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Physiology of Sporeforming Bacteria Associated with Insects

II. Lipids of Vegetative Cells[1]

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Lipid composition was studied in two strains each of mid-log phase cells of *Bacillus thuringiensis*, *B. larvae*, *B. popilliae*, *B. alvei*, and *B. lentimorbus*. Total lipids varied from 2.5 to 3.5% of the cell dry weight of *B. thuringiensis* to 4.3 to 5.0% of *B. popilliae*. Phospholipids in the organisms examined ranged from 55 to 79% of total lipids; neutral lipids averaged from 13 to 45%. Common phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and lysophosphatidylethanolamine. 1,2-Diglycerides, methyl esters, free fatty acids, and hydrocarbons were found in all the organisms studied. Branched-chain fatty acids constituted more than 50% of the total fatty acids in *B. thuringiensis*, *B. larvae*, *B. popilliae*, and *B. alvei*, whereas, in *B. lentimorbus*, normal-chain acids constituted more than 50%. Anteiso-C_{15} (12-methyltetradeconoate) was the most abundant acid (30 to 50%) in *B. alvei*, *B. larvae*, *B. popilliae*, and *B. lentimorbus*. In contrast, *B. thuringiensis* contained more iso-C_{13} (7%), iso-C_{15} (17%), normal-C_{16} (24%), and iso-C_{17} (18%) than anteiso-C_{15} (6%). The distribution of individual fatty acids was similar in the phospholipids and neutral lipids of each organism. However, the total amount of iso, anteiso, and normal isomers differed.

Membrane synthesis is an important feature of the cellular metabolism of bacteria. Bacterial membranes contain lipid structures that appear to be flexible and vary in composition according to age of cells, cultural conditions, nutritional requirements, and metabolic activity (17). Studies on lipid composition of several aerobic sporeforming bacteria have shown that certain lipid components are common to these organisms (1, 5, 14, 19, 21, 22, 27).

Currently, we are investigating the lipid metabolism of sporeforming bacteria associated with insect diseases. Several of these organisms are beneficially significant because of their potential use as biological insecticides. Our laboratory has shown that large populations of *Bacillus popilliae* and *B. lentimorbus*, causative agents of milky disease in Japanese beetle larvae, can be grown readily in artificial culture and that such cells are both pathogenic and sporogenic when injected into larvae (24). However, *B. popilliae* sporulates only slightly in artificial culture (25) and *B. lentimorbus* not at all. Spore formation is the key to use of these bacteria for biological control of Japanese beetles. Limited information is available on their biochemistry.

In this communication, results are presented of comparative biochemical studies with the following bacteria; *B. larvae*, cause of American foulbrood in honey bees; *B. thuringiensis*, producer of a toxin lethal to *Lepidoptera* larvae; and *B. alvei*, a bacterium sometimes affiliated with European foulbrood in honey bees. *B. thuringiensis* and *B. alvei* grow and sporulate under cultural conditions conducive only to vegetative growth of *B. popilliae*, *B. lentimorbus*, and *B. larvae*. The latter three organisms require complex media and remain viable for only a short time (6, 26, 30). Previously (4), we compared the primary routes of carbohydrate catabolism in vegetative cells and found that the extent of participation of concurrent pathways differed in all these organisms. Also, *B. lentimorbus* and *B. popilliae* did not exhibit an operational tricarboxylic acid cycle. The purpose of the present study was to investigate whether the differences in metabolism, nutritional requirements, and capacity of these five organisms to sporulate were reflected in the lipid composition during

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vegetative growth. To date, there has been no report on their phospholipid and neutral lipid composition. We describe here the phospholipid and neutral lipid compositions as well as the pattern and relative abundance of fatty acids in each of the lipid fractions.

MATERIALS AND METHODS

Organisms and cultural conditions. The organisms studied were obtained from the ARS Culture Collection: *B. thuringiensis* NRRL NRS-996, NRRL B-2172; *B. larvae* NRRL B-2605, NRRL B-3555; *B. popilliae* NRRL B-2309, NRRL B-2309M; *B. alvei* NRRL B-384, NRRL B-385; *B. lentimorbus* NRRL B-2522, NRRL B-2530.

Cultures of all organisms were grown in liquid medium (designated MD) composed of 1.5% yeast extract, 0.6% K_2HPO_4, and 0.2% glucose. A 10% inoculum of cells previously transferred three times was used to obtain active growth. Flask cultures were aerated by rotary agitation of 250 rev/min at 28 C. Cultures were examined at 2-hr intervals during a 72-hr incubation period. Appropriate cell dilutions of the culture at each sample time were spread on MD agar plates and incubated at 28 C for 3 to 7 days. The number of viable cells was calculated from the resultant colony count.

Cells for lipid extraction were harvested by centrifugation at mid-log phase of growth, washed three times in 0.85% NaCl, lyophilized to constant weight, and stored in N_2 atmosphere at −18 C.

Extraction of lipids. Duplicate samples of lyophilized cells (500 mg) of each organism were transferred to 125-ml glass-stoppered flasks and suspended in 40 ml of chloroform-methanol (2:1, v/v). Flasks were flushed with N_2 and allowed to stand for 12 hr at 23 C in the dark. After solvent-cell suspensions were filtered through methanol-extracted filter paper (Whatman no. 1), the residue was washed two times with 5 ml of extraction solvent. Combined extracts and washings were dried under N_2 at 23 C and re-extracted with 10 ml of chloroform-methanol (2:1, v/v). Nonlipid residue was removed by filtration in a Swinny syringe (Gelman Instrument Co., Ann Arbor, Mich.) fitted with a methanol-extracted, 13-mm filter disc (no. 740-E, Schleicher & Schuell Co., Keene, N.H.). The residue was washed twice with 2 ml of chloroform-methanol and then discarded. Extracts were washed by the method of Folch et al. (7), dried in vacuo, and weighed. Per cent of total lipids as cell dry weight was determined.

Column chromatography. Partially purified, extractable lipids were separated into neutral lipids and phospholipids by micro silicic acid chromatography (11). Two grams of silicic acid (Bio Sil HA, −325 mesh, BioRad Laboratories, Richmond, Calif.) was placed in a column (5 by 200 mm) fitted with a sintered glass filter; height of the column bed was 170 mm. The packed column was washed with 50 ml of anhydrous diethyl ether overnight. Lipid samples (5 to 20 mg), dissolved in 0.1 ml of hexane-ether (1:1, v/v), were quantitatively transferred to the column. Neutral lipids were eluted first with ether (7 ml). phospholipids were then eluted with methanol (7 ml); Both lipid fractions were evaporated under N_2 and dried in vacuo, and their dry weights were determined.

Thin-layer chromatography. Phospholipids were separated on thin layers (0.25 mm) of activated Silica Gel G on glass plates (20 by 20 cm). The thin-layer chromatography plates were developed in the following solvent systems (v/v): (i) chloroform-methanol-water (90:20:2); (ii) chloroform-methanol-water-ammonium hydroxide (90:20:2:1); (iii) chloroform-methanol-acetic acid-water (100:50:14:6). The following compounds (all from Supelco, Inc., Bellefonte, Pa.) were used as standards: phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), lysophosphatidylcholine, and phosphatidylinositol. Phosphatidylcholine was kindly supplied by T. Kaneshiro of this Laboratory.

Neutral lipids were separated on long (20 by 34 cm), activated Silica Gel G thin-layer plates (8) in a one-dimensional, double-development solvent system (v/v): (iv) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2), and (v) diethyl ether-hexane (6:94). The following standards (The Hormel Institute, Austin, Minn.) were used: monopalmitin, cholesterol oleate, methyl oleate, triolein, cholesterol, and oleic acid. Both 1,2- and 1,3-dipalmitin were purchased from Anspec Co., Ann Arbor, Mich.

Lipids were detected on thin-layer chromatography plates by exposing to iodine vapor, by charring after spraying with 30% H_2SO_4 and by examining the plates under ultraviolet irradiation after spraying with 2,7-dichlorofluorescein (Applied Science Laboratories, State College, Pa.). Phospholipids were detected by the reagent of Vaskovsky and Kostetsky (32). Periodate-Schiff reagents (29) were used to detect vicinal hydroxyl groups; ninhydrin (0.5% in water-saturated acetone) was used to detect amino groups; and a modified Dragendorff solution (2) was used to detect choline.

Acid hydrolysis. Phospholipids were hydrolyzed by refluxing in 6 N HCl for 24 hr. The samples were dried under N_2, dissolved in water, and chromatographed on Whatman no. 1 filter paper in *n*-butyl alcohol–pyridine–water (6:4:3, v/v). Alkaline silver nitrate reagent (31) was used to detect sugars. Amino acids were determined on an automatic amino acid analyzer.

Methyl ester preparation. Total extractable lipids were hydrolyzed and methylated with boron trifluoride-methanol reagent (20). The methanolysis method of C. Litchfield, Texas A&M University, College Station (*personal communication*), was followed to prepare methyl esters of neutral lipids and phospholipids. One milliliter of 0.5 N methanolic KOH was added to the lipids (5 to 15 mg) dissolved in 0.5 ml of anhydrous ether. This mixture was allowed to stand at 23 C for 20 min and then was neutralized with 1 N HCl. Methyl esters were extracted with three 1-ml portions of hexane. Combined hexane extracts were dried under N_2, and the residues were dissolved in 0.1 ml of hexane for gas-liquid chromatography. Water-soluble phosphate esters were irrigated on Whatman

no. 1 filter paper with 90% phenol in the first direction and with *n*-butyl alcohol–propionic acid–water (142:71:100, v/v) in the second direction.

Bromine addition. Bromine derivatives of the total monounsaturated fatty methyl esters were prepared by the method of Brian and Gardner (3). Samples were dissolved in 2 ml of anhydrous diethyl ether and cooled in an ice bath. To each sample was added 1 ml of cold bromine reagent (1 ml of bromine in 5 ml of ether). Excess bromine was removed by repeated evaporation from 1-ml portions of hexane at 40 to 45 C under nitrogen. Bromine derivatives were dissolved in 0.1 ml of hexane for gas-liquid chromatography.

Gas-liquid chromatography. Fatty acid methyl esters were separated and determined on polar and nonpolar columns with an F&M model 810 dual column chromatograph equipped with hydrogen flame detectors. Operating parameters for both polar and nonpolar columns were: injection and detector ports, 235 C; carrier gas, helium; sample size, 1 or 2 μliters; range and attenuation, $10^2 \times 2$.

Dual stainless steel columns [20 ft (6 m) by ⅛ inch (0.32 cm)] packed with ethylene glycol adipate (7%) on high-performance Chromosorb G (AW-DMCS), 80 to 100 mesh (Hewlett-Packard, Skokie, Ill.), were operated isothermally at 200 C. Carrier gas flow rate was adjusted to 12 to 15 ml/min.

Dual nonpolar columns [6 ft (1.8 m) by ⅛ inch (0.32 cm)] packed with SE-30 (2.5%) on Chromosorb G (AW-DMCS), 80 to 100 mesh, were used to chromatograph bromine derivatives of the monounsaturated fatty acid methyl esters. Carrier gas flow rate was 25 ml/min, and the temperature was programmed from 150 to 200 C at 4 C/min.

Fatty acids of *Bacillus* lipids were tentatively identified by comparing their retention times to those of authentic standards. When no authentic standard was available (iso-C_{12}, iso-C_{13}, anteiso-C_{13}, iso-C_{17}), identification was made by comparing the linear relationship of the log retention time to the number of carbon atoms within a homologous series of long-chain fatty acids (9).

Standard fatty acids. Standards of branched-chain fatty acid methyl esters were obtained from Anspec Co. Two admixtures (BC-L and BC-1) contained the following acids: i (iso)-C_{14}, n (normal)-C_{14}, a (anteiso)-C_{15}, n-C_{15}, i-C_{16}, n-C_{16}, a-C_{17}, i-C_{18}, n-C_{18}, a-C_{19}, i-C_{20}, n-C_{20}, and a-C_{21}. An admixture of normal fatty acids (n-C_8, n-C_{10}, n-C_{12}, n-C_{14}, n-C_{16}, n-C_{18}, n-$C_{18:1}$, n-$C_{18:2}$) and n-$C_{16:1}$ came from The Hormel Institute.

RESULTS

Total extractable lipids. The amount of lipids extracted from mid-log phase cells of each organism is summarized in Table 1. On a dry-weight basis, the cellular lipids ranged from 2.5% in *B. thuringiensis* B-2172 to 5.0% in *B. popilliae* B-2309M. Phospholipids predominated in all organisms examined and ranged from 55% of total lipids in *B. alvei* B-384 to 79% in *B. larvae* B-3555. Neutral lipids averaged 13% of total lipids in *B. larvae* B-2605 to 45% in *B. alvei* B-384.

Phospholipids. A thin-layer chromatogram tracing of the phospholipids in one strain of each organism is presented in Fig. 1. Similar chromatograms were obtained from strains of both species. Table 2 summarizes the identification of the components. Compound **a** was identified as DPG because it had the same mobility as standard DPG, reacted with I_2 vapors and molybdate stain, and yielded diglycerylphosphorylglycerol on alkaline hydrolysis. Compound **c** was identified as PG because it reacted with I_2 vapors, molybdate stain, and periodate-Schiff reagents. It exhibed an R_F value similar to standard PG and, upon alkaline hydrolysis, yielded glycerolphosphorylglycerol. Compounds **d** and **g** are PE and LPE, respectively. Both components exhibited R_F values similar to authentic standards in solvent systems i, ii, and iii and yielded glycerolphosphorylethanolamine on alkaline hydrolysis. They reacted with I_2 vapors, molybdate stain, and were ninhydrin positive. PE, present in all the organisms, could be detected in *B. larvae*, *B. alvei*, and *B. lentimorbus* only after threefold concentration of the phospholipid extracts.

Compound **e** reacted with alkaline $AgNO_3$ and may be a glycolipid. However, glycerol and a phosphate ester, which had an R_F value similar to α-glycerol phosphate, were the only products detected after acid hydrolysis. Components **b**, **f**, **i**, and **j** also are unidentified. They could be detected only upon exposure to I_2 vapors or upon charring after spraying with 30% H_2SO_4. Their acid or alkaline hydrolysis products were not

TABLE 1. *Extractable lipids of Bacillus species*

Organism	NRRL strain[a]	Percentage of total lipids (cell dry wt)	Percentage of total lipids[b] found as	
			NL	PL
B. thuringiensis...	NRS-996	3.5	42	56
	B-2172	2.5	40	56
B. larvae.....	B-2605	3.8	15	74
	B-3555	3.9	13	79
B. popilliae.....	B-2309	4.3	15	70
	B-2309M	5.0	23	68
B. alvei.....	B-384	3.2	45	55
	B-385	4.0	25	73
B. lentimorbus....	B-2522	4.0	24	75
	B-2530	4.6	25	68

[a] All strains are from the ARS Culture Collection, Peoria, Ill. Organisms were grown in MD medium (see Materials and Methods) and harvested at mid-log phase.

[b] NL, neutral lipid; PL, phospholipid.

present in sufficient quantity to identify. Compound **h**, also unidentified, reacted with molybdate stain and was ninhydrin positive. Its R_F value in solvent iii was close to that of standard PS. However, its R_F values in solvents i and ii were different from PS. The quantity of acid and alkaline hydrolysis products was insufficient to analyze.

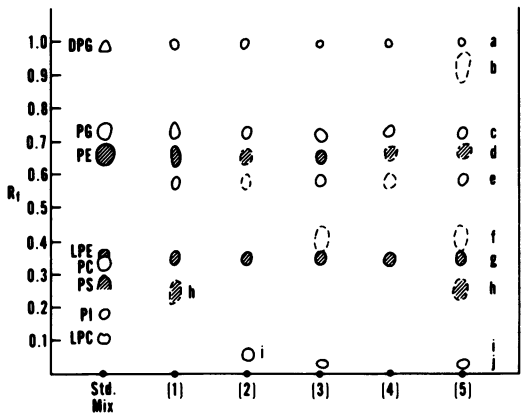

FIG. 1. *Thin-layer chromatogram tracing of the phospholipids of B. thuringiensis (1), B. larvae (2), B. popilliae (3), B. alvei (4), and B. lentimorbus (5). Plates were developed in solvent iii. Components were detected with I_2 vapors. Shaded spots are ninhydrin positive. Dotted lines represent components present in trace amounts. Lettered components: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine, PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine.*

TABLE 2. *Characterization and tentative identification of phospholipids*

Spot	Staining reaction						Mild alkaline hydrolysate[a]	Identification
	I_2	Ninhydrin	Periodate-Schiff	Molybdate	Dragendorff	Alkaline $AgNO_3$		
a	+	−	−	+	−	−	GPGPG	DPG
b	+	−	−	−	−	−		?
c	+	−	+	+	−	−	GPG	PG
d	+	+	−	+	−	−	GPE	PE
e	+	−	−	−	−	+		?
f	+	−	−	−	−	−		?
g	+	+	−	+	−	−	GPE	LPE
h	+	+	−	+	−	−	?	?
i	+	−	−	−	−	−		?
j	+	−	−	−	−	−		?

[a] GPGPG, diglycerylphosphorylglycerol; DPG, diphosphatidylglycerol; GPG, glycerolphosphorylglycerol; PG, phosphatidylglycerol; GPE, glycerolphosphorylethanolamine; PE, phosphatidylethanolamine; LPE, lyso PE.

Analyses of the phospholipid acid hydrolysates on the amino acid analyzer revealed minor amounts of a number of amino acids in each organism studied. The predominant amino acids were lysine, arginine, aspartic acid, serine, glutamic acid, alanine, valine, isoleucine, and leucine.

Neutral lipids. Figure 2 is a thin-layer chromatogram tracing of neutral lipids in one strain of the five organisms. Both strains of each organism produced similar chromatograms. 1,2-Diglycerides, hydrocarbons, methyl esters, and free fatty acids appeared to be common to all the organisms. No monoglycerides were detected. Components which migrated with authentic triglyceride and 1,3-diglyceride standards were observed in extracts of *B. thuringiensis*, *B. lentimorbus*, and *B. alvei*. However, the identity of these components cannot be based on R_F values alone and, therefore, will have to be investigated further.

Fatty acid composition of total lipids. All strains examined contained saturated fatty acids of 12 to 18 carbons (Table 3). Only minor quantities of unsaturated acids were detected. Chromatographic patterns were similar for both strains

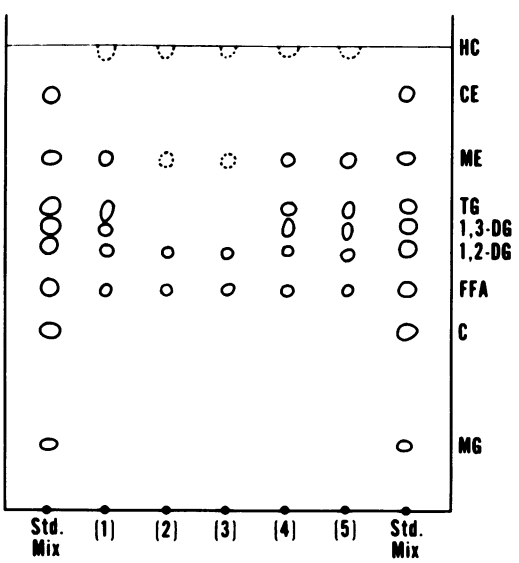

FIG. 2. *Thin-layer chromatogram tracing of the neutral lipids of B. thuringiensis (1), B. larvae (2), B. popilliae (3), B. alvei (4), and B. lentimorbus (5). Plates were developed in a one-dimensional, double-development system with solvents iv and v. Components were detected with I_2 vapors and by charring after spraying with 30% H_2SO_4. Dotted lines represent components present in trace amounts. Lettered components: HC, hydrocarbon; CE, cholesterol ester; ME, methyl ester; TG, triglyceride; 1,3- and 1,2-DG, 1,3- and 1,2-diglyceride; FFA, free fatty acid; C, cholesterol; MG, monoglyceride.*

TABLE 3. *Per cent relative abundance of fatty acids in total lipids of five Bacillus species*

Organism	Strain	Fatty acid																
		i-12[a]	n-12	i-13	a-13	n-13	i-14	n-14	i-15	a-15	n-15	i-16	n-16	n-16:1	i-17	a-17	n-17	n-18	n-18:1
B. thuringiensis	NRS-996	0.4	0.4	6.8	1.7		4.6	5.1	19.0	5.9	0.4	7.6	22.4		19.0	2.1	0.4	3.4	0.8
	B-2172	0.8	0.8	7.4	2.1		3.3	3.4	14.2	5.1	0.4	5.9	25.2		16.7	4.2	1.1	7.6	1.7
B. larvae	B-3555		Tr[b]		Tr		Tr	2.7	4.1	46.3	1.4	2.4	33.0		Tr	7.1	Tr	3.0	Tr
	B-2605		Tr				Tr	2.7	5.5	31.1	1.2	5.8	31.1		Tr	10.9	Tr	11.7	Tr
B. popilliae	B-2309		0.7		0.2		1.6	5.2	1.8	40.1	1.4	6.8	35.8	Tr	2.3	1.8		2.5	
	B-2309M		0.4	0.2	1.0		0.5	3.6	2.9	44.7	1.6	2.4	38.9	Tr	3.0	4.3	0.1	2.2	0.8
B. alvei	B-384		Tr	Tr	Tr		0.6	3.4	5.7	36.1	2.1	5.7	26.4		3.1	4.9	Tr	5.7	4.1
	B-385		Tr		Tr		Tr	1.0	4.8	49.3	Tr	1.0	23.5		6.0	8.5	Tr	2.0	
B. lentimorbus	B-2522		2.6	0.2	2.6		0.8	20.6	3.6	30.4	0.8	0.8	26.2	0.8	1.5	1.0	1.0	4.6	2.3
	B-2530		1.6	Tr	1.5	Tr	0.8	15.5	2.2	37.5	0.7	0.9	26.6	1.7	1.3	2.1	0.2	4.2	2.8

[a] i, iso; n, normal; a, anteiso.
[b] Tr, trace.

of each organism; however, some quantitative differences occurred. Branched-chain acids accounted for more than 50% of the total in *B. thuringiensis*, *B. larvae*, *B. popilliae*, and *B. alvei*, whereas, in *B. lentimorbus*, normal-chain acids constituted more than 50% of the total.

Anteiso-C_{15} was the predominant fatty acid (30.4 to 49.3% of the total) in *B. larvae*, *B. popilliae*, *B. alvei*, and *B. lentimorbus;* n-C_{16} was the second most abundant (23.5 to 35.8%). In addition, *B. lentimorbus* contained 15.5 to 20.6% n-C_{14}. In contrast, *B. thuringiensis* contained only small amounts of a-C_{15} (5.1 to 5.9%) with greater quantities of i-C_{13} (6.8 to 7.4%), i-C_{15} (14.2 to 19.0%), i-C_{16} (5.9 to 7.6%), n-C_{16} (22.4 to 25.2%), and i-C_{17} (16.7 to 19.0%) than a-C_{15}. *B. alvei* contained an unidentified compound which accounted for 2.3 to 4% of the total fatty acids. This unknown had a retention time intermediate to n-C_{16} and i-C_{17} on the adipate column. Co-chromatography with standard n-$C_{16:1}$ indicated that it was not palmitoleic acid; the unknown was not removed by bromine addition. Therefore, we believe that it is neither an unsaturated acid nor a cyclopropane acid. In addition to the acids listed in Table 3, most organisms contained a compound which had a retention time on the SE-30 column corresponding to that of i-C_{20}. This compound was not detected with the polar adipate column.

Fatty acid composition of phospholipids and neutral lipids. The qualitative distribution of individual fatty acids was similar in the phospholipids and neutral lipids of each organism. However, the total amount of i-, a-, and n-isomers differed (Table 4). Iso homologues predominated in the phospholipids and neutral lipids of *B. thuringiensis*, and anteiso homologues predominated in *B. larvae*, *B. popilliae*, and *B. alvei*. Normal homologues were most abundant in the phospholipids and neutral lipids of *B. lentimorbus*.

DISCUSSION

The foregoing data reveal that *B. thuringiensis*, *B. alvei*, *B. larvae*, *B. lentimorbus*, and *B. popilliae* have certain phospholipids in common. These include DPG, PG, PE, and LPE. Differences in the quantity of each component were apparent. Although several other *Bacillus* species have been reported to contain some of these same compounds (1, 5, 19, 21, 22), it is not yet established whether their presence is characteristic of all aerobic sporeforming bacteria. Kates et al. (18) reported phosphatidylcholine and lysophophatidylcholine in *Bacillus cereus*, but we were unable to detect either compound. Lang and Lundgren (19) found a glycolipid component in *B. cereus* that contained glucose and an unidentified sugar. Interestingly, the unidentified compound we found (component **e**, Fig. 1) that reacted with alkaline $AgNO_3$ did not contain sugar. The only products detected after acid hydrolysis were glycerol and α-glycerophosphate, indicating that α-glycerophosphate is bound to glycerol within the cell. Also, the presence of α-glycerophosphate and the complete absence of monoglycerides in all the organisms studied may indicate that biosynthesis of their phospholipids and neutral lipids occurs via formation of phosphatidic acid from α-glycerophosphate and fatty acid coenzyme A esters. The presence of amino acids in the phospholipid acid hydrolysates of all the organisms is as yet not understood. Possibly, they occur as part of nonlipid contaminating material or bound as aminoacylphosphatidyl glycerides (22). PG amino acid esters have been demonstrated in *B. megaterium* (23) and *B. cereus* (12, 19). Whether the unidentified ninhydrin-positive

TABLE 4. *Distribution[a] of iso, anteiso, and normal isomers of phospholipid (PL) and neutral lipid (NL) fatty acids*

Organism	NRRL strain	PL			NL		
		Iso	Anteiso	Normal	Iso	Anteiso	Normal
B. thuringiensis	NRS-996	59	18	22	59	16	25
	B-2172	63	13	23	60	14	25
B. larvae	B-2605	22	51	27	21	52	26
	B-3555	14	54	32	11	62	25
B. popilliae	B-2309	23	35	42	14	56	31
	B-2309M	14	53	34	9	60	31
B. alvei	B-384	18	54	24	15	51	31
	B-385	15	55	25	16	49	29
B. lentimorbus	B-2522	9	38	53	8	37	53
	B-2530	9	35	56	10	39	50

[a] Relative percentage of total fatty acids.

compound (component h, Fig. 1) of *B. thuringiensis* and *B. lentimorbus* is esterified with an amino acid is yet to be determined.

1,2-Diglycerides were the common neutral lipids in all the organisms. Components were detected in *B. thuringiensis*, *B. alvei*, and *B. lentimorbus* that exhibited mobility on thin-layer chromatography plates similar to authentic triglyceride and 1,3-diglyceride. Work is now in progress to further characterize these compounds. To our knowledge, no study has been reported that demonstrates the existence of triglycerides in bacilli.

Differences in relative amounts of phospholipids and neutral lipids in vegetative cells of each organism may suggest variability in the metabolic activities that are precursory to early stages of sporulation. The percentage of neutral lipids in *B. thuringiensis* and *B. alvei* was higher than in *B. larvae*, *B. lentimorbus*, and *B. popilliae*. As mentioned earlier, the latter three organisms are highly fastidious and do not sporulate appreciably in artificial culture. Possibly the higher quantity of neutral lipids in *B. thuringiensis* and *B. alvei* indicates a bias for the synthesis of neutral lipids in these organisms. Whether increased neutral lipid synthesis during vegetative growth reflects a greater capacity for these two organisms to sporulate poses an interesting question. Scandella and Kornberg (28) analyzed *B. megaterium* vegetative cells and spores, and found that the neutral lipids, relative to phospholipids, were four times more abundant in spores than in vegetative cells. The significance of neutral lipids for the structure and function of vegetative cells and spores is not established.

In examining the total fatty acids of several bacilli, Kaneda (15) reported much greater quantities of unsaturated acids in *B. thuringiensis* than we found. We detected greater quantities of normal-chain acids; also, n-C_{18} acids, not reported by Kaneda (14–16), were present in all the organisms. Furthermore, he found branched-chain acids to predominate in *B. lentimorbus* (16); we found normal-chain acids to be more abundant. Cells of *B. larvae*, *B. lentimorbus*, and *B. popilliae* studied by Kaneda were from 71- to 222-hr cultures; cells of *B. thuringiensis* and *B. alvei* were from 16-hr cultures. In our work, all organisms were harvested for lipid extraction at mid-log phase (6 to 10 hr).

The significance of the quantitative distribu- of i-, a-, and n-fatty acids is not clearly understood, although this relationship has been interpreted as reflecting the abundance of available precursors for synthesis of the appropriate branched-chain acid (13). In *B. alvei*, *B. larvae*, *B. lentimorbus*, and *B. popilliae*, a-fatty acids predominated in both phospholipids and neutral lipids. In contrast, *B. thuringiensis* contained much greater quantities of i-fatty acids. Because iso acids were more prevalent in both the phospholipids and neutral lipids of *B. thuringiensis* than in the other bacilli examined, we believe that there must be a correspondingly greater capacity for biosynthesis of iso acids in *B. thuringiensis* and that synthesis of a specific branched-chain acid does not depend entirely on the availability of precursors. The qualitative distribution of fatty acids in the phospholipids and neutral lipids of each organism studied resembled that of its total lipids. Apparently there was no selective incorporation of a particular fatty acid into either of the lipid fractions.

The relationship of lipid synthesis to central metabolic pathways in these organisms is not clear. Apparently lipid synthesis in *B. lentimorbus* and *B. popilliae* does not depend upon tricarboxylic acid cycle activity, because these organisms lack an operational tricarboxylic acid cycle (4). Reduced nicotinamide adenine dinucleotide phosphate necessary for synthesis of fatty acids is supplied by the pentose phosphate pathway.

Before the overall significance of phospholipids and neutral lipids to the sporulation process can be fully realized, it will be necessary to determine their relative abundance and distribution in sporulating and nonsporulating cells throughout the sporulation and culture cycles. Further characterization of the cellular lipids, together with enzyme activities related to their synthesis and breakdown, could provide insight into the organization of plasma and spore membranes.

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