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Characteristic Lipids of *Bordetella pertussis*: Simple Fatty Acid Composition, Hydroxy Fatty Acids, and an Ornithine-Containing Lipid

YOHKO KAWAI[1]* AND ATSUKO MORIBAYASHI[2]

First Department of Bacteriology[1] and Department of Technology,[2] National Institute of Health, Shinagawa-ku, Tokyo 141, Japan

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The lipids and fatty acids of *Bordetella pertussis* (phases I to IV) were analyzed by thin-layer chromatography, gas-liquid chromatography, and mass spectrometry and compared with those of *B. parapertussis* and *B. bronchiseptica*. The major lipid components of the three species were phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, lysophosphatidylethanolamine, and an ornithine-containing lipid. The ornithine-containing lipid was characteristic of the genus *Bordetella*. The fatty acid composition of the total extractable cellular lipids of *B. pertussis* was mostly hexadecanoic and hexadecenoic acids (90%) in a ratio of about 1:1. The hexadecenoic acid of *B. pertussis* was in the *cis*-9 form. The fatty acid composition of the residual bound lipids was distinctly different from that of the extractable lipids, the residual bound lipids being mainly 3-hydroxytetradecanoic, tetradecanoic, and 3-hydroxydecanoic acids, with 3-hydroxydodecanoic acid occurring in some strains. It was determined that the 3-hydroxy fatty acids were derived from lipid A. The fatty acid composition of the total extractable cellular lipids of *B. parapertussis* and *B. bronchiseptica*, mainly composed of hexadecanoić and heptadecacyclopropanoic acids, differed from that of *B. pertussis*. Although the fatty acid composition of the residual bound lipids of *B. parapertussis* was similar to that of the residual bound lipids of *B. pertussis*, 2-hydroxydodecanoic acid was detected only in the bound lipids of *B. bronchiseptica*.

In a previous study (16), American Type Culture Collection strains ATCC 190 and ATCC 6627, originally accepted as *Bordetella pertussis*, were identified as a new species of *Pseudomonas* (*P. pertucinogena*) on the basis of morphology, biochemical and physiological characteristics, and DNA homology. This identification was further supported by our later study (15) of the lipid composition of the strains, and some preliminary observations on the lipid composition of *B. pertussis* were also made for comparison in that report. However, reports of systematic studies of the lipids of *B. pertussis* have been scarce to date. Only Thiele and Schwinn (29) have reported on the total extractable cellular lipids and the fatty acids of one *B. pertussis* strain.

There are four known antigenic types of *B. pertussis* which are related by surface properties, virulence, and toxigenicity. Furthermore, there are three known species of *Bordetella*, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. This situation prompted us to undertake a more extended study of the lipids in the three *Bordetella* species and in the four antigenic types of *B. pertussis*.

MATERIALS AND METHODS

Organisms. The sources of the eight *B. pertussis* strains used in this study, representing the four antigenic types of this species, have been described elsewhere (14, 16). The *B. parapertussis* and *B. bronchiseptica* strains were provided by the Kitasato Institute, Tokyo. The organisms, cultured on slants of Bordet-Gengou medium, were transferred to the liquid medium described by Sutherland and Wilkinson (26), as slightly modified by Morse and Bray (21), or to the solid medium described by Cohen and Wheeler (2). In the liquid medium, 0.15% soluble starch was used instead of Dowex resin, and *B. pertussis* was incubated at 36°C for 72 h in Roux bottles placed on their sides. *B. pertussis* and *B. parapertussis* were cultured at 36°C for 46 h and *B. bronchiseptica* was cultured at 36°C for 24 h on the solid medium in eggplant-type bottles until the organisms entered into the stationary phase of growth. In general, growth phases are not clear on solid medium. The liquid medium and the solid medium described here are substantially the same, but in the liquid medium, nicotinamide replaced the yeast extract used in the solid medium to deter-

mine whether the lipid of the yeast extract contaminated the bacterial lipid. In liquid medium, thin-layer culture was required.

Organisms cultured until the late-log phase were collected from the liquid medium by centrifugation and were washed four times with 0.9% sodium chloride. A 1-liter culture of *B. pertussis* ATCC 8467 yielded 0.280 g (dry cell weight). The organisms harvested from the solid medium were suspended in 0.9% sodium chloride, filtered through six layers of gauze, and washed three times with 0.9% sodium chloride. A 1-liter culture slant of *B. pertussis* ATCC 8467 yielded 0.466 g (dry cell weight). Similar cell yields were obtained from the other *B. pertussis* strains. A 1-liter culture slant each of *B. parapertussis* and *B. bronchiseptica* yielded, respectively, 0.77 and 1.00 g (dry cell weight).

Preparation of lipids and fatty acids. The methods for preparing total neutral solvent-extractable cellular lipids (total extractable cellular lipids), residual bound lipids, and fatty acid methyl esters have been described in detail previously (15). The separation of fatty acids as methyl esters was carried out by methanolysis with 5% hydrochloric acid–methanol at 105°C for 3 h (extractable lipid) or 5 h (bound lipid). The quantity of fatty acid methyl esters was determined by weight and by quantitative analysis gas-liquid chromatography (GLC) results.

To obtain the ornithine-containing lipid, the extractable lipid was developed as a band on a Merck Silica Gel F_{254} (Merck & Co., Inc.) thin-layer chromatography (TLC) plate with the solvent system chloroform-methanol-water (65:25:4 [vol/vol]), and the resulting lipid bands were visualized with iodine vapor and ninhydrin reagent at both margins of the TLC plate. The ornithine-containing lipid thus confirmed was extracted with chloroform-methanol (2:1 [vol/vol]) in an ultrasonic vibrator. The ornithine-containing lipid was then treated with phospholipase A from snake venom to remove the phosphatidylethanolamine located just above the lipid, and TLC was repeated. The purified ornithine-containing lipid appeared as only one spot on the TLC plate with either the chloroform-methanol-water (65:25:4 [vol/vol]) or the chloroform-methanol-acetic acid (65:25:10 [vol/vol]) solvent system. The purified ornithine-containing lipid was hydrolyzed for amino acid analysis with 6 N hydrochloric acid at 110°C for 24 h.

Lipid A from *B. pertussis* was prepared from the residual extractable lipid-free cells by the method of Kamio et al. (10). The residue containing lipopolysaccharide was partly hydrolyzed with 5% trichloroacetic acid at 90°C for 15 min, and the resulting precipitate was refluxed at 80°C for 1 h with chloroform-methanol (1:3 [vol/vol]) to extract lipid A. This crude lipid A was purified by TLC with the solvent system chloroform-methanol-acetic acid-water (25:15:1:2 [vol/vol]) on prewashed Merck Silica Gel F_{254} TLC plates. After the color was developed with iodine vapor or by charring with 50% sulfuric acid, the main component band (R_f, 0.90) was scraped off and extracted with chloroform-methanol (2:1 [vol/vol]). Fatty acid methyl esters of lipid A were prepared as described for the bound lipid and analyzed by GLC. About 16 mg of fatty acid methyl esters of lipid A were obtained from 1 g (dry cell weight) of *B. pertussis* ATCC 8467 (phase I) and *B. pertussis* Sakurayashiki (phase III).

Hexadecenoic acid, one of the major fatty acids in the total extractable cellular lipids of *B. pertussis*, was examined to determine the position of the unsaturated bond. The fatty acid methyl ester mixture, obtained by methanolysis of the extractable lipids, was separated by TLC with the solvent system hexane-ether (95:5 [vol/vol]) on a silver nitrate-coated Merck Silica Gel F_{254} TLC plate prewashed with the same solvent. The TLC plate was visualized at the margins by charring with 50% sulfuric acid, and the hexadecenoic acid band detected was extracted with ether. The hexadecenoic acid was subjected to the oxidative degradation reaction by the method of Scheuerbrandt and Bloch (25). The reaction products were methylated with diazomethane and analyzed by GLC at 175°C for dicarboxylic acids and at 82°C for monocarboxylic acids. The geometrical configuration of the double bond in hexadecenoic acid was determined by infrared (IR) absorption spectrometry.

Analysis of lipids. To separate the lipid components of the total extractable cellular lipids, two-dimensional TLC was carried out on Merck Silica Gel F_{254} TLC plates, first with the solvent system chloroform-methanol-water (65:25:4 [vol/vol]) in the vertical direction and second with the solvent system chloroform-methanol-acetic acid (65:25:10 [vol/vol]) in the horizontal direction.

GLC was carried out by using a Shimazu GC-7A apparatus with a hydrogen flame ionization detector. The glass-coiled column (2 m long) was packed with 25% diethylene glycol succinate or 1.5% SE-30 (Applied Science Laboratories, Inc.) on Chromosorb W. The column temperature was 160 or 180°C. Combined GLC and mass spectrometry (MS) were carried out at 170°C on a Hitachi RMU-6MG apparatus with a glass-coiled column (0.5 m long) coated with 1% OV-1 (Applied Science Laboratories, Inc.) on Chromosorb W. The ionization energy was 20 eV, and the molecular separator and ion source temperature was 200°C. The composition of saturated and monoenoic fatty acids was compared with that of quantitative fatty acid methyl ester standard mixtures (Applied Science Laboratories) of the National Institute of Health type (9). 3-Hydroxytetradecanoic and 2-hydroxyoctadecanoic acids were analyzed quantitatively. Other hydroxy fatty acids were identified by extrapolation from the chromatographs, with reference to the relationship between the number of carbon atoms of the saturated fatty acids and the column yields of the fatty acids by GLC.

Individual phospholipids were identified by TLC and by mild alkaline hydrolysis (3) as described previously (15). For quantitative analysis of lipid phosphorus on the TLC plate, each spot was located with iodine vapor, scraped off, and analyzed by the method of Bartlett (1). To determine the distribution of ester and amide linkages, bound lipid was subjected to mild alkaline hydrolysis in 0.3 N potassium hydroxide–methanol at 37°C for 4 h. After the reaction, ester-linked fatty acids were obtained by hexane extraction at an acidic pH. The methanolic layer was centrifuged, and hydroxy fatty acids, e.g., 3-hydroxydecanoic acid, were obtained from the supernatant. The ester-linked fatty acids thus separated were methylated by diazomethane. The separation of amide-linked fatty acids from the precipitate as methyl esters was carried out

by methanolysis with 5% hydrochloric acid–methanol at 105°C for 5 h, with subsequent extraction with hexane.

Amino acids were analyzed by the ninhydrin colorimetric method on a Hitachi KLA-3B amino acid analyzer equipped with a column packed with amberlite CG-120 type III ion-exchange resin. The following column conditions were used: (i) column (0.9 by 50 cm) at 30°C, with 0.38 N sodium citrate buffer (pH 4.26); and (ii) column (0.6 by 10 cm) at 55°C, with 0.2 N sodium citrate buffer (pH 5.28).

IR absorption spectra were obtained by the KBr tablet method on a Nihonbunko DS-301 apparatus and by oiling the samples to the sodium chloride plate on a Nihonbunko IR-G apparatus.

Chemicals. All chemicals were of the highest purity available commercially. Authentic standards of phospholipids and fatty acids were obtained from Serdary Research Laboratories, Inc., and Applied Science Laboratories, respectively. Phospholipase A obtained from the venom of *Crotalus adamanteus* (30, 31) was purchased from Worthington Diagnostics.

RESULTS

General analysis of total extractable cellular lipids. For both liquid and solid cultures, about 140 and 120 mg of total extractable cellular lipids were obtained from 1 g (cell dry weight) each of *B. pertussis* cells and *B. parapertussis* or *B. bronchiseptica* cells, respectively. Total extractable cellular lipids from eight strains of *B. pertussis* (phases I to IV), two strains of *B. parapertussis*, and two strains of *B. bronchiseptica* were analyzed by two-dimensional TLC. When the TLC was performed with lipids from some strains of *B. pertussis* that were cultured both in the liquid and on the solid media, no difference was detected in the TLC patterns between the two culture methods. This lipid analysis was carried out with *B. parapertussis* and *B. bronchiseptica* cultured on slants. The TLC patterns obtained were almost the same among the four phases of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (Fig. 1). Individual phospholipids on the TLC plates were identified on the basis of R_f values and reactive functional groups, as compared with those from authentic standards. The phospholipid structures were verified by the mild alkaline hydrolysis method described by Dawson (3). The hydrolysates of individual phospholipids were analyzed by paper chromatography with the solvent system phenol-water-acetic acid-ethanol (80:20:10:12 [vol/vol]). The R_f values of the hydrolysates were 0.63 (a), 0.23 (b), 0.40 (c), and 0.63 (e), confirming a, b, c, and e as phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, and lysophosphatidylethanolamine, respectively.

The percent composition of individual phospholipids was determined, with phosphatidylethanolamine representing 60 to 70% of the total phospholipids, followed by cardiolipin (Table 1).

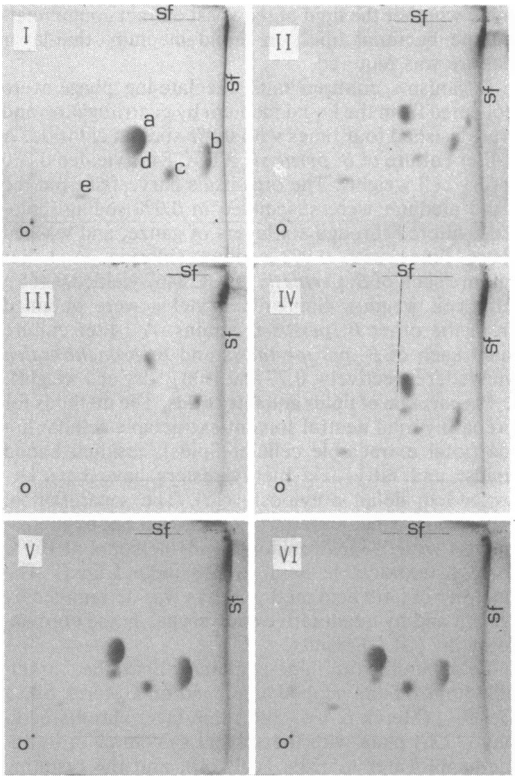


FIG. 1. Two-dimensional thin-layer chromatograms of total extractable cellular lipids of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. The solvent systems were chloroform-methanol-water (65:25:4 [vol/vol]) in the vertical direction and chloroform-methanol-acetic acid (65:25:10 [vol/vol]) in the horizontal direction. The plates were visualized by charring with 50% sulfuric acid. (I) *B. pertussis* ATCC 8467 (phase I); (II) *B. pertussis* Kudo (phase II); (III) *B. pertussis* 3B (phase III); (IV) *B. pertussis* Kudo (phase IV); (V) *B. parapertussis* 21619; (VI) *B. bronchiseptica* 214. O, Origin; sf, solvent front; a, phosphatidylethanolamine; b, cardiolipin; c, phosphatidylglycerol; d, ornithine-containing lipid; e, lysophosphatidylethanolamine.

The phosphate-negative, ninhydrin-positive lipid (Fig. 1, spot d, and Fig. 2, spot d) was studied in further detail. This aminolipid was purified as described above, and its amino acid was analyzed. Since ornithine overlapped with lysine in the usual amino acid analysis system, another system was also used. The amino acid contained in the lipid was identified as ornithine for all 12 of the *Bordetella* strains. These data suggest that the three *Bordetella* species have the ornithine-containing lipid in common.

Changes in lipids during growth of *B. pertussis* ATCC 8467. The yields and changes in the lipid profile of *B. pertussis* ATCC 8467 (phase I) during individual growth phases were investigat-

TABLE 1. Phospholipid composition of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*

Phospholipid	Phospholipid composition[a] of:			
	B. pertussis ATCC 8467 (phase I)	*B. pertussis* 3B (phase III)	*B. parapertussis* 21619	*B. bronchiseptica* 214
Phosphatidylethanolamine	8.86	7.85	7.20	7.74
Cardiolipin	2.27	3.60	2.68	1.87
Phosphatidylglycerol	1.40	0.72	0.92	1.10
Lysophosphatidylethanolamine	0.08	0.26	Tr	0.86
Lysocardiolipin	Tr	0.14	Tr	Tr
Lysophosphatidylglycerol	Tr	0.02	Tr	Tr

[a] The amount of phospholipid was calculated from the moles percent of phosphorus (determined by the method of Bartlett [1]) and the fatty acid composition of individual phospholipids and is expressed as a percentage of the dry weight of cells.

ed. The bacteria were cultured in the liquid medium containing nicotinamide instead of yeast extract. Phospholipids constituted 90.0% and fatty acid methyl esters constituted about 9.6% of the total extractable cellular lipids during all growth phases (Fig. 3A). Figure 3B-1 shows that phospholipid compositions were almost the same for all growth phases, although slight differences were observed in the early logarithmic and stationary phases. It was observed in both total extractable cellular lipids and phosphatidylethanolamine that fatty acid compositions were almost constant for all growth phases, with hexadecanoic and hexadecenoic acids constituting about 90%, in a ratio of 1:1 (Fig. 3B-2 and 3B-3). This simple fatty acid composition of *B. pertussis* is very characteristic, as compared with that of other bacterial lipids.

Fatty acid composition of total extractable cellular lipids. Fatty acid composition studies were carried out with solid-cultured cells, because no difference in the lipids and fatty acids was detected between liquid and solid cultures of some *B. pertussis* strains tested. Phospholipids constituted 90.0% of the total extractable cellular lipids of *B. pertussis*. The quantity of fatty acid methyl esters obtained from the total extractable cellular lipids of *B. pertussis* was 96 mg from 1 g (cell dry weight).

The quantitative fatty acid composition shown in Table 2 indicates that the extractable lipids derived from *B. pertussis* were composed of hexadecanoic and hexadecenoic acids (90%) in a ratio of 1:1. The structure of the two fatty acids was studied by TLC, GLC, MS, and IR absorption spectrometry. Furthermore, the hexadecenoic acids of *B. pertussis* ATCC 8467 (phase I) and *B. pertussis* Sakurayashiki (phase III) were subjected to the oxidative degradation reaction, and azelaic acid was obtained as the dicarboxylic acid. The IR absorption spectrum of hexadecenoic acid indicated the *cis* form when compared with authentic standards. From these data, the hexadecenoic acid of *B. pertussis* was determined to be in the *cis*-9 form. The distribution of fatty acids on the 1 and 2 positions of the phospholipids was then determined with snake venom phospholipase A. In *B. pertussis* ATCC 8467 (phase I), the major fatty acids in the 1 position of phosphatidylethanolamine were hex-

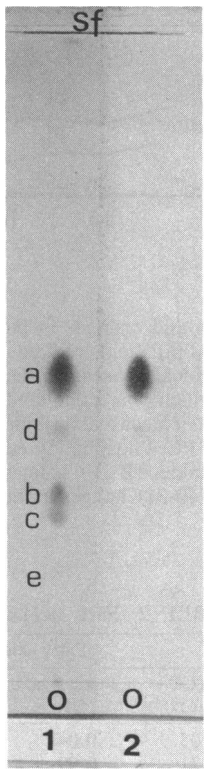


FIG. 2. Separation of the ornithine-containing lipid from phospholipids and neutral lipids by TLC. The solvent system was chloroform-methanol-water (65:25:4 [vol/vol]). Total extractable cellular lipid patterns of *B. pertussis* ATCC 8467 were visualized with iodine vapor (lane 1) and with ninhydrin reagent (lane 2). See the legend to Fig. 1 for definitions.

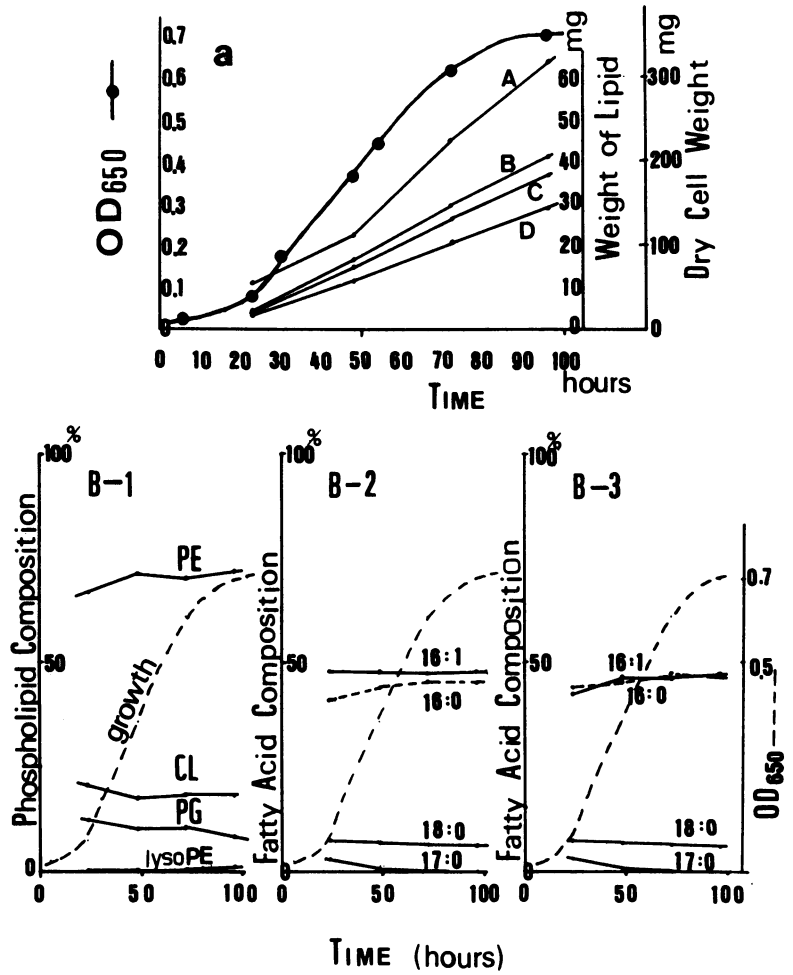


FIG. 3. Yields and changes in the lipid profile during growth of *B. pertussis* ATCC 8467. OD_{650}, Optical density at 650 nm. (a) Yields of lipids at different times during bacterial growth. An 800-ml culture was used for each of the individual phases. (A) Dry cell weight; (B) weight of total extractable cellular lipids; (C) weight of phospholipids, which was calculated on the basis of lipid and phosphorus analyses; (D) weight of fatty acid methyl esters, which was determined by GLC analysis. (B-1) Changes in phospholipid composition during bacterial growth. PE, Phosphatidylethanolamine; CL, cardiolipin; PG, phosphatidylglycerol; lysoPE, lysophosphatidylethanolamine. (B-2) Changes in fatty acid composition of the total extractable cellular lipids during bacterial growth. (B-3) Changes in fatty acid composition of phosphatidylethanolamine during bacterial growth.

TABLE 2. Fatty acid composition of total extractable cellular lipids of *B. pertussis*

Fatty acid	Fatty acid composition[a] of extractable lipids of *B. pertussis* strain (phase):							
	ATCC 8467 (I)	Fukasawa (I)	Mikami (II)	Kudo (II)	3B (III)	Sakuraya-shiki (III)	Kudo (IV)	Sakuraya-shiki (IV)
14:0	0.05	0.04	0.07	0.06	0.05	0.05	0.07	0.03
15:0	0.21	0.09	0.16	0.24	0.17	0	0.27	0.16
15:1	0.16	0.04	0.07	0.17	0.14	0	0.29	0.10
16:0	3.97	4.36	4.23	3.67	4.06	4.49	3.88	3.88
16:1	4.54	4.58	4.57	4.65	4.62	4.51	4.33	4.76
17:0	0.29	0.10	0.11	0.53	0.22	0	0.41	0.27
18:0	0.37	0.38	0.36	0.34	0.33	0.55	0.36	0.41

[a] Expressed as a percentage of the dry weight of cells.

TABLE 3. Distribution of fatty acids on the 1 and 2 positions of phospholipids of *B. pertussis* ATCC 8467

Fatty acid	Distribution[a] of fatty acids on indicated positions of phospholipids					
	Phosphatidylethanolamine		Cardiolipin		Phosphatidylglycerol	
	1	2	1	2	1	2
14:0	0.04	0.03	0.03	0.11	0.10	0.07
14:1	0.01	0.02	0.01	0.01	0.03	0.02
15:0	0.12	0.02	0.08	0.10	0.18	0.11
15:1	0	0.09	0.02	0.05	0.01	0.05
16:0	3.49	0.28	3.35	1.89	3.04	2.18
16:1	0.12	4.18	0.04	1.92	0.05	1.21
17:0	0.27	0.07	0.30	0.20	0.36	0.21
18:0	0.73	0.12	0.96	0.56	0.92	0.95
18:1	0.02	0	0	0	0.10	0

[a] Expressed as a percentage of the dry weight of cells.

adecanoic and octadecanoic acids, whereas the major fatty acid in the 2 position was 9-hexadecenoic acid. In cardiolipin and phosphatidylglycerol, the major fatty acids in the 1 position were similar to those of phosphatidylethanolamine, whereas the major fatty acids in the 2 position were 9-hexadecenoic, hexadecanoic, and octadecanoic acids (Table 3).

The yield of fatty acid methyl esters from total extractable cellular lipids of *B. parapertussis* and *B. bronchiseptica* was 83 mg from 1 g (cell dry weight). The fatty acid composition of the total extractable cellular lipids derived from these two species was found to be mainly hexadecanoic acid and methylene-hexadecanoic acid (Table 4). The methylene-hexadecanoic acid was studied further by IR absorption spectrometry and MS. In the IR absorption spectra of a mixture of fatty acids from the extractable lipids of the three species (Fig. 4), an absorption band at 1,020 cm^{-1}, based on the skeleton structure of cyclopropane, was observed for lipids of *B. parapertussis* and *B. bronchiseptica*, but not for lipids of *B. pertussis*. With another method and apparatus, described above, an absorption band at 3,050 cm^{-1}, based on the —CH_2— group of cyclopropane, was observed for lipids of *B. parapertussis* and *B. bronchiseptica*, but not for lipids of *B. pertussis*. From these IR absorption spectrum data, the presence of heptadecacyclopropanoic acid in *B. parapertussis* and *B. bronchiseptica* was suggested. The results of the MS analysis were not satisfactory.

TABLE 4. Fatty acid composition of total extractable cellular lipids of *B. parapertussis* and *B. bronchiseptica*

Fatty acid	Fatty acid composition[a] of extractable lipids of:			
	B. parapertussis		*B. bronchiseptica*	
	17903	21619	5376	214
14:0	0.11	0.14	0.10	0.11
15:0	0.11	0.06	0.10	0.14
16:0	4.57	4.78	4.84	4.67
16:1	0.30	0.22	0.66	0.48
17:0	0.08	0.07	0.09	0.09
17:1	0.14	Tr	0.05	0.06
17CA[b]	2.08	2.14	1.75	1.91
18:0	0.12	0.13	0.09	0.10
18:2	0.81	0.77	0.64	0.75

[a] Expressed as a percentage of the dry weight of cells.

[b] CA, Cyclopropanoic acid.

Fatty acid composition of residual bound lipids. The quantities of fatty acid methyl esters obtained from the bound lipids of *B. pertussis* and

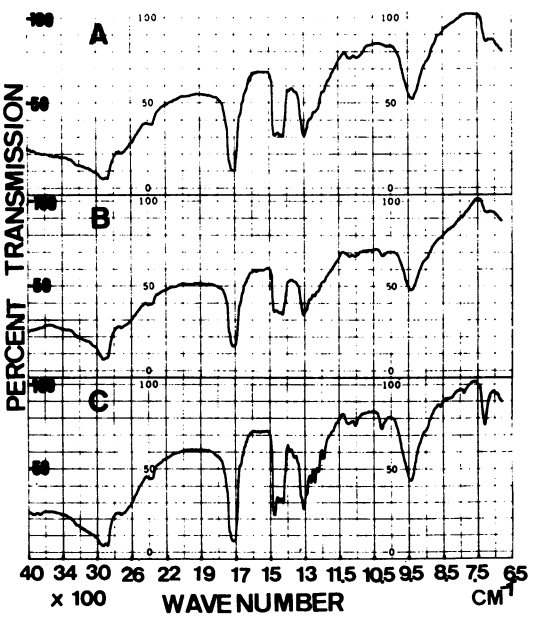


FIG. 4. IR absorption spectra of mixtures of fatty acids from total extractable cellular lipids prepared from (A) *B. pertussis* Sakurayashiki (phase III), (B) *B. parapertussis* 17903, and (C) *B. bronchiseptica* 214. These spectra were obtained by the KBr tablet method on a Nihonbunko DS-301 apparatus.

TABLE 5. Fatty acid composition of bound lipids of *B. pertussis*

Fatty acid	Fatty acid composition[a] of bound lipids of *B. pertussis* strain (phase):						
	ATCC 8467 (I)	Fukasawa (I)	Kudo (II)	3B (III)	Sakuraya-shiki (III)	Kudo (IV)	Sakuraya-shiki (IV)
3-OH 10:0	0.28	0.41	0.32	0.11	0.35	0.46	0.75
3-OH 11:0	0.01	0	0.16	0.01	0	0.14	0
3-OH 12:0	0.10	0.10	0.52	0.12	0.12	0.45	0.15
3-OH 13:0	0.05	0	0.05	0.06	0	0.03	0
3-OH 14:0	1.12	0.97	0.47	1.34	1.30	0.41	0.82
14:0	0.52	0.59	0.46	0.46	0.40	0.48	0.39
15:0	0.05	0.03	0.09	0.05	0	0.10	0.04
16:0	0.12	0.16	0.16	0.11	0.12	0.16	0.09
16:1	0.06	0.02	0.05	0.05	0.01	0.07	0.06
18:0	Tr	Tr	Tr	Tr	Tr	Tr	Tr

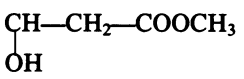
[a] Expressed as a percentage of the dry weight of cells.

B parapertussis or *B. bronchiseptica* were 23 and 20 mg from 1 g (cell dry weight), respectively. Unlike the fatty acids of extractable lipids, most of the fatty acids of the bound lipids of *B. pertussis* were 3-hydroxy acids. The major fatty acids of the bound lipids were 3-hydroxytetradecanoic, tetradecanoic, and 3-hydroxydecanoic acids, with 3-hydroxydodecanoic acid occurring in some strains (Table 5). These characteristics remained stable during strain preservation and subculturing, independent of phase change. The fatty acid composition of lipid A is shown in Table 6. 3-Hydroxytetradecanoic acid was the main component of both lipid A and the bound lipids. Hydroxy fatty acids of the bound lipids were determined to be derived from lipid A.

Although the fatty acid composition of the bound lipids of *B. parapertussis* was similar to that of the bound lipids of *B. pertussis*, the bound lipids of *B. bronchiseptica* had a very different composition, containing a large amount of 2-hydroxydodecanoic acid (Table 7).

Based on the results of mild alkaline hydrolysis of the bound lipids, it was presumed that 3-hydroxytetradecanoic acid was derived mainly from the amide bond, but that 3-hydroxydecanoic, tetradecanoic, and hexadecanoic acids and a part of 3-hydroxytetradecanoic acid were from the ester linkage of lipid A in the three *Bordetella* species. 2-Hydroxydodecanoic acid was presumed to be from the ester bond.

Identification of hydroxy fatty acids by MS. The mass spectra (5, 20, 22, 33) of the major hydroxy fatty acids in the bound lipids of the three *Bordetella* species are shown in Fig. 5. In the case of methyl-3-hydroxytetradecanoate (Fig. 5A), the base peak at *m/e* 103 due to



and a fragment peak at *m/e* 74 due to C_3—C_2

TABLE 6. Fatty acid composition of lipid A of *B. pertussis*

Fatty acid	Fatty acid composition[a] of lipid A of *B. pertussis* strain (phase):	
	ATCC 8467 (I)	Sakurayashiki (III)
3-OH 10:0	0.09	0.05
3-OH 11:0	0.02	Tr
2-OH 12:0	0.04	0.04
3-OH 12:0	0.11	0.09
3-OH 13:0	0.04	Tr
3-OH 14:0	0.64	0.72
3-OH 15:0	0.01	Tr
14:0	0.05	0.05
14:1	0.02	0.03
15:0	0.02	0.01
16:0	0.41	0.42
16:1	0.04	0.03
17:0	0.01	0.01
18:0	0.09	0.09
18:1	0.04	0.06

[a] Expressed as a percentage of the dry weight of cells.

TABLE 7. Fatty acid composition of bound lipids of *B. parapertussis* and *B. bronchiseptica*

Fatty acid	Fatty acid composition[a] of bound lipids of:			
	B. parapertussis		*B. bronchiseptica*	
	17903	21619	5376	214
12:0	0.01	Tr	Tr	0.04
14:0	0.43	0.53	0.17	0.19
15:0	0.01	Tr	0	0
16:0	0.23	0.14	0.03	0.06
2-OH 12:0	0.04	0.06	0.85	0.82
3-OH 10:0	0.82	0.54	0.03	0.04
3-OH 14:0	0.41	0.66	0.92	0.80
Unknown	0.06	0.06		

[a] Expressed as a percentage of the dry weight of cells.

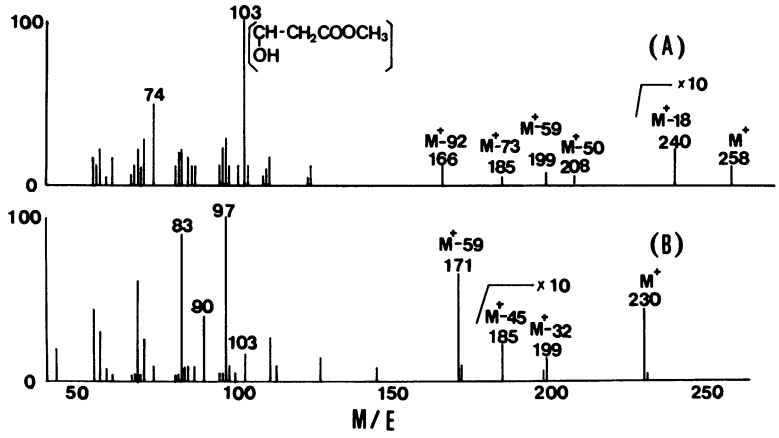


FIG. 5. Mass spectra of major hydroxy fatty acid methyl esters obtained from the bound lipids of *Bordetella* species. (A) Methyl-3-hydroxytetradecanoate; (B) methyl-2-hydroxydodecanoate.

cleavage showed high intensity. Although the molecular ion at *m/e* 258 (M^+) was of low intensity, some peaks at *m/e* 240 (M^+-18, loss of water), *m/e* 208 (M^+-50, loss of water and methanol), *m/e* 199 (M^+-59, loss of $COOCH_3$), and *m/e* 166 (M^+-92, loss of water and CH_2COOCH_3) verify the molecular structure. Methyl-2-hydroxydodecanoate, indicated in Fig. 5B, had a molecular ion at *m/e* 230 (M^+) of relatively high intensity. Characteristic fragment ions were detected at *m/e* 90 (C_2—C_3 cleavage), *m/e* 103 (C_3—C_4 cleavage), *m/e* 171 (C_1—C_2 cleavage, M^+-59), *m/e* 199 (M^+-32, loss of methanol), and *m/e* 185 (M^+-45, $\underset{\underset{OH}{|}}{CH}$—$CH_2$—$H^+$).

DISCUSSION

In view of the fact that very limited information is available concerning the lipids of the genus *Bordetella*, the lipids of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were analyzed and compared.

As a result of this study, we determined some of the characteristics of the lipids of *B. pertussis*. An ornithine-containing lipid which constitutes about 3% of the total extractable cellular lipids is characteristic of *Bordetella* species. This aminolipid may have some important function in the living organism, because its structure possesses both hydrophilic and lipophilic parts, and unlike phospholipids, it is not hydrolyzed by phospholipase. The presence of an ornithine-containing lipid in some other species of bacteria has been documented (7, 17–19, 27, 28, 32), although its physiological function is not clear.

Furthermore, it is characteristic of *B. pertussis* that the fatty acid composition of the total extractable cellular lipids which constitute the bacterial membrane is simple, being composed of hexadecanoic and *cis*-9-hexadecenoic acids (90%) in a ratio of about 1:1. The fatty acid composition was almost independent of the growth and antigenic phases. Hexadecenoic acid may be important for membrane fluidity and function in this organism, as it is the only major unsaturated fatty acid.

It was determined that the 3-hydroxy fatty acids of the bound lipids of *B. pertussis* are derived from lipid A. The ester-linked and the amide-linked fatty acids of the bound lipids of *B. pertussis* were examined, because it has been reported that ester-linked fatty acids in lipid A carry most of the endotoxic activity (18a). It was found that only 3-hydroxytetradecanoic acid was in amide linkage.

B. pertussis and *B. parapertussis* are similar in the fatty acids of their bound lipids, although different in the fatty acids of their extractable lipids, but *B. pertussis* and *B. bronchiseptica* are different in the fatty acids of both their extractable lipids and bound lipids. *B. pertussis* and *B. parapertussis* are similar in their small, lustrous colony forms and in their poor growth on common media. On the other hand, *B. bronchiseptica* is motile, with flagella, forms large, opaque colonies, and grows very well on common media. In view of these facts, the data on lipids of *Bordetella* correspond well with the bacterial relationships based on morphological and biochemical characteristics.

In 1973, Thiele and Schwinn (29) reported the lipid composition of one *B. pertussis* strain. In agreement with their data, we found that the free-lipid content of *B. pertussis* is higher than that of other bacteria. However, there are some discrepancies between the results found by the two research groups. Although Thiele and

Schwinn stated that the major phospholipids were phosphatidylethanolamine, cardiolipin, and phosphatidylserine, we could not detect phosphatidylserine. Thiele and Schwinn proposed an ethanediol structure without hydroxy fatty acid as the chemical structure of the ornithine-containing lipid of *B. pertussis*. We propose a structure in which the carboxyl end of 3-hydroxyhexadecanoic acid is in amide linkage with the α-amino group of ornithine, and the β-hydroxyl group of 3-hydroxyhexadecanoic acid is esterified to a second hexadecanoic acid. The ornithine-containing lipid of *B. pertussis* will be described in detail in a separate paper. The fatty acid composition of phospholipids described by Thiele and Schwinn differs from that determined from our data.

In any case, the conclusion of Thiele and Schwinn (29) that the lipid pattern of *B. pertussis* is not like that of *Brucella* species is compatible with the description in *Bergey's Manual of Determinative Bacteriology* (23) of *Bordetella* as a genus of uncertain affiliation.

Kasai studied the lipid composition of endotoxins derived from one strain each of *B. pertussis* (phase I) and *B. bronchiseptica*, relative to their biological activities (12). He stated that the lipopolysaccharide of *Bordetella* species is characterized by a high content of nitrogen, phosphorus, and lipids, as compared with *Escherichia coli*. Moreover, Haeffner et al. (8) reported the presence of branched hydroxy fatty acids as components of the endotoxin of *B. pertussis*. However, we could not confirm this observation despite extensive analysis of almost all of the fatty acids of the bound lipids and lipid A.

Kawahara et al. (13) found that when *Pseudomonas ovalis*, in which 80% of the lipid A is composed of 3-hydroxydecanoic, 3-hydroxydodecanoic, and 2-hydroxydodecanoic acids, was cultured in an N_2 atmosphere containing $^{18}O_2$, the molecular oxygen from $^{18}O_2$ was specifically incorporated into the 2-hydroxyl group of the 2-hydroxy fatty acid but not into 3-hydroxy fatty acids. They proposed a direct hydroxylation biosynthesis of the 2-hydroxy fatty acid from the nonpolar fatty acid of the same chain length. After Yano et al. studied the distribution of 2-hydroxy fatty acids and the phenomenon found by Kawahara et al. (13) in various bacteria, they suggested that 2-hydroxy fatty acids are present only in strictly aerobic bacteria and stated that the phenomenon occurs widely in such bacteria as *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Cytophaga*, and *Flexibacter* species (I. Yano, E. Yabuuchi, A. Yamamoto, and Y. Kishimoto, Abstr. Annu. Meet. Jpn. Soc. Bacteriol. 1980, p. 257; E. Yabuuchi, Annu. Meet. Jpn. Soc. Bacteriol. 1981, p. 70). In view of these facts, it is interesting that a 2-hydroxy fatty acid which is not present in facultative aerobic bacteria such as *Enterobacteriaceae* and *Vibrionaceae* (6, 11, 24, 34) was detected in only *B. bronchiseptica* of the genus *Bordetella*. Since the biological characteristics of *B. bronchiseptica* are considerably different from those of the other two species of *Bordetella*, the organism being motile, with flagella, and showing rapid growth on culture media (23), the mechanism of fatty acid biosynthesis may also be different from that of the others.

While this paper was being written, the cellular fatty acid composition of *B. bronchiseptica*, analyzed by GLC, was reported by Dees et al. (4). Their data show that 2-hydroxydodecanoic acid is one of the major fatty acids in the five strains of *B. bronchiseptica* they tested and are in good agreement with our data.

It is worth mentioning that changes in phase from I to IV in *B. pertussis* do not affect the lipid composition.

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