholipids; 11, 12, lysylphospholipids. Lipid 8 is referred to as PL_U; lipids 11 and 12 are referred to as $LysPL_X$.

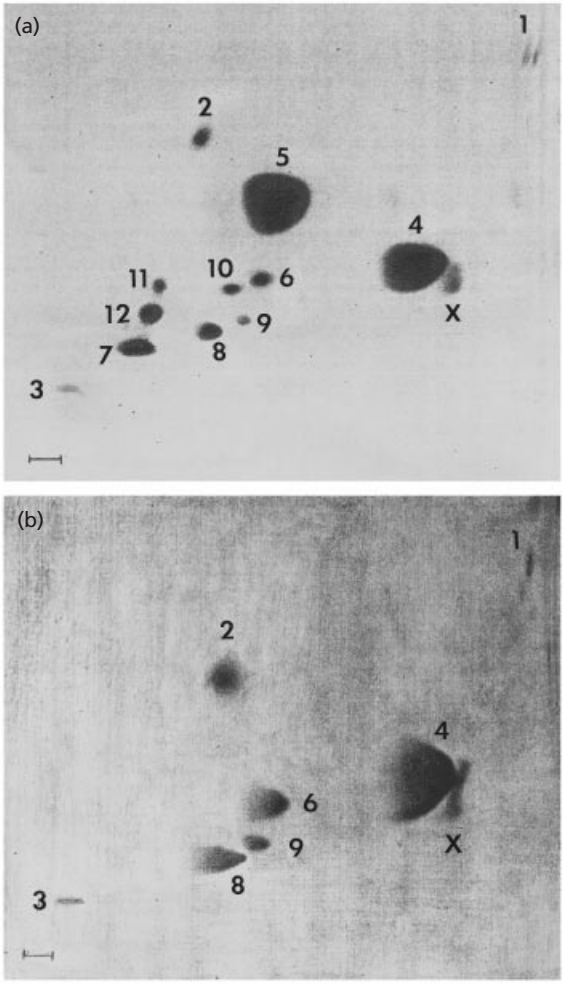

Fig. 3. Polar lipids of *L. innocua* before (a) and after selective aminoacyl ester cleavage (b). The sample was applied on the line at the lower left. TLC conditions, solvents, staining and identification of the lipids are as given in Fig. 1. Compound X, tentatively identified as polyprenol phosphate (see text).

Table 1. Relative abundance of phospholipids

Values are percentage of total lipid phosphorus. The figures in parentheses are those used in Figs 2 and 3.

Lipid source	Ptd_2Gro (4)	$LysPtd_2Gro$ (5)	PtdGro (6)	LysPtdGro (7)	Gro*P*-Gal-Glc-$acyl_2Gro$ (3′ and 3)	PL_U (8)	$LysPL_X$ (11 and 12)
L. welshimeri	31·8	37·4	4·1	10·8	8·1	2·6	3·0
*L. innocua**	30·4	47·3	1·4	8·6	2·6	1·0	3·1
	39·1	33·4	2·9	12·3	2·9	2·9	3·8
L. monocytogenes	34·9	12·3	6·4	5·4	4·3	22·8	8·0
L. seeligeri	51·4	12·0	6·8	2·9	1·1	11·7	8·7

* Stationary phase cells (first row), late exponential phase cells (second row).

seeligeri, it contributed 22·8 and 11·7 %, respectively, to the lipid phosphorus. In *L. welshimeri* and *L. innocua*, it was also detectable but less abundant. In addition, there were two ninhydrin-positive lipids (Figs 2 and 3; lipids 11 and 12) which, quantified together, contributed 3–9 % to the lipid phosphorus (Table 1; $LysPL_X$). Measured from thin-layer plates, each of them contained L-lysine in a molar ratio to phosphorus of approximately 0·3. When the total lipid extract of *L. innocua* was subjected to selective aminoacyl ester cleavage, only Ptd_2Gro, PtdGro and PL_U were detected besides the unaffected glycolipids (Fig. 3b). Similarly, after the same treatment of the lipid extract from *L. monocytogenes*, only these three phospholipids and the glycolipids were seen (data not shown). It is therefore hypothesized that the aminoacyl phospholipids 11 and 12 ($LysPL_X$) are L-lysyl derivatives of lipid 8 (PL_U). It should be further noted that lipids 11 and 12 showed a relative mobility to lipid 8 that was similar to the relative mobility of lipid 5 to lipid 4.

Fractionation of lipids

Crude lipid extracts were fractionated by column chromatography on DEAE-cellulose (Rouser *et al.*, 1967; Landgraf, 1976). An example of separation is summarized in Table 2 and illustrated in Fig. 4. Two aminophospholipids (APL I and APL II) eluted along with Ptd_2Gro in fractions 7 and 8. They were not further studied. In fraction 8, an anionic lipid which ran much faster than Ptd_2Gro also appeared, showing a mobility relative to Ptd_2Gro of 1·56 (Fig. 4a). As outlined below, it was tentatively identified as a polyprenol phosphate.

Structural analysis

For analysis, individual lipids were finally purified by chromatography on silica gel columns (Pasteur pipettes) or by preparative TLC using solvent A or B (data not shown).

Glyceroglycolipids and glycerophosphoglycolipids. In all species, the glycolipids (lipids 1 and 2 in Figs 2 and 3) were minor components. The content of lipid 1 varied between 0·5 and 3 mol % and that of lipid 2 varied between 2 and 9 mol % of total polar lipids.

Lipid 1 contained equimolar amounts of D-glucose and glycerol and lipid 2 contained equimolar amounts of D-galactose, D-glucose and glycerol. On TLC, lipids 1 and 2 co-chromatographed with Glc(α1-3)$acyl_2Gro$ and Gal(α1-2)Glc(α1-3)$acyl_2Gro$ (solvents A and B), respectively. The deacylation products had the same mobility as the deacylated reference lipids (solvent D). On TLC of the lipid extract from *L. welshimeri* (Fig. 2) and *L. monocytogenes* (not shown), lipids 3 and 3′ appeared as a double band. Both components reacted positively with 1-naphthol/H_2SO_4 and were faintly stained by the Dittmer–Lester reagent. Lipid 3 showed the same chromatographic mobility (silica gel, solvent A) as the recently described *sn*-Gro-1-*P*-6Gal(α1-2)Glc(α1-3)$acyl_2Gro$ (Fischer & Arneth-Seifert, 1998). The faster moving component, lipid 3′, was also ninhydrin-positive. When scraped off the TLC plate and analysed after hydrolysis in HCl (2 M, 100 °C, 2·5 h), lipid 3′ contained D-alanine in a molar ratio to phosphorus of 0·8. Lipids 3 and 3′ isolated together by preparative TLC (Fischer, 1977b) released on acid hydrolysis (2 M HCl, 100 °C, 2·5 h) glycerol, Gro*P*, D-glucose, D-galactose, D-alanine and fatty acids in molar ratios to phosphorus (1·00) of 0·92:0·90:0·96:0·90:0·37:2·1. Hydrolysis with 98 % (v/v) acetic acid yielded a phosphate-free, ninhydrin-negative glycolipid with the co-chromatographic mobility of Gal(α1-2)Glc(α1-3)$acyl_2Gro$ (solvent A). The α-Gro*P*, released by alkali hydrolysis (0·5 M NaOH, 100 °C, 2 h), was not oxidized by *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) indicating a *sn*-glycero-1-phosphate residue in the parent compound (Fischer *et al.*, 1973a, b; Brotherus *et al.*, 1974; Fischer & Landgraf, 1975; Joutti *et al.*, 1976). The structure proposed for lipid 3 is *sn*-Gro-1-*P*-[Gal(α1-2)Glc(α1-3)]-$acyl_2Gro$; approximately one-third of it is esterified with D-alanine, apparently on the Gro*P* moiety.

Phospholipids. Lipids 4 and 5 contained glycerol, fatty acids and phosphorus in molar ratios close to 3:4:2. Lipid 5 contained, in addition, 1 mol equivalent L-lysine (Table 3). Mild alkaline treatment (0·1 M sodium borate, pH 9·8, 37 °C, 8 h) of lipid 5 released the L-lysine residue and yielded a ninhydrin-negative lipid with the chromatographic mobility of Ptd_2Gro (TLC,

Table 2. Fractionation of the crude lipid extract of *L. innocua*

Crude lipid extract (600 μmol phosphorus) of *L. innocua* was fractionated by chromatography on a column (2 × 19 cm) of DEAE-cellulose (acetate form). The column was eluted at a flow rate of 200 ml h^{-1} by a stepwise gradient of methanol MeOH in $CHCl_3$, followed by a stepwise gradient of ammonium acetate NH_4OAc (adjusted to pH 5 with acetic acid) in $CHCl_3$/methanol (2:1, v/v). Fractions of 200 ml were collected and analysed for phosphorus and by TLC for lipid composition (solvent A).

Fraction no.	Eluant		Effluent	
	$CHCl_3$/MeOH (v/v)	NH_4OAc [g(100 ml)$^{-1}$]	Phosphorus (μmol)	Lipid (elution maxima)
1	1:0	–	–	'Pigments'
2	95:5	–	–	Glc-acyl$_2$Gro
3	9:1	–	7·7	Gal-Glc-acyl$_2$Gro
4	2:1	–	38·4	LysPtdGro
5	2:1	0·05	122·7	LysPtd$_2$Gro
6	2:1	0·1	62·7	LysPtd$_2$Gro
7	2:1	0·2	130·6	Ptd$_2$Gro, APL I, APL II
8	2:1	0·4	193·6	Ptd$_2$Gro, APL I, polyprenol-*P*
9	2:1	0·6	63·8	Gro*P*-Gal-Glc-acyl$_2$Gro, minor phospholipids

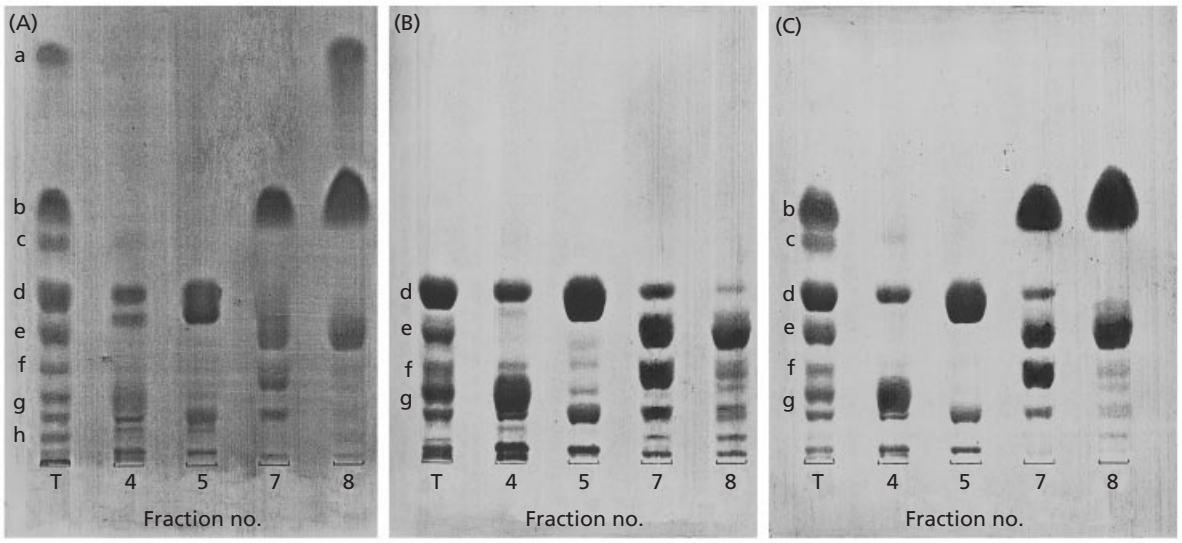

Fig. 4. Fractionation of a lipid extract from the type strain of *L. innocua* on DEAE-cellulose. TLC on silica gel, developed with solvent B. The same plate was stained in sequence with iodine vapour (A), ninhydrin (B) and with the Dittmer–Lester reagent (lipid phosphorus) (C). For elution scheme and fraction numbers, see Table 2. T, Total lipid extract. Lipids: a, polyprenol phosphate; b, Ptd$_2$Gro; c, PtdGro; d, LysPtd$_2$Gro; e, APL I; f, APL II (APL were not further characterized); g, LysPtdGro; h, Gro*P*-Gal-Glc-acyl$_2$Gro (eluted in fraction no. 9; not shown).

solvent B). Hydrolysis with 98 % (v/v) acetic acid of lipid 4 and the lysine-free lipid 5 resulted in the formation of 2 mol equivalents diacylglycerol (acyl$_2$Gro) and 1 mol equivalent glycero-1,3-bisphosphate, which were separated by phase partitioning and identified by compositional analysis. Native lipid 5 resisted hydrolysis with 98 % (v/v) acetic acid, indicating that the lysyl residue was linked to *O*-2 of the middle glycerol moiety.

Alkali hydrolysis (0·5 M NaOH, 100 °C, 3 h) of deacylated lipid 4 and 5 released glycerol, glycerobisphosphate, and β- and α-Gro*P* in molar proportions of 0·6:0·08:0·6:0·4. As shown in Table 3, approximately 70 % of the released α-Gro*P* was oxidized by *sn*-glycerol-3-phosphate dehydrogenase (Lang, 1984) which suggests that, as in ox heart Ptd$_2$Gro (LeCocq & Ballou, 1964), three of the four Gro*P* bonds are linked to the *sn*-3 position and one is linked to the *sn*-1

Table 3. Phospholipid analyses (values shown are molar ratios)

Constituent	Ptd_2Gro	$LysPtd_2Gro$	LysPtdGro
Phosphorus	2·00	2·00	1·00
Glycerol	2·80	2·75	1·88
Fatty acids	3·95	4·33	2·18
L-Lysine	–	1·16	1·23
sn-Gro-3-*P**/α-Gro*P*	0·68	0·72	0·48

* Released by alkali hydrolysis; see text for explanation.

Table 4. Fatty acid composition of total lipids and phospholipids of *L. welshimeri*

Values are mol%. Fatty acid abbreviations are given in the following form: n-14:0 (non-branched), tetradecanoate; i-15:0 (iso-branched), 13-methyltetradecanoate; a-15:0 (anteiso-branched), 12-methyltetradecanoate.

	n-12:0	n-14:0*	i-15:0	a-15:0	i-16:0	n-16:0	i-17:0	a-17:0	n-18:0
Total lipid	–	0·8	10·7	53·4	1·8	2·8	3·6	22·2	3·8
Ptd_2Gro	1·2	1·6	11·6	54·2	2·0	3·4	4·2	22·6	0·5
$LysPtd_2Gro$	1·1	2·6	10·2	48·9	2·1	7·3	3.8	21·3	2·5
LysPtdGro	–	2·3	9·4	48·6	2·5	7·7	3·3	21·6	4·5
Gro*P*-Gal-Glc-$acyl_2Gro$		4·3	8·9	30·4	2·5	10·1	3·3	33·2	6·3

* In addition, small amounts (<0·5 mol%) of i-14:0, n-14:1 and n-16:1 were detected.

Table 5. Molecular species of Ptd_2Gro and $LysPtd_2Gro$ from *L. welshimeri* identified by FAB-MS in negative ion mode

Fatty acid combinations are given as numbers of carbon atoms of branched fatty acids (16:1, n-16:1).

Ptd_2Gro		$LysPtd_2Gro$		Fatty acid combinations
Mass (Da)	$[M-H^+]^-$ *m/z*	Mass (Da)	$[M-H^+]^-$ *m/z*	
1380	1379	1508	1507	15/17/17/17
1352	1351	1480	1479	15/15/17/17
1336	1335	1464	1463	15/15/16:1/17
1324	1323	1452	1451	15/15/15/17
1308	1307	1436	1435	15/15/15/16:1

position (Fischer, 1977b). Accordingly, both phosphatidyl residues of lipid 4 and the lysine-free lipid 5 were hydrolysed by the stereospecific phospholipase A_2 (EC 3.1.1.4) (Haas *et al.*, 1968), yielding the monodeacyl and finally the dideacyl derivative (TLC, solvent A). On the basis of these results, it is proposed that the structures for lipids 4 and 5 are: bis(*sn*-3-phosphatidyl)-1′,3′-glycerol and 2′-*O*-L-lysyl-bis(*sn*-3-phosphatidyl)-1′,3′-glycerol.

Lipid 7 contained glycerol, fatty acids, phosphorus and L-lysine in molar ratios close to 2:2:1:1. Mild alkaline treatment released the lysine residue and a ninhydrin-negative phospholipid which, on TLC, co-chromatographed with authentic PtdGro (solvents A and B). In contrast to the parent compound, the lysine-free derivative reacted positively with the periodate/Schiff reagent for glycol groups (Shaw, 1968). Alkali hydrolysis (0·5 M NaOH, 100 °C, 3 h) released equimolar amounts of glycerol and Gro*P* (α- and β-isomer, 2:3). Approximately, half of the α-Gro*P* reacted with *sn*-glycerol-3-phosphate dehydrogenase and therefore the other half was the *sn*-1-isomer (see above). The susceptibility of the lysine-free lipid 7 to the stereospecific phospholipase A_2 (EC 3.1.1.4) (Haas *et al.*, 1968) assigns the *sn*-3-configuration to the phosphatidyl moiety. Lipid 7 has therefore the proposed structure 2′(3′)-*O*-L-lysyl-*sn*-3-phosphatidyl-*sn*-1′-glycerol.

In Fig. 2, lipid 7 appears as a double spot which may reflect lysyl-positional isomers on the glycerol moiety

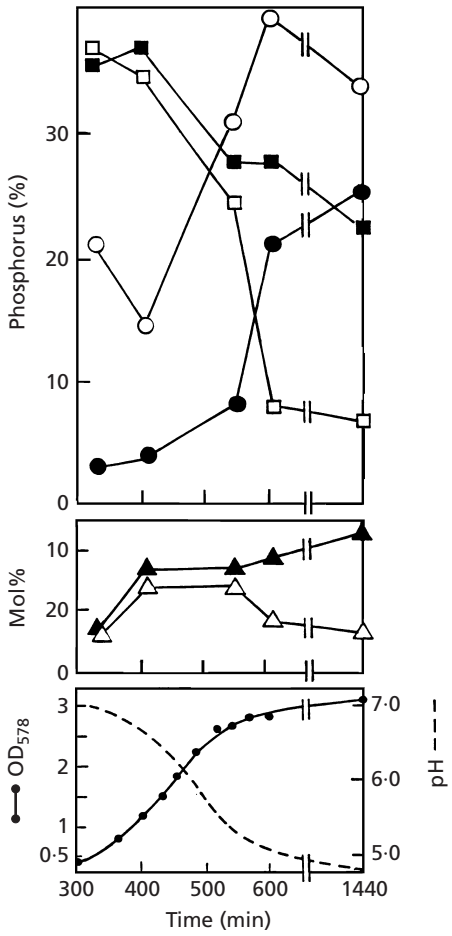

Fig. 5. Changes in lipid composition during growth of *L. welshimeri*. Growth was followed by measuring optical density (OD_{578}) and pH (bottom graph). Phospholipids (top graph): □, PtdGro; ■, LysPtdGro; ○, Ptd_2Gro; ●, $LysPtd_2Gro$. Glycolipids and Gro*P*-glycolipid (middle graph): △, Glc-acyl$_2$Gro and Gal-Glc-acyl$_2$Gro; ▲, Gro*P*-Gal-Glc-acyl$_2$Gro. Lipids were separated by two-dimensional TLC as in Fig. 1. Spots were faintly stained with iodine vapour, scraped off into screw-capped reaction vessels after evaporation of iodine and heated in 2 M HCl (100 °C, 3 h). Samples of the hydrolysates were analysed for phosphorus and/or D-glucose and D-galactose. The content of phospholipids is given as a percentage of total lipid phosphorus and the content of glycolipids is given as mol % of total polar lipids.

which are not interconvertible, except in the presence of a strong acid catalyst such as HCl (Tocanne *et al.*, 1974).

Tentative identification of polyprenol phosphate. Compound X in Fig. 3 proved to be chromatographically identical to compound a in Fig. 4 when the second dimension was developed with solvent B instead of solvent C on two-dimensional TLC. In solvents A and C, it moved more slowly than octadecaprenol phosphate and was close to decaprenol phosphate. The anionic character, revealed on elution from DEAE-cellulose (Fig. 4a), was caused by phosphate which was identified after charring (Schnitger *et al.*, 1959). Like the reference polyprenol phosphates, it did not react on TLC with the Dittmer–Lester reagent for lipid phosphorus (Fig. 4c) and was stained with the anisaldehyde reagent (Stahl, 1967) at room temperature, yielding a red colour. Colour development without heating is specific for polyprenol phosphates.

Fatty acid composition. The fatty acid composition of total lipids and individual phospholipids of *L. welshimeri* is shown in Table 4. Besides small amounts of even-numbered saturated straight-chain fatty acids, the prevailing fatty acid species were anteiso-branched (a-15:0, a-17:0) accompanied by less abundant iso-branched fatty acids (i-15:0, i-16:0, and i-17:0). The fatty acid patterns of total lipids, Ptd_2Gro, $LysPtd_2Gro$ and LysPtdGro were quite similar; in the Gro*P*-glycolipid a-17:0 was enriched at the cost of a-15:0. In total lipids of *L. innocua* i-15:0, a-15:0, i-17:0 and a-17:0 amounted to 13, 40, 6 and 32 mol %, respectively. A similar fatty acid pattern was reported for five listeria species (Feresu & Jones, 1988) and, in another study, the major polar lipid of *L. monocytogenes* was shown to contain 11 % i-15:0, 45 % a-15:0 and 3 % i-17:0 (Mastronicolis *et al.*, 1996). In an earlier study by Kosaric & Carroll (1971) into the phospholipids of *L. monocytogenes* anteiso and iso fatty acids were quantified together as branched fatty acids (br). These authors studied the positional distribution of the fatty acids and found br-15:0 (59 ± 6 mol %) preferentially linked to *O*-2, br-17:0 (30 ± 4 mol %) to *O*-1 of the glycerol moieties.

Molecular species. Ptd_2Gro and $LysPtd_2Gro$ proved to be heterogeneous on negative ion fast atom bombardment-mass spectrometry (FAB-MS), as shown in Table 5. Five species can be discriminated owing to different fatty acid combinations. The molecular masses of the lysyl-substituted species are, as expected, 128 Da higher than the corresponding species of Ptd_2Gro. The most abundant species had molecular masses of m/z 1336 and 1464, respectively. The fragmentation patterns obtained by FAB-MS that corroborate the proposed structures are published elsewhere (Peter-Katalinić & Fischer, 1998).

Growth-dependent changes in the lipid composition

Fig. 5 illustrates the changes in the polar lipid composition during bacterial growth: PtdGro and LysPtdGro were gradually replaced by Ptd_2Gro and $LysPtd_2Gro$. The content of Ptd_2Gro increased up to the onset of the stationary phase of growth and then decreased. The content of $LysPtd_2Gro$ rose slowly from a low level during exponential growth, increased rapidly from transition to the stationary phase, and then continued increasing at a reduced rate. Glycolipids and the Gro*P*-glycolipid were detectable during all stages of growth. In the stationary phase, the relative content of the former decreased and that of the latter approached a maximum. Similar growth-dependent alterations were observed in the lipid composition of *L. innocua*, except that Ptd_2Gro was present

in larger proportions from the beginning (data not shown).

DISCUSSION

Compared with previous work (Kosaric & Carroll, 1971), a novel aspect of listerial lipids is the occurrence of at least four lysyl-containing phospholipids, which contribute between 25 and 50% to the lipid phosphorus. They may have been lost in previous work by spontaneous hydrolysis when the pH was not considered during extraction and purification of the lipids (Houtsmüller & van Deenen, 1965; Gould & Lennarz, 1970). In a recent study into the polar lipids of *L. monocytogenes*, two ninhydrin-positive phospholipids that have not been further characterized were identified by TLC, which together constitute 15% of the polar lipids on a weight basis (Mastronicolis *et al.*, 1996).

The most abundant aminoacyl-phospholipid in our study was $LysPtd_2Gro$. To the best of our knowledge, $LysPtd_2Gro$ has so far not been found in nature. Looking through thin-layer chromatograms documented in earlier work, $LysPtd_2Gro$ could not be detected in the lipid extracts of bacilli, enterococci, *Lactobacillus casei*, lactococci, *Staphylococcus aureus*, *Streptococcus pyogenes*, group B streptococci, *Streptococcus pneumoniae* and vagococci (Fischer, 1977a, 1982, 1997; Fischer *et al.*, 1978; Nakano & Fischer, 1977; Schleifer *et al.*, 1985; Fischer & Arneth-Seifert, 1998). The occurrence of $LysPtd_2Gro$ in *L. monocytogenes*, *L. welshimeri*, *L. innocua* and *L. seeligeri* indicates that it is a characteristic lipid component of the *L. monocytogenes* line of descent (Collins *et al.*, 1991). Further studies on the lipid pattern of *Listeria grayi* and *Listeria murrayi*, members of the other line of descent, are necessary to decide whether $LysPtd_2Gro$ can serve as a chemotaxonomic marker for the genus *Listeria*. Noteworthy is the absence of $LysPtd_2Gro$ from enterococci which show the highest degrees of 16S rRNA sequence similarity with members of the genus *Listeria* (Collins *et al.*, 1991).

$LysPtd_2Gro$ is a novel member of the recently discovered lipid class of substituted cardiolipins. Other representatives are α-D-glucopyranosylcardiolipin, isolated from group B streptococci (Fischer, 1977a), and *Vagococcus fluvialis* (Fischer & Arneth-Seifert, 1998), and D-alanylcardiolipin, which is a major membrane lipid of *V. fluvialis* (Fischer & Arneth-Seifert, 1998).

Ptd_2Gro, PtdGro and glyceroglycolipids are common components of the cytoplasmic membrane of Gram-positive bacteria (see review by O'Leary & Wilkinson, 1988). Less widespread are LysPtdGro and D-AlaPtdGro (O'Leary & Wilkinson, 1988). *L. monocytogenes* has been reported to contain an unusual phospholipid which was proposed to be $(PtdGro)_2P$ (Kosaric & Carroll, 1971). PL_U, detected in the present study, had chromatographic properties similar to those of $(PtdGro)_2P$. It was abundant in *L. monocytogenes* and *L. seeligeri*, but it was also detectable as a minor component in *L. welshimeri* and *L. innocua* (Table 1). In all four strains, there were in addition two L-lysylphospholipids ($LysPL_X$), which might be derivatives of PL_U. It is desirable to elucidate the structures of these rare lipids because, along with $LysPtd_2Gro$, they are prospective chemotaxonomic markers for listeriae.

In addition to Glc(α1-3)$acyl_2$Gro and Gal(α1-2)-Glc(α1-3)$acyl_2$Gro, *sn*-Gro-1-*P*-[Gal(α1-2)Glc(α1-3)]-$acyl_2$Gro was identified. *sn*-Glycero-1-phosphoglycolipids are widespread among Gram-positive bacteria and parallel the occurrence of LTA. Structurally, they are the lowest homologue of the respective LTA and are thought to be the first intermediate in LTA biosynthesis (see review by Fischer, 1990). Although listerial LTA contain Gal(α1-2)Ptd-6Glc(α1-3)$acyl_2$Gro as a second lipid anchor (Uchikawa *et al.*, 1986; Ruhland & Fiedler, 1987; Fischer *et al.*, 1990), phosphatidyl derivatives of the glycolipid and Gro*P*-glycolipid were not detectable. Such derivatives, namely Glc(α1-2)Ptd-6Glc(α1-3)$acyl_2$Gro (Fischer *et al.*, 1973b) and *sn*-Gro-1-*P*-6Glc(α1-2)Ptd-6Glc(α1-3)$acyl_2$Gro (Fischer & Landgraf, 1975), have been isolated from enterococci, which, like listeriae, possess a phosphatidylglycolipid-anchored LTA species (Fischer, 1990). In *L. welshimeri*, a D-alanyl derivative of the Gro*P*-glycolipid was also found. Alanyl ester-containing Gro*P*-glycolipids have been detected previously in *Bacillus licheniformis* and *Lactococcus lactis* (Fischer, 1982), which suggests that during LTA synthesis D-alanine is added to the growing chain (Fischer, 1990).

The growth-dependent alterations in the lipid composition of listeriae are remarkable, particularly towards the end of exponential growth. There was a sharp increase in levels of Ptd_2Gro and $LysPtd_2Gro$, accompanied by a moderate decline in the level of LysPtdGro and a dramatic decrease in the level of PtdGro. The increase in the level of Ptd_2Gro and the concomitant decrease in the level of PtdGro may have been caused, at least in part, by the accelerated synthesis of Ptd_2Gro (De Siervo & Salton, 1971; Short & White, 1972):

$$2\text{PtdGro} \rightarrow \text{Ptd}_2\text{Gro} + \text{Gro}.$$

An increase in the content of Ptd_2Gro at the cost of PtdGro has been observed in *Staphylococcus aureus* during the end of exponential growth (Short & White, 1971), growth at high salt concentration (Kanemasa *et al.*, 1972), on autoplast formation (Okabe *et al.*, 1980) and after energy poisoning (Koch *et al.*, 1984).

Concerning the synthesis of $LysPtd_2Gro$, one might suggest that it is formed from Ptd_2Gro rather than from LysPtdGro. The L-lysyl donor in this reaction may be L-Lys-tRNA, as it is in the synthesis of LysPtdGro (Lennarz *et al.*, 1966; Gould & Lennarz, 1967).

The quantitatively most important reaction of PtdGro is the Gro*P* donor function in LTA chain synthesis (Glaser & Lindsay, 1974; Emdur & Chiu, 1974;

Cabacungan & Pieringer, 1981; Taron *et al*., 1983; Koch *et al*., 1984):

(i) PtdGro + glycolipid → Gro*P*-glycolipid + $acyl_2$Gro

(ii) Gro*P*-glycolipid + *n*PtdGro →

$$(GroP)_{n+1}\text{-glycolipid} + n\text{acyl}_2\text{Gro}.$$

The increasing level of Gro*P*-glycolipid at the end of bacterial growth may indicate a hold in LTA chain synthesis.

Little is known about the physiological role of individual lipids in membrane properties. In experiments using bilayer membranes and liposomes, LysPtdGro, compared to PtdGro, led to a decreased permeability for cations and an increased permeability for anions and low M_r non-electrolytes (Hopfer *et al*., 1970; Haest *et al*., 1972). Similar changes in permeability were observed in *Staphylococcus aureus* cells when, in response to decreasing pH in the medium, the content of LysPtdGro in the membrane increased at the cost of PtdGro. It has therefore been thought that the positively charged LysPtdGro could play a role in inhibiting protons entering the cells from acid media (Haest *et al*., 1972). The situation in *Staphylococcus aureus* is, however, relatively simple because the cytoplasmic membrane contains alternating amounts of PtdGro and LysPtdGro and only small amounts of Ptd_2Gro (Haest *et al*., 1972).

It has been shown recently that vagococci possess a different but similarly complex lipid pattern to listeriae containing D-AlaPtd_2Gro as aminoacyl derivative of Ptd_2Gro (Fischer & Arneth-Seifert, 1998). On transition to the stationary growth phase, the content of D-AlaPtd_2Gro increased from 10 to 26 mol%, but in this case the accumulation of the aminoacyl derivative was accompanied by a decline in Ptd_2Gro from 37 to 12 mol%. Simultaneously, again in contrast to listeriae, the proportions of LysPtdGro increased from 10 to 21 mol% and the proportion of PtdGro remained fairly constant at a low level of 4–6 mol% (Fischer & Arneth-Seifert, 1998). Considering the different lipid composition and the different changes during bacterial growth, one is tempted to speculate that listeriae and vagococci have diverse demands on the properties of their membranes and that these requirements are met by the particular lipid composition.

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REFERENCES

Baer, E. & Kates, M. (1950). Migration of esters of glycerophosphoric acid. II. The acid and alkaline hydrolysis of L-α-lecithins. *J Biol Chem* **185**, 615–623.

Brotherus, J., Renkonen, O., Herrmann, J. & Fischer, W. (1974). Novel stereoconfiguration in lysobisphosphatidic acid of cultured BHK-cells. *Chem Phys Lipids* **13**, 178–182.

Brückner, H., Wittner, R. & Godel, H. (1991). Fully automated separation of DL-amino acids derivatized with *O*-phthaldialdehyde together with *N*-isobutyryl-cysteine. Application to food samples. *Chromatographia* **32**, 383–388.

Cabacungan, E. & Pieringer, R. A. (1981). Mode of elongation of the glycerophosphate polymer of membrane LTA of *Streptococcus faecium* (*Streptococcus faecalis* ATCC 9790). *J Bacteriol* **147**, 75–79.

Collins, M. D., Wallbanks, S., Lane, D. J., Shah, J., Nietupski, R., Smida, J., Dorsch, M. & Stackebrandt, E. (1991). Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol* **41**, 240–246.

Deroo, P. W. (1969). MSc thesis, University of Western Ontario.

De Siervo, A. J. & Salton, M. R. J. (1971). Biosynthesis of cardiolipin in the membranes of *Micrococcus lysodeikticus*. *Biochim Biophys Acta* **239**, 280–292.

Emdur, L. I. & Chiu, T. H. (1974). Turnover of phosphatidylglycerol in *Streptococcus sanguis*. *Biochem Biophys Res Commun* **59**, 1137–1144.

Feresu, S. B. & Jones, D. (1988). Taxonomic studies on *Brochothrix*, *Erysipelothrix*, *Listeria* and atypical lactobacilli. *J Gen Microbiol* **134**, 1165–1183.

Fischer, W. (1977a). The polar lipids of group B streptococci. I. Glycosylated diphosphatidylglycerol, a novel glycophospholipid. *Biochim Biophys Acta* **487**, 74–88.

Fischer, W. (1977b). The polar lipids of group B streptococci. II. Composition and positional distribution of fatty acids. *Biochim Biophys Acta* **487**, 89–104.

Fischer, W. (1982). D-Alanine ester-containing glycerophosphoglycolipids in the membrane of Gram-positive bacteria. *Biochim Biophys Acta* **711**, 372–375.

Fischer, W. (1990). Bacterial phosphoglycolipids and LTAs. In *Glycolipids*, *Phosphoglycolipids and Sulfoglycolipids*, pp. 123–234. Edited by M. Kates (vol. 6 of *Handbook of Lipid Research*, edited by D. J. Hanahan). New York & London: Plenum.

Fischer, W. (1997). Pneumococcal lipoteichoic acid and teichoic acid. *Microbial Drug Resistance* **3**, 309–325.

Fischer, W. & Landgraf, H. R. (1975). Glycerophosphoryl phosphatidyl kojibiosyl diacylglycerol, a novel phosphoglucolipid from *Streptococcus faecalis*. *Biochim Biophys Acta* **380**, 227–244.

Fischer, W. & Arneth-Seifert, D. (1998). D-Alanylcardiolipin, a major component of the unique lipid pattern of *Vagococcus fluvialis*. *J Bacteriol* **180**, 2950–2957.

Fischer, W., Ishizuka, I., Landgraf, H. R. & Herrmann, J. (1973a). Glycerophosphoryl diglucosyl diglyceride, a new phosphoglycolipid from streptococci. *Biochim Biophys Acta* **296**, 527–545.

Fischer, W., Landgraf, H. R. & Herrmann, J. (1973b). Phosphatidyldiglucosyl diglyceride from streptococci and its relationship to other polar lipids. *Biochim Biophys Acta* **306**, 353–367.

Fischer, W., Nakano, M., Laine, R. A. & Bohrer, W. (1978). On the relationship between glycerophosphoglycolipids and lipoteichoic acids in Gram-positive bacteria. I. The occurrence of phosphoglycolipids. *Biochim Biophys Acta* **528**, 288–297.

Fischer, W., Koch, H. U. & Haas, R. (1983). Improved preparation of LTAs. *Eur J Biochem* **133**, 523–530.

Fischer, W., Mannsfeld, T. & Hagen, G. (1990). On the basic structure of poly(glycerophosphate) LTAs. *Biochem Cell Biol* **68**, 33–43.

Fischer, W., Peter-Katalinić, J., Hartmann, R. & Egge, H. (1994). (*S*)-2-Amino-1,3-propanediol-3-phosphate-carrying diradylglyceroglycolipids. Novel major membrane lipids of *Clostridium innocuum*. *Eur J Biochem* **223**, 879–892.

Glaser, L. & Lindsay, B. (1974). The synthesis of LTA carrier. *Biochem Biophys Res Commun* **59**, 1131–1136.

Gould, R. M. & Lennarz, W. J. (1967). Biosynthesis of aminoacyl derivatives of phosphatidylglycerol. *Biochem Biophys Res Commun* **26**, 510–515.

Gould, R. M. & Lennarz, W. J. (1970). Metabolism of phosphatidylglycerol and lysylphosphatidylglycerol in *Staphylococcus aureus*. *J Bacteriol* **104**, 1135–1144.

Grassl, M. & Supp, M. (1985). D-Alanine. In *Methods of Enzymatic Analysis*, 3rd edn, vol. 8 (*Metabolites 3: Lipids, Amino Acids and Related Compounds*), pp. 336–340. Edited by H. U. Bergmeyer, J. Bergmeyer & M. Grassl. Weinheim: VCH.

de Haas, G. H., Postema, N. M., Nieuwenhuizen, W. & van Deenen, L. L. M. (1968). Purification and properties of phospholipase A from porcine pancreas. *Biochim Biophys Acta* **159**, 103–117.

Haest, C. W. M., de Gier, J., Op Den Kamp, J. A. F., Bartels, V. & van Deenen, L. L. M. (1972). Changes in permeability of *Staphylococcus aureus* and derived liposomes with varying lipid composition. *Biochim Biophys Acta* **255**, 720–733.

Hether, N. W. & Jackson, L. L. (1983). LTA from *Listeria monocytogenes*. *J Bacteriol* **156**, 809–817.

Hopfer, U., Lehninger, A. L. & Lennarz, W. J. (1970). The effect of the polar moiety of lipids on the ion permeability of bilayer membranes. *J Membrane Biol* **2**, 41–58.

Houtsmüller, U. M. T. & van Deenen, L. L. M. (1964). On the accumulation of amino acid derivatives of phosphatidylglycerol in bacteria. *Biochim Biophys Acta* **84**, 96–98.

Houtsmüller, U. M. T. & van Deenen, L. L. M. (1965). On the amino acid esters of phosphatidyl glycerol from bacteria. *Biochim Biophys Acta* **106**, 564–576.

Joutti, A., Brotherus, J., Renkonen, O., Laine, A. & Fischer, W. (1976). The stereochemical configuration of lysobisphosphatidic acid from rat liver, rabbit lung, and pig lung. *Biochim Biophys Acta* **450**, 206–209.

Kanemasa, Y., Yoshioka, T. & Hayashi, H. (1972). Alteration of the phospholipid composition of *Staphylococcus aureus* cultured in medium containing NaCl. *Biochem Biophys Acta* **280**, 444–450.

Kates, M. (1986). *Techniques of Lipidology*, 2nd edn, pp. 396–399. New York: Elsevier.

Koch, H. U., Haas, R. & Fischer, W. (1984). The role of LTA biosynthesis in membrane lipid metabolism of growing *Staphylococcus aureus*. *Eur J Biochem* **138**, 357–363.

Kosaric, N. & Carroll, K. K. (1971). Phospholipids of *Listeria monocytogenes*. *Biochim Biophys Acta* **239**, 428–442.

Kritchevski, D. & Kirk, M. C. (1952). Detection of steroids in paper chromatography. *Arch Biochem Biophys* **35**, 346.

Landgraf, H. R. (1976). *Fettsäuremuster und positionsspezifische Verteilung der Fettsäuren in den polaren Lipiden von Streptococcen*. PhD thesis, University Erlangen-Nürnberg.

Lang, G. (1984). L-(−)Glycerol 3-phosphate. In *Methods of Enzymatic Analysis*, 3rd edn, vol. 6 (*Metabolites 1: Carbohydrates*), pp. 525–531. Edited by H. U. Bergmeyer, J. Bergmeyer & M. Grassl. Weinheim: VCH.

LeCocq, J. & Ballou, C. E. (1964). On the structure of cardiolipin. *Biochemistry* **3**, 976–980.

Lennarz, W. J., Nesbitt, J. A., III & Reiss, J. (1966). The participation of SRNA in the enzymatic synthesis of *O*-L-lysyl phosphatidylglycerol in *Staphylococcus aureus*. *Proc Natl Acad Sci USA* **55**, 934–941.

Mastronicolis, S. K., German, J. B. & Smith, G. M. (1996). Diversity of the polar lipids of the food-borne pathogen *Listeria monocytogenes*. *Lipids* **31**, 635–640.

Nakano, M. & Fischer, W. (1977). The glycolipids of *Lactobacillus casei* DSM 20021. *Hoppe-Seyler's Z Physiol Chem* **358**, 1439–1453.

Okabe, A., Hirai, Y., Hayashi, H. & Kanemasa, Y. (1980). Alteration in phospholipid composition of *Staphylococcus aureus* during formation of autoplast. *Biochim Biophys Acta* **617**, 28–35.

O'Leary, W. M. & Wilkinson, S. G. (1988). Gram-positive bacteria. In *Microbial Lipids*, vol. 1, pp. 117–201. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press.

Peter-Katalinić, J. & Fischer, W. (1998). α-D-Glucopyranosyl, D-alanyl, and L-lysylcardiolipin from Gram-positive bacteria. Analysis by fast atom bombardment mass spectrometry. *J Lipid Res* **39**, 2286–2292.

Rouser, G., Kritchevsky, D. & Yamamoto, A. (1967). Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids. In *Lipid Chromatographic Analysis*, vol. 1, pp. 99–161. Edited by G. Marinetti. New York: Marcel Dekker.

Ruhland, G. J. & Fiedler, F. (1987). Occurrence and biochemistry of LTAs in the genus *Listeria*. *Syst Appl Microbiol* **9**, 40–46.

Schleifer, K. H., Kraus, J., Dvorak, C., Kilpper-Bälz, R., Collins, M. D. & Fischer, W. (1985). Transfer of *Staphylococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. *Syst Appl Microbiol* **6**, 183–195.

Schnitger, H., Papenberg, K., Ganse, E., Czok, R., Bücher, T. & Adam, H. (1959). Chromatographie phosphathaltiger Metabolite eines menschlichen Leberpunktats. *Biochem Z* **332**, 167–185.

Shaw, N. (1968). The detection of lipids on thin-layer chromatograms with the periodate Schiff reagent. *Biochim Biophys Acta* **164**, 435–438.

Shaw, N. & Stead, A. (1972). Bacterial glycophospholipids. *FEBS Lett* **21**, 249–253.

Short, S. A. & White, D. C. (1971). Metabolism of phosphatidylglycerol, lysylphosphatidylglycerol and cardiolipin of *Staphylococcus aureus*. *J Bacteriol* **108**, 219–226.

Short, S. A. & White, D. C. (1972). Biosynthesis of cardiolipin from phosphatidylglycerol in *Staphylococcus aureus*. *J Bacteriol* **109**, 820–826.

Stahl, E. (1967). *Dünnschicht-Chromatographie*, 2nd edn. Berlin/Heidelberg/New York: Springer.

Taron, D. J., Childs, W. C., III & Neuhaus, F. C. (1983). Biosynthesis of D-alanyl-LTA: role of diglyceride kinase in the synthesis of phosphatidylglycerol for chain elongation. *J Bacteriol* **154**, 1110–1116.

Tocanne, J. F., Verheij, H. M., Op den Kamp, J. A. F. & van Deenen, L. L. M. (1974). Chemical and physicochemical studies of lysylphosphatidylglycerol derivatives. Occurrence of A2′ → 3′ lysyl migration. *Chem Phys Lipids* **13**, 389–403.

Uchikawa, K.-I., Sekikawa, I. & Azuma, I. (1986). Structural studies on LTAs from four *Listeria* strains. *J Bacteriol* **168**, 115–122.