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Fatty Acid Composition of the Complex Lipids of *Staphylococcus aureus* During the Formation of the Membrane-bound Electron Transport System

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In *Staphylococcus aureus*, 64 fatty acids could be separated by gas-liquid chromatography. The fatty acids consisted of normal, iso, and anteiso saturated fatty acids of from 10 to 21 carbon atoms. Of the total fatty acids, 2 to 4% were normal, iso, and anteiso monoenoic fatty acids. Positional isomers of the normal monoenoic fatty acids could be detected. The fatty acids could be extracted, leaving 1 to 2% of the total fatty acids in the residue. The proportions of the fatty acids in the residue and the total lipids differed significantly. The lipid extract contained less than 0.12% free fatty acid. Between 5 and 10% of the lipid fatty acids were associated with neutral lipids. The majority of the fatty acids were associated with the complex lipids: mono- and diglucosyl diglyceride, phosphatidyl glycerol, lysyl phosphatidyl glycerol, and cardiolipin. The proportions of the fatty acids changed markedly between bacteria grown anaerobically (no membrane-bound electron transport system) and those grown aerobically (containing a functional electron transport system). In each of the complex lipids, the proportions of the fatty acids, as well as the magnitude and direction of change in the molar quantity of the fatty acids per bacterium, changed dramatically between these growth conditions. Since the glucosyl diglycerides and phospholipids were formed from the same pool of diglyceride intermediates, the marked differences in fatty acids indicate that acyl transferase activities must be an important part of complex lipid metabolism in *S. aureus*.

During the formation of the membrane-bound respiratory system in *Staphylococcus aureus*, cytochromes *b* and *a* and cytochrome oxidase *o* are formed into a functional electron transport system (3). At the same time, there is a 1.2-fold coordinate increase in the two glucolipids, a 1.6-fold increase in vitamin K_2 and cardiolipin, and a 2-fold increase in phosphatidyl glycerol in the electron transport membrane (3). The fatty acid composition of these complex lipids was examined to see whether special classes of lipids were synthesized when the membrane was modified to carry out electron transport. The results of this study indicate that there is an extensive modification of the fatty acid composition of all the complex lipids.

The fatty acid composition of *S. aureus* can be modified by growth under different conditions (14). Aeration of the growth medium increases the proportion of unsaturated fatty acids; when cultures are agitated, the proportion of unsaturated fatty acids is 10%, whereas this value is only 6% when cells are incubated statically. A decrease in the *p*H of the medium from 7.2 to 6.0 results in a 3% increase in the proportion of the unsaturated fatty acids. When the *p*H at which bacteria are grown is changed from 6.0 to 8.0, the proportion of C_{20} increases from 32 to 49% of the fatty acids. During these changes in growth conditions, the proportions of C_{14}, C_{16}, iso-C_{15}, and anteiso-C_{15} decrease 2 to 5%. About 5% of the total fatty acids are unsaturated in *S. aureus* grown in synthetic medium. The proportion increases to 27% when 5% human serum is added to the medium or to 37% when the bacteria are isolated from the peritoneum of guinea pigs. Macfarlane (10) found the fatty acid patterns of the complex lipids to be similar. The major fatty acids reported by Macfarlane (10) were essentially those found in aerobically growing bacteria in this study. Examination of the change in fatty acids, especially the minor fatty acids in

the complex lipids, during the formation of the electron transport system, however, suggests that extensive transacylation must be a part of the metabolism of the complex lipids.

MATERIALS AND METHODS

Materials. Materials used were as described previously (3, 18, 19). The methyl oleate, methyl *cis*-vaccenate, methyl *trans*-vaccenate, and methyl elaidiate were supplied by the Hormel Institute, Austin, Minn.

Growth of bacteria. The strain, medium, harvesting procedure, and method of preservation of the *S. aureus* used in this study have been described (3, 19). Bacteria were harvested at a density of 0.05 mg (dry weight)/ml after anaerobic growth in 2-liter Florence flasks containing 1.5 liters of medium. These flasks were fitted with ports to allow gassing of the medium with deoxygenated nitrogen (17). Bacteria were grown aerobically in low-form Erlenmeyer flasks (16) shaken vigorously with gyratory agitation.

Dry weight. Dry weights were determined as described previously (19).

Fatty acid isolation. Fatty acids were isolated after saponification and were esterified as described (18). The methyl esters were hydrogenated in methanol at room temperature in the presence of a 5% platinum catalyst on charcoal by one atmosphere of hydrogen for 1 hr (18). The mercuric adducts of the monoenoic fatty acid esters were synthesized and concentrated by silicic acid chromatography by the procedures of Goldfine and Block (4).

Lipid extraction. Lipids were extracted by the modified Bligh and Dyer (1) method that involves acidification of the growth medium before harvest and heating bacteria suspended in isopropanol (19). Free fatty acids were separated from a portion of the total lipid extract by partitioning three times against 0.47 M Na_2CO_3. The combined aqueous phases were extracted with diethyl ether and then acidified with HCl to *p*H 2.0. The acidified combined aqueous phases were extracted with three portions of ether to yield the free fatty acids. The neutral and complex lipids were separated by silicic acid chromatography (18). The glucolipids were separated from the phospholipids by a modification of the Vorbeck and Marinetti procedure (15) that is described in the text. The two glucolipids and the three phospholipids were then separated by the thin-layer chromatographic method previously described (19). Fatty acid methyl esters were quantitatively released from the isolated lipids by mild alkaline methanolysis (19). Phosphate and glucose were estimated as described by White and Frerman (19).

Fatty acid determination. Total fatty acids were determined by the colorimetric procedure of Novak (11) as modified by W. J. Lennarz, with palmitic acid as standard (18).

Fatty acids are designated as follows: the subscript of C indicates the number of carbon atoms in the chain; the prefix a- (a-C) indicates an anteiso branched acid; the prefix i- indicates an iso branched acid; and the superscript = indicates a monounsaturated fatty acid.

Gas chromatography. A dual-column gas chromatograph (model 402, F and M Scientific, Division of Hewlett-Packard Co., Avondale, Pa.) with glass columns in the paperclip configuration, 4 mm in internal diameter and 3.59 meters long, were packed with 15% (w/v) ethylene glycol adipate on 60/80 mesh Gas Chrome P prepared by Applied Science Co. (State College, Pa.). Columns were run isothermally at 156 C with the flash heater at 190 C and the detector at 240 C. The flow rate of the helium carrier gas was 120 ml/min, with a head pressure of 60 psi. Hydrogen at a head pressure of 18 psi (flow rate, 45 ml/min) and air at a head pressure of 32 psi (flow rate, 350 ml/min) gave detector operating characteristics essentially as described previously (18). Under these operating conditions, the resolution of i-C_{15} and a-C_{15} was 4.20. The efficiency of the column for C_{16} was 3,480 theoretical plates.

Columns packed with 6.8% diethylene glycol succinate on 80/100 mesh chromosorb W gave a resolution of 3.88 and an efficiency of 2,881 theoretical plates under these operating conditions.

The percentage composition was calculated from the areas of the response to each methyl ester. The area was estimated as the product of the peak height and the width at half the peak height. Repeated analysis by gas chromatography of portions of the same preparation of fatty acid methyl esters shows that the proportions of each ester calculated as the percentage of the total varies ±0.5%.

RESULTS

Total fatty acids. In aerobically grown *S. aureus* cells 5.95% of the dry weight consists of fatty acids. The fatty acids represent 235 μmoles per g (dry weight) of cells, determined colorimetrically and calculated as palmitic acid. In anaerobically grown *S. aureus* cells, 2.98% of the dry weight consists of fatty acids; i.e., there are 115 μmoles of fatty acid/g (dry weight) of cells, calculated as palmitic acid.

Identification of the fatty acids. When fatty acid methyl esters prepared from aerobically grown *S. aureus* were separated on a polar gas-liquid chromatograph column long enough to resolve i-C_{15} and a-C_{15}, a total of 64 recognizable components were detected. The major components of this mixture had retention times corresponding to authentic i-C_{15}, a-C_{15}, C_{16}, i-C_{17}, a-C_{17}, C_{18}, and C_{20}. These were the major fatty acids in *S. aureus* as reported by Macfarlane (10). Figure 1 is a James plot (5) of the logarithm of the relative retention time versus the number of carbon atoms of these 64 components. This plot shows three characteristic classes of methyl esters.

(i) The first class is composed of a series of homologous members which, when plotted, have parallel slopes. These consist of iso branched

saturated esters (C_{10} to C_{21}), anteiso branched saturated esters (C_{10} to C_{21}), and normal saturated esters (C_{10} to C_{21}).

(ii) The second class contains components which, when plotted, fall on a line of slightly greater slope; at longer relative retention times, these components correspond to a homologous series of monoenoic normal fatty acid esters (C_{10} to C_{19}). Two monoenoic isomers with the olefinic double bond at different positions are found between C_{12} and C_{19}. The two C_{18} normal monoenoic methyl esters have retention times corresponding to methyl oleate and methyl *cis*-vaccenate. The resolution between authentic methyl oleate and methyl *cis*-vaccenate was 2.38. The resolution between the two C_{18} monoenoic fatty acid esters from *S. aureus* averaged 2.82. As predicted from the experience with polar Golay columns (12), the ester with the unsaturation closer to the carbonyl had the shorter retention time. The column used for this study could not distinguish between methyl oleate and methyl elaidiate, methyl *cis*-vaccenate, or methyl *trans*-vaccenate.

(iii) Components with a slightly longer retention time than the iso and anteiso branched fatty acid esters were provisionally designated as branched monoenoic fatty acid methyl esters from their retention times by gas-liquid chromatography. These components can be hydrogenated and the mercuric adducts concentrated, as would be expected for monoenoic fatty acids. The positions of the first and second classes of fatty acid esters correspond to those of authentic compounds.

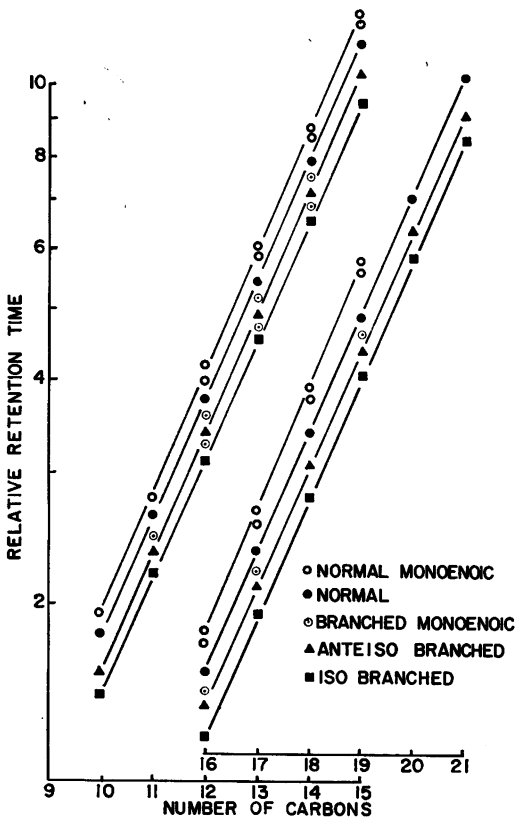

FIG. 1. *Logarithm of the relative retention time versus the number of carbon atoms in the gas-liquid chromatographic analysis of fatty acid methyl esters from Staphylococcus aureus. The liquid phase of the column was ethylene glycol adipate, the temperature of the column was 156 C, and the other conditions were as described in Materials and Methods.*

Similar patterns and James plots can be constructed for the response of a polar 6.8% diethylene glycol column. Fatty acids of chain lengths greater than C_{14} gave relative areas essentially identical to those from the analysis of the ethylene glycol column. When the total fatty acid mixture was applied to a nonpolar 3.8% SE-30 column, the relative responses overlapped and could not be compared to the responses from the polar columns.

Further evidence as to the presence of unsaturated fatty acids in *S. aureus* lipid preparations was obtained in two ways. The monoenoic fatty acid esters were concentrated relative to the saturated esters by chromatography on silicic acid of the mercuric adducts. This treatment caused the proportion of unsaturated to saturated fatty acid esters to increase from 2.8% to 44.6% of the total fatty acid esters. There was a 2- to 20-fold relative concentration of the individual unsaturated fatty acid esters (Table 1). Hydrogenation of the sample, as described below, resulted in a marked decrease in the unsaturated fatty acid esters with increases in the expected saturated homologues.

The total fatty acid mixture was hydrogenated as described in Materials and Methods. The data of Table 1 indicate a 2- to 20-fold decrease in the relative concentrations of each of the monoenoic fatty acid esters. The total unsaturated esters decreased from 2.8% of the total fatty acid esters to 0.3%. A 2% error in the estimation of each pair of esters could result in a 12% error in the relative increase or decrease in the ratios. This could account for the differences in the degree of concentration or depletion between the different fatty acid esters in Table 1. The changes in the proportions of the methyl esters after concentration of the mercuric adducts or depletion by

TABLE 1. *Unsaturated fatty acids of Staphylococcus aureus*[a]

A Fatty acids	B Increase in unsaturated	C Decrease in unsaturated
11-11 =	1.4	1.1
12, i-12,i =	1.9	19.0
12,a-12,a =		4.4
12 -12 =	2.9	1.4
13,i -13,i =	3.5	1.9
13,a-13,a =	8.8	15.9
13 -13 =	8.9	10.0
14,i -14,i =	1.9	20.5
14,a-14,a =	20.2	2.7
14 -14 =	1.4	5.1
15 -15 =	2.8	2.8
16,a-16,a =	1.4	7.0
17 -17 =	18.2	1.8
18 -18 =	13.8	13.0
19 -19 =	13.7	15.4

[a] Methyl esters of fatty acids of aerobically grown *S. aureus* were separated by gas-liquid chromatography as in Fig. 1. The ratio of the response of the saturated to the homologous unsaturated fatty acid methyl ester from the total fatty acids of aerobically grown bacteria (column A) was calculated and compared to the ratio of the responses of the same fatty acid methyl ester pairs (column B) after concentration of the unsaturated fatty acid methyl esters by silicic acid chromatography of the mercuric adducts (4), and (column C) after hydrogenation in methanol. The numbers in column B represent the *increase* in relative proportion of the unsaturated fatty acid methyl esters. The figures in column C represent the *decrease* in relative proportions of unsaturated fatty acid methyl esters after hydrogenation. Fatty acid methyl esters are designated as the number of carbon atoms with = for monoenoic, a for anteiso, and i for iso branched fatty acids.

hydrogenation of the individual monoenoic methyl esters are illustrated in Table 2.

Proportions of the total fatty acid esters in aerobically and anaerobically grown cells. The twofold increase in the total fatty acid per cell between anaerobically and aerobically grown cells was accompanied by a marked change in the fatty acid composition (Table 2). In anaerobically growing *S. aureus*, C_{18} and C_{20} comprised 59% of the total fatty acids. These plus i-C_{11}, C_{14}, a-C_{15}, C_{16}, and C_{19} comprised 91% of the total fatty acids. The unsaturated fatty acids represented 4.3% of the total. In aerobically growing cells, a-C_{15} and C_{20} comprised 58% of the total, and these plus C_{16}, a-C_{17}, i-C_{17}, C_{18}, a-C_{19}, i-C_{19}, and C_{19} comprised 91% of the total fatty acids. The

TABLE 2. *Proportions of the fatty acid methyl esters in aerobically and anaerobically grown Staphylococcus aureus*

Fatty acid[a]	Percentage of the total fatty acids					
	Aerobic				Anaerobic	
	Total	Residue	Unsaturated	Hydrogenated	Total	Residue
10,i				0.08	0.39	0.84
10,a				0.04	0.12	0.41
10		2.73	0.48	0.03	0.33	1.41
10 =		0.34	0.75	0.04	0.04	2.21
11,i	0.05	0.79	1.17	0.11	8.40	
11,a	0.07	0.17	0.72	0.01	0.10	0.53
11,a =			1.24			
11	0.05	0.10	0.37	0.06	0.02	13.58
11 =	0.01	0.03	0.97	0.03	0.07	1.24
12,i	0.01	0.44	0.48	0.07	0.18	2.03
12,i =	0.01	0.21	1.03	0.01		0.47
12,a	0.04	0.69		0.02	0.02	
12,a =	0.06	0.16	0.31		0.07	0.38
12	0.04	0.62	0.46	0.05	0.10	0.81
12, =		0.35	1.16	0.01	0.17	2.59
12 =	0.04					
13,i	0.05	0.40	0.74	0.38	0.51	0.66
13,i =		0.41				0.62
13,a	0.32	0.10		0.11	0.23	0.62
13,a	0.07		0.11			
13	0.24	0.83	0.28	0.11	0.36	0.29
13 =	0.14	0.58	0.29	0.01	0.04	1.77
13 =			1.10		0.20	
14,i	0.52	0.68	0.11	1.64	0.63	0.74
14,i	0.05					
14,a	0.20	0.46	0.09	0.01	0.03	
14,a		1.14	0.75			
14	0.71	1.21	2.62	0.78	8.11	3.58
14 =	0.14	0.22	0.29	0.09	0.17	2.03
14 =	0.27	1.44	1.84		0.47	
15,i	3.46	1.01	1.84	11.10	3.28	2.42
15,a	36.32	36.91	12.41	35.88	11.32	16.66
15	0.33	0.10	0.64	0.06	0.59	0.96
15 =	0.31	0.44	1.03	0.05	0.29	2.65
15 =	0.44	1.06	2.90		0.78	1.59
16,i	0.86	0.88	0.46	0.58	0.62	0.68
16a	0.05	0.07			0.06	0.26
16	4.23	2.65	7.47	3.56	1.28	8.04
16 =	0.38	0.18	5.22	0.05	0.63	0.49
16 =		0.35				1.34
17,i	3.54	2.08	1.29	2.21	0.25	0.19
17,a	8.50	6.96	5.16	7.44	0.42	0.10
17,a =					0.02	0.10
17	0.80	0.63	0.23	0.53	0.10	0.82
17 =	0.04	0.16	0.86	0.06	0.19	1.00
17 =	0.13	0.14				
18,i	0.50	0.39		0.32	0.02	
18,a		0.01				
18	2.71	16.32	8.49	14.82	21.41	13.43
18 =	0.37	0.04	17.58	0.02	0.61	0.74
18 =	0.12	0.18	3.45		0.34	1.03

Continued

TABLE 2—*Continued*

Fatty Acid[a]	Percentage of the total fatty acids					
	Aerobic				Anaerobic	
	Total	Residue	Unsaturated	Hydrogenated	Total	Residue
19,i	2.87	2.26	0.46	2.12	0.05	
19,a	4.95	4.74	2.87	3.26		
19,a =			3.45			
19	2.78	2.15		1.70	2.62	0.37
19 =	0.10	0.13	0.11		0.02	0.66
19 =						
20,i	0.17	0.24	0.57	0.06		
20,a						
20	21.67	3.80	5.17	12.15	37.65	17.51
21,i	0.50	0.77		0.10		0.07
21,a	0.55	0.64		0.19		
21	0.08	0.18	0.69	0.05	0.53	0.44
Total fatty acids	235.0	4.9			115.0	1.48

[a] Fatty acid methyl esters are designated as the number of carbon atoms, with = for monoenoic, a for anteiso, and i for iso branched fatty acids. With the aerobically grown bacteria, the residue indicates the fatty acids left after the third extraction, unsaturated indicates the fatty acid methyl esters that were concentrated as mercuric adducts, and hydrogenated indicates the fatty acid esters after hydrogenation of the total fatty acid esters in the first column. Values are expressed as percentage of the total fatty acid. Blanks indicate that less than 0.01% was present. The bottom line indicates the total fatty acids measured colorimetrically and expressed as micromoles per gram (dry weight).

unsaturated fatty acids comprised 2.8% of the total fatty acids.

Extraction of the lipids. The maximal yields of lipid phosphate, lipid glucose, lipid lysine, lipid glycerol, and lipid acyl ester were achieved by adjusting the *p*H of the growth medium to 2.0 before harvest, heating the bacterial pellet after centrifugation in isopropanol, and then extracting by a modified Bligh and Dyer procedure (19). The data of Table 3 indicate that the extraction procedure was effective in removing 98 to 99% of the total fatty acids in the bacterium. Repeated experiments with one-tenth the concentration of bacteria and half the volume of solvent used for the experiment reported in Table 3 indicated that, with careful washing of the pellet after the initial extraction, less than 0.5% of the extractable lipid fatty acids were not recovered after a single extraction. This was the usual scale of the extraction.

Further evidence for the completeness of the extraction procedure lies in the differing proportions of the fatty acids in the residue and the total lipids. With both aerobically and anaerobically grown bacteria, the residue contained 2- to 20-fold higher proportions of the fatty acids between i-C_{10} and $C_{12}^{=}$ and the monounsaturated fatty acids $C_{12,}^{=}$ $C_{13}^{=}$, $C_{14}^{=}$, $C_{15}^{=}$, a-$C_{16}^{=}$, $C_{16}^{=}$, $C_{17}^{=}$, $C_{18}^{=}$, and $C_{19}^{=}$. The relative increase in monoenoic fatty acid esters in the residue was greater in anaerobically grown cells. The proportion of unsaturated fatty acids in aerobically grown cells between total lipid and residue increased from 2.8% to 7.8%. In anaerobically grown cells the proportions of unsaturated to saturated fatty acids between total lipid and residue increased from 4.4% to 21.5%. The relative proportions of the fatty acids in the residue compared to the total increased in the aerobically grown bacteria and decreased in the anaerobically grown bacteria for i-C_{11}, a-C_{12}, and C_{21}; the opposite was true of C_{15}, C_{16}, C_{17}, and C_{18}^{-} (Table 2). The pro-

TABLE 3. *Extraction of the fatty acids of Staphylococcus aureus*

Fraction[a]	Aerobically grown[b]	Anaerobically grown[c]
	%	%
Residue	2.1	1.48
Extract 1	96.7	98.4
Extract 2	3.1	1.2
Extract 3	0.2	0.4
Recovery	98.2	108

[a] *S. aureus* was grown aerobically and anaerobically as described in Materials and Methods. A portion of the bacteria was saponified, and the fatty acids were determined colorimetrically (11). A second portion was extracted with the modified Bligh and Dyer procedure described previously (19), the lipid extract was saponified, and the fatty acids were determined colorimetrically. The residue of the first extract was extracted twice more by the Bligh and Dyer procedure (1), without heating in isopropanol, the lipids were saponified, and the fatty acids were determined colorimetrically. The residue after these extractions was then saponified and the fatty acids were determined colorimetrically. Recovery gives the comparison of the fatty acids measured after saponification of the whole bacteria compared with the sum of the fatty acids from the extract and the residue. Palmitic acid was used to standardize the colorimetric assay. A total of 0.913 mg (dry weight) of aerobically grown cells and 0.700 mg (dry weight) of anaerobically grown cells was extracted.

[b] The total fatty acids in aerobically grown cells was 54.5 μmoles.

[c] The total fatty acids in anaerobically grown cells was 16.6 μmoles.

portions of fatty acids in the residues of aerobically and anaerobically grown *S. aureus* after extraction differed significantly from the total lipids and from each other.

Free fatty acids. The data in Table 4 show that the percentage of the total lipid fatty acids not esterified in either aerobically or anaerobically grown *S. aureus* was less than 0.12%.

Neutral and phospholipid fatty acids. Neutral lipids were separated from the glucolipids and phospholipids by chromatography on silicic acid (18). The neutral lipid fraction contained 5.2% of the lipid fatty acids in anaerobically grown *S. aureus*. When the bacteria formed the electron transport system and grew aerobically, the proportion of neutral lipid fatty acids increased to 9.2% (Table 4).

Separation of glucolipids and phospholipids. Glucolipids and phospholipids were recovered from several thin-layer plates that were chromatographed in the two-dimensional system developed previously (19). The lipid was carefully dried by successive evaporations in the rotary evaporator in benzene-absolute ethyl alcohol (4:1) and was subjected to silicic acid chromatography. The elution scheme illustrated in Fig. 2 was then applied. There was no detectable contamination of glucolipids by phospholipids or of phospholipids by glucolipids.

Fatty acids of the glucolipids. The evidence of significant differences in the proportions of the fatty acids of the monoglucosyl and diglucosyl diglyceride and the difference in the magnitude of the change in various fatty acids that occurred with the formation of the electron transport system indicate clearly that the monoglucosyl diglyceride is not an artifact of the isolation procedure. Monoglucosyl diglyceride contained significantly higher proportions of a-C_{11}, a-C_{12}, a-$C_{12}^{=}$, and $C_{15}^{=}$ and a significantly lower proportion of i-C_{10}, $C_{12}^{=}$, and $C_{14}^{=}$ than did the diglucosyl diglyceride in both aerobically and anaerobically grown bacteria. The fatty acid composition of the complex lipids are given in Table 5. There were significant differences in the fatty acid composition of the glucolipids of bacteria grown anaerobically and bacteria grown aerobically. The absolute amount of each fatty acid per gram (dry weight) in each complex lipid with aerobic and anaerobically grown bacteria is given in Table 6. These data are summarized in Table 7, where the significant differences in the composition between anaerobically and aerobically grown bacteria can be more readily observed. The absolute amount of 11 fatty acids *increased* at least twofold in one glucolipid and *decreased* at least twofold in the other glucolipid during the shift to aerobic growth.

Fatty acids of lysyl phosphatidyl glycerol. There were differences in the proportions of fatty acids in lysyl phosphatidyl glycerol and phosphatidyl

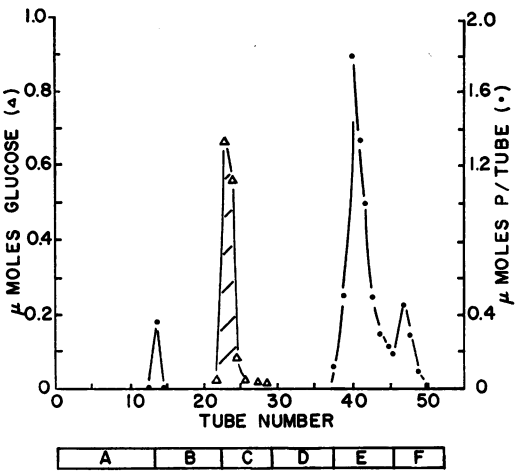

FIG. 2. *Separation of glucolipids and phospholipids from Staphylococcus aureus. Glucolipids and phospholipids were recovered from two-dimensional thin-layer plates as one fraction, dried by successive evaporations in vacuo in benzene-absolute ethyl alcohol (4:1, v/v), and applied to a 1.75 cm × 14 cm column of silicic acid (Unisil, 60–100 mesh). The following solvents were used: (A) chloroform; (B) chloroform-acetone, 1:1; (C) acetone; (D) chloroform-methanol, 98:2; (E) chloroform-methanol, 2:1; and (F) methanol. A 60-ml amount of each solvent was used at a 1 ml/min flow rate, and 7-ml fractions were collected. Recovery of the lipid phosphate was 100%; recovery of the lipid glucose was 92%.*

TABLE 4. *Comparison of the distribution of fatty acids between free and esterified and neutral and complex lipids in anaerobically and aerobically grown Staphylococcus aureus*

Fatty acids[a]	Amt of fatty acid (μmoles/g, dry wt)	
	Anaerobically grown	Aerobically grown
Total lipid	154.0	244.0
Free fatty acid	0.19	0.18
Neutral lipid	7.6	32.1
Complex lipid	140.2	212.3

[a] Lipid extracts were treated with Na_2CO_3, and the free fatty acids were recovered as described in Materials and Methods. Neutral lipids and complex lipids (glucolipids and phospholipids) were separated by silicic acid chromatography and saponified (18). The recovery from the column was 96 and 92% for the anaerobically and aerobically grown samples, respectively. Fatty acids were assayed colorimetrically with palmitic acid as the standard (11, 18).

glycerol. Lysyl phosphatidyl glycerol contained three- to fivefold higher proportions of i-C_{11}, C_{11}, i-C_{12}, a-C_{12}, $C_{12}^{=}$, a-$C_{14}^{=}$, $C_{14}^{=}$, a-$C_{16}^{=}$, and C_{19}, as well as lower proportions of C_{14} and-a-C_{19}, in both aerobically and anaerobically growing cells than were found in phosphatidyl glycerol. In these experiments, there was a 16% decrease in the lysyl phosphatidyl glycerol content per bacterium during the shift to aerobic growth. Even so, there was at least a twofold increase in the absolute amount of six fatty acids in this lipid. The absolute amounts of 10 fatty acids decreased at least twofold in lysyl phosphatidyl glycerol and increased at least twofold in phosphatidyl glycerol during the shift to aerobic growth. These data are illustrated in Tables 5, 6, and 7.

Fatty acids of cardiolipin. A comparison of the proportions of fatty acids of cardiolipin and phosphatidyl glycerol showed that cardiolipin contained significantly higher proportions of i-C_{11}, C_{11}, $C_{11}^{=}$, C_{15}, i-C_{16}, a-$C_{16}^{=}$, i-C_{17}, and a-C_{17}, and significantly lower proportions of i-C_{13}, a-C_{13}, i-C_{15}, C_{18}, $C_{18}^{=}$, and a-C_{19} than phosphatidyl glycerol in both aerobically and anaerobically grown cells. During the formation of the electron transport system, the cardiolipin increased about 1.7-fold (3). Despite this *increase*, there was at least a twofold *decrease* in the content of 11 fatty acids in the cardiolipin. For seven of these fatty acids, there was at least a twofold *increase* in the absolute amount of fatty acid in the phosphatidyl glycerol with the shift to aerobic growth (Tables 5–7).

Fatty acids of phosphatidyl glycerol. The total fatty acid composition of phosphatidyl glycerol reflects the twofold increase in the phosphatidyl glycerol. Even though there was a twofold increase in the content of phosphatidyl glycerol per bacterium, the absolute content of C_{10}, $C_{11}^{=}$, i-C_{13}, $C_{13}^{=}$, i-C_{14}, and $C_{16}^{=}$ decreased at least twofold (Tables 5–7).

DISCUSSION

The fatty acid analyses reported in this paper were obtained by growing portions of the same inoculum of *S. aureus* aerobically and anaerobically. Repeated analyses of portions of the same sample showed the proportions of the fatty acid methyl esters, calculated as percentage of the total fatty acid esters, to be accurate to 1% for each component. Consequently, the differences, both in the proportions of esters between the lipids and in the changes in proportions of the esters in each lipid with the formation of the electron transport system, are valid. The reproducibility of the proportions of the fatty acids in the isolated lipids was examined by comparing the fatty acids of the isolated lipids of three independently grown *S. aureus* cultures that were incubated aerobically and two cultures incubated anaerobically. The maximal variability in the proportions of fatty acid methyl esters with some lipid was ±3 to ±5%. The usual variability in proportions was between ±0.2 to ±1% for each ester in the same lipid. The pattern of fatty acids from the same strain of *S. aureus* grown in the same way was reproducible.

Examination by gas-liquid chromatography of the fatty acid methyl ester patterns of the lipids of *S. aureus*, with particular attention to the minor components, revealed the following facts.

(i) The fatty acids of *S. aureus* are numerous if the minor components are included in the examination. The fatty acids include saturated normal, iso, and anteiso branched fatty acids. In addition, 3 to 4% of the fatty acids are normal, iso, and anteiso branched monoenoic fatty acids. Positional isomers of normal monoenoic fatty acids can be identified between C_{12} and C_{19} by their retention times. Normal monoenoic positional isomers of C_{14}, C_{16}, and C_{18} have been documented in streptococci (12). The fatty acids in *S. aureus* were identified by the relative retention times of their methyl esters after gas-liquid chromatography. The monoenoic fatty acids were concentrated by chromatography of their mercuric adducts and saturated by hydrogenation, yielding the predicted products.

(ii) The extraction procedure developed previously (19) removed 98 to 99% of the total fatty acids. The residue that remained after the extraction contained markedly different proportions of the fatty acids than were found in the lipids. Both proportions of the shorter fatty acids i-C_{10} to $C_{12}^{=}$ and the monoenoic fatty acids increased markedly in the residue. The extraction procedure was effective in removing esterified fatty acids from the membrane.

(iii) An examination of the lipids indicated that there were essentially no free fatty acids in *S. aureus*. The neutral lipids accounted for 5 to 9% of the lipid fatty acids. The major portion of the fatty acids were part of the complex lipids which are mono- and diglucosyl diglyceride, phosphatidyl glycerol, lysyl phosphatidyl glycerol, and cardiolipin.

(iv) We have shown that the shift from anaerobic to aerobic growth in *S. aureus* involves the formation of an electron transport system. The formation of this membrane-bound system is accompanied by a coordinate 1.3-fold increase in the glucosyl diglycerides, a 1.6-fold increase in vitamin K_2 and cardiolipin, and a 2-fold increase in phosphatidyl glycerol (3). During the formation of the electron transport system, there is a

TABLE 5. *Proportions of the fatty acids in the lipids of Staphylococcus aureus grown aerobically and anaerobically*

Fatty acid[a]	Percentage of total fatty acids									
	Aerobically grown					Anaerobically grown				
	LPG[b]	PG	C	MGDG	DGDG	LPG	PG	C	MGDG	DGDG
9									0.07	
10,i				0.1	0.86	0.19			0.03	0.85
10,a	0.15			0.12	0.09	0.06		2.11	0.21	0.05
10	0.42		0.20	0.08	0.22	0.28	0.10	2.19	0.18	0.06
10 =	0.17		0.11	0.07	0.02			0.42	0.06	
11,i	0.14	0.07	0.40	0.15	0.13	0.16	0.02	0.15	0.19	0.18
11,a	0.21	0.14	0.10	0.53	0.11	0.28	0.17	1.08	0.31	0.02
11	0.27	0.11	0.17	0.17	0.17	0.84	0.01	0.88	0.08	0.03
11 =	0.32	0.02	0.05	0.37	0.05		0.06	0.20	0.07	0.06
12,i	0.45	0.11	0.03	0.09	0.15	0.52	0.01	0.56	0.09	0.16
12,i		0.04		0.14				0.13		0.01
12,a	0.28	0.15	0.13	0.26	0.08	0.56	0.08	0.45	0.13	0.04
12,a =	0.55	0.03		0.33			0.03		0.04	
12	0.41	0.31	0.02	0.07	0.19	0.25	0.06	0.22	0.17	0.26
12 =	0.65	0.18	0.05	0.09	0.85	0.15	0.11	0.58	0.18	0.23
12 =	0.21			0.23		0.57	0.09			
13,i	0.23	0.23	0.18	0.24	0.03	0.41	3.81	0.13	0.61	0.90
13,i =	0.64					0.23	0.44		0.31	
13,a	0.05	0.20	0.05	0.21	0.33	0.21	0.20	0.03	0.07	0.91
13,a =	0.17	0.08	0.04	0.07						
13	0.21	0.43	0.24	0.31	0.03	0.21	0.14	0.66	0.15	0.31
13 =	0.10	0.36	0.12	0.07	0.29	1.27	1.11	0.05	0.04	0.09
13 =			0.09	0.29				0.06	0.12	
14,i	0.58	0.22	0.40	4.7	0.42	0.73	2.68	0.80	1.14	2.28
14,i =		0.09	0.07		0.04			0.67		
14,a	0.01	0.45	0.32	0.11	0.50	1.09		1.00	0.26	0.30
14,a =	0.22					2.66				
14	0.53	0.59	0.35	1.23	0.66	1.74	3.46	3.30	7.39	4.14
14 =	1.29	0.58	0.10	0.46	0.46		0.34	2.23	0.55	0.11
14 =	1.13	0.66	0.45	0.81	6.18					0.10
15,i	5.07	8.49	6.28	7.43	10.73	3.47	11.17	4.48	3.07	5.91
15,a	25.26	34.01	40.94	31.37	33.87	10.50	28.02	23.55	13.46	14.71
15	0.24	0.24	0.98	0.27	0.11	0.65	0.50	0.64	0.71	0.63
15 =	2.92	0.38	0.16	0.21	0.16	1.29	0.15	1.98		0.20
15 =		0.59	0.33	0.66	0.25				0.53	0.18
16,i	1.50	0.73	0.87	0.58	0.49	0.71	0.42	1.86	0.37	0.30
16,a	0.16	0.02	0.02	0.07	0.03	0.33	0.02		0.03	0.06
16,a =	0.71	0.12	0.21	0.34	0.11	0.14	0.07	0.40	0.11	1.53
16	3.98	2.09	1.82	1.34	0.56	8.50	3.95	5.44	11.43	9.88
16 =	1.83	0.14	0.12	5.07	2.56	1.44	0.92	2.12	0.64	0.80
16 =										
17,i	0.34	1.12	2.55	6.45	3.93	0.66	0.54	0.60	0.20	0.26
17,a	7.24	1.64	7.16	6.83	8.87	1.03	1.10	1.46	0.41	0.55
17	0.61	0.51	0.27	0.73	0.34	1.03	0.64	1.66	0.72	8.26
17 =	0.56	1.24	0.03	0.33		1.30	0.03	0.11	0.06	0.10
17 =		0.07		0.14					0.07	
18,i	0.83	0.68	0.52	0.34	0.36	0.03	0.02	0.03	0.03	
18,a	0.03	0.01	0.03			0.03	0.01	0.01	0.01	0.01
18	9.57	12.68	9.98	10.0	11.52	26.13	21.44	13.44	20.60	27.60
18 =	0.42	0.03		0.58		1.30	0.09		0.38	0.29
18 =				0.16					0.06	0.16

Continued

TABLE 5.—(*Continued*)

Fatty acid[a]	Percentage of total fatty acids									
	Aerobically grown					Anaerobically grown				
	LPG[b]	PG	C	MGDG	DGDG	LPG	PG	C	MGDG	DGDG
19,i	3.27	2.80	3.07	1.77	0.28	0.75	0.21	0.01	0.03	0.19
19,a	5.78	7.93	5.67	2.28	0.42	0.21	0.30	0.05	0.07	0.10
19	3.12	1.66	1.23	0.96	1.58	4.37	0.98	3.32	2.28	2.51
19 =		1.07	0.72	0.15	0.64	0.55	0.01	0.07	0.07	0.05
19 =		0.01	0.04		0.03					
20,i		0.28	0.40	0.12				0.07		
20,a		0.01					0.01			
20	16.56	15.91	13.07	12.0	12.43	22.17	16.51	20.49	31.68	21.10
21,i		0.78							0.03	
21,a		0.80							0.26	0.19
21		0.02		0.23	0.10				0.15	0.26
Total	14.0	95.0	31.0	5.6	14.0	18.8	50.0	18.0	4.5	11.0

[a] Fatty acids are indicated as in Table 2. The last line indicates the micromoles of each lipid per gram (dry weight).

[b] LPG, PG, C, MGDG, and DGDG indicate lysyl phosphatidyl glycerol, phosphatidyl glycerol, cardiolipin, monoglucosyl diglyceride, and diglucosyl diglyceride.

doubling of the total fatty acids. In the course of this doubling of the total fatty acids, there are 3- to 200-fold increases in branched and unsaturated fatty acids of 13, 17, 19, 20, and 21 carbon atoms, as well as decreases in the molar content per bacterium of several fatty acids. The residue left after extraction of the lipids also exhibits changes in the proportions of the fatty acids before and after the formation of the electron transport system. The absolute amounts of the individual fatty acids in each of the complex lipids of the bacteria show striking changes during membrane modification. The proportions of fatty acids in mono- and diglycosyl diglycerides differ in both aerobically and anaerobically grown *S. aureus*. During the formation of the electron transport system, there is a coordinate formation of these two lipids, yet there are marked changes in the absolute amounts of the different fatty acids in the two lipids. This difference rules out the possibility that the monoglucosyl diglyceride is generated during the isolation of the lipids from the diglucosyl diglyceride. It is likely that the diglucosyl diglyceride is synthesized from the monoglucosyl diglyceride in *S. aureus* as the dimannosyl diglyceride is formed from monomannosyl diglyceride and guanosine diphosphate (GDP)-mannose (9). Since the manomannosyl diglyceride is formed from the reaction of GDP-mannose and diglyceride (9), the fatty acid patterns of both lipids should be identical.

Phosphatidyl glycerol is the source of the fatty acids of lysyl phosphatidyl glycerol as the lipid is formed by the condensation of lysyl-soluble ribonucleic acid and phosphatidyl glycerol in *S. aureus* (8). However, in this study we have found that the proportions of the fatty acids in the lysyl phosphatidyl glycerol and phosphatidyl glycerol are different. In addition, there are different changes in the absolute amounts of the individual fatty acids during the formation of the electron transport system between these two lipids.

Cardiolipin is formed by the condensation of phosphatidyl glycerol and cytosine diphosphate (CDP)-diglyceride in *E. coli* (13). A CDP-diglyceride also reacts with L-α-glycerol phosphate to form phosphatidyl glycerol phosphate (2). Consequently, the fatty acid pattern of the three major phospholipids should reflect the fatty acid pattern of the CDP-diglyceride and these patterns should be identical. The CDP-diglyceride is formed from CTP and phosphatidic acid. Phosphatidic acid amounting to 1% of the lipid phosphate has been detected in the lipids of *S. aureus* labeled with ^{32}P (F. E. Frerman, *unpublished data*). Insufficient material has accumulated to allow fatty acid analysis. However, the phosphatidic acid in *Haemophilus parainfluenzae* has a different fatty acid pattern from the phosphatidyl glycerol, phosphatidyl ethanolamine, and cardiolipin (D. C. White, *unpublished data*).

It appears likely that the complex lipids of *S. aureus* are formed by pathways similar to those demonstrated in other organisms. Diglyceride is the precursor of phosphatidic acid and thus of the phospholipids, and is itself the precursor of

TABLE 6. *Changes in absolute amounts of fatty acids in the complex lipids of Staphylococcus aureus during the shift from anaerobic to aerobic growth*[a]

Fatty acid	LPG		PG		C		MGDG		DGDG	
	An → aer		An → aer		An → aer		An → aer		An → aer	
10,i	35.1						1.4	5.6	93.5	120.4
10,a	11.3	21.0			379.8		9.5	6.7	5.5	12.6
10	52.6	58.8	50.0		394.2	65.1	8.1	4.5	6.6	30.8
10 =		23.8			75.6	34.1	2.7	3.9		2.8
11,i	30.1	19.4	10.0	66.5	27.0	124.0	8.6	8.4	19.8	18.2
11,a	52.6	29.4	85.0	133.0	194.4	31.0	14.0	29.7	2.2	15.4
11	157.9	37.8	5.0	104.5	158.4	52.7	3.6	9.5	3.3	23.8
11 =		44.8	30.0	19.0	36.0	15.5	3.2	20.7	6.6	7.0
12,i	97.8	63.0		104.5	100.8	9.3	4.1	5.0	17.6	21.0
12,i =			5.0	38.0	23.4			7.8	1.1	
12,a	105.3	39.2	40.0	142.5	81.0	40.3	5.9	14.6	4.4	11.2
12,a =		77.0	15.0	28.5			1.8	18.5		
12	47.0	57.4	30.0	294.5	39.6	6.2	7.7	3.9	28.6	26.6
12 =	28.2	91.0	55.0	71.0	104.4	15.5	8.1	5.0	25.3	119.0
12 =	107.2	29.4	45.0					12.9		
13,i	77.1	32.2	1,905.0	218.5	23.4	55.8	27.5	13.4	99.0	4.2
13,i =	43.2	89.6	220.0				14.0			
13,a	39.5	7.0	100.0	190.0	5.4	15.5	3.2	11.8	100.1	46.2
13,a =		23.8		76.0		12.4		3.9		
13	39.5	29.4	70.0	408.5	118.8	74.4	6.8	17.4	34.1	4.2
13 =	238.8	14.0	555.0	342.0	9.0	37.2	1.8	3.9	9.9	40.6
13 =					10.8	27.9	5.4	16.2		
14,i	137.2	81.2	1,340.0	209.0	144.0	124.0	51.3	263.2	250.8	58.8
14,i =				85.5	120.6	21.7				5.6
14,a	204.9	1.4		427.5	180.0	99.2	11.7	6.2	33.0	70.0
14,a =	500.1	30.8								
14	327.1	74.2	1,730.0	560.5	594.0	108.5	332.6	68.9	455.4	92.4
14 =		180.6	170.0	551.0	401.4	31.0	24.8	25.8	12.1	64.4
14 =		158.2		627.0		139.5		45.4	11.0	865.2
15,i	652.4	709.6	5,585.0	8,065.5	806.4	1,946.8	138.2	416.1	65.0	1,502.2
15,a	1,974.0	3,536.0	14,010.0	32,309.0	4,239.0	12,691.4	605.1	1,756.7	1,618.1	4,741.8
15	122.2	33.6	250.0	228.0	115.2	303.8	32.0	15.1	69.3	15.4
15 =	242.5	408.8	75.0	361.0	356.4	49.6		11.8	22.0	22.4
15 =				560.5		102.3	23.9	37.0	19.8	35.0
16,i	133.5	210.0	210.0	693.5	334.8	269.7	16.7	32.5	33.0	68.6
16,a	62.0	140.0	10.0	19.0		6.2	1.4	3.9	6.6	4.2
16,a =	26.3	99.4	35.0	114.0	72.0	65.1	5.0	19.0	168.3	15.4
16	1,598.0	557.2	1,975.0	1,985.5	979.2	564.2	514.4	75.0	1,086.8	78.4
16 =	270.7	256.2	460.0	133.0	381.6	37.2	28.8	283.9	88.0	358.4
16 =										
17,i	124.1	47.6	270.0	1,064.0	108.0	790.5	9.0	361.2	28.6	550.2
17,a	193.6	1,013.6	550.0	1,558.0	262.8	2,219.6	18.5	382.5	60.5	1,241.8
17	693.6	85.4	320.0	484.5	298.8	83.7	32.4	40.9	908.6	47.6
17 =	244.4	78.4	15.0	1,178.0	19.8	9.3	2.7	18.5	11.0	
17 =				66.5			3.2	4.8		
18,i	5.6	116.2	10.0	646.0	5.4	161.2	1.4	19.0		50.4
18,a	5.6	4.2	5.0	9.5	1.8	9.3	0.5		1.1	
18	4,912.4	1,139.0	10,720.0	12,046.0	241.8	3,093.8	927.0	560.0	836.0	1,612.8
18 =	244.4	58.8	45.0	28.5			17.1	32.5	3,036.0	
18 =							2.7	9.0	31.9	
19,i	141.0	457.8	105.0	2,660.0	1.8	951.7	1.4	99.1	17.6	39.2

Continued

TABLE 6.—*Continued*

Fatty acid	LPG		PG		C		MGDG		DGDG	
	An → aer		An → aer		An → aer		An → aer		An → aer	
19,a	39.5	809.2	150.0	7,533.5	9.0	1,757.7	3.2	127.7	20.9	58.8
19	821.6	436.8	490.0	1,577.0	597.6	381.3	102.6	53.8	11.0	221.2
19 =	103.4		5.0	1,016.5	12.6	223.2	3.2	8.4	276.1	89.6
19 =				9.5		12.4			5.5	4.2
20,i				266.0	12.6	124.0		6.7		
20,a			5.0	9.5						
20	4,168.0	2,318.0	8,255.0	15,114.5	3,688.2	4,051.7	1,425.6	672.0	2,321.0	1,740.2
21,i				741.0			1.4			
21,a				760.0			11.7		20.9	
21				19.0			6.8	12.9	28.6	14.0

[a] Values are given in millimicromoles of fatty acid per g of bacterial dry weight. Values were calculated from the total fatty acid content of each lipid and the percentage given in Table 5. An → aer indicates the shift from anaerobic to aerobic growth. LPG, PG, C, MGDG, and DGDG indicate lysyl phosphatidyl glycerol, phosphatidyl glycerol, cardiolipin, monoglucosyl diglyceride, and diglucosyl diglyceride. Fatty acids are indicated as in Table 2.

TABLE 7. *Changes in the amounts of fatty acid per gram (dry weight) in the complex lipids of Staphylococcus aureus during the shift from anaerobic to aerobic growth*[a]

Fatty acid	MGDG	DGDG	LPG	PG	C
10	D	I	N	D	D
11,i	N	N	D	I	I
11	I	I	D	I	D
11, =	I	N	I	D	D
12,i	N	N	D	I	D
12,a	I	I	D	I	D
12, =	D	I	I	I	D
13,i	D	D	D	D	I
13,a	I	D	D	I	I
13	I	D	N	I	N
13, =	I	I	D	D	I
14,i	I	D	D	D	I
14,a	D	I	D	I	D
14, =	N	I	I	I	D
15	D	D	D	N	I
15, =	I	N	I	I	D
16,a =	I	D	I	I	N
16, =	I	I	N	D	D
17,i	I	I	D	I	I
17, =	I	D	D	I	I
18	D	I	D	I	I
19	D	I	I	I	D
19, =	I	D	D	I	I

[a] Data are taken from Table 6. D indicates a 2- to over 200-fold decrease in the amount of fatty acid per gram (dry weight) in the lipid, I indicates a 2- to over 120-fold increase in the molar content of fatty acid per gram (dry weight) in the lipid, and N indicates a less than 2-fold change in the absolute molar content of the fatty acid per gram (dry weight) in the lipid. MGDG, DGDG, LPG, PG, and C indicate monoglucosyl diglyceride, diglucosyl diglyceride, lysyl phosphatidyl glycerol, phosphatidyl glycerol, and cardiolipin.

the glucolipids. Consequently, the fatty acid pattern of the diglyceride should be reflected in the fatty acid patterns of all the complex lipids of *S. aureus*. However, the experimental data reveal that not only are the fatty acid patterns of the complex lipids different but the changes in the absolute amounts of the individual fatty acids during membrane formation are different.

A seemingly inescapable conclusion from the data in this study is that the diglyceride portion of these complex lipids must undergo extensive rearrangements of the fatty acids after the lipid has been synthesized. Mammalian acyl transferase enzymes having various positional and fatty acyl coenzyme A specificities have been described (6, 7). These enzymes are capable of postsynthetic modifications of the fatty acids of complex lipids. It is hoped that direct studies of the metabolism of the lipids can lead to some understanding of the mechanism and the function of these changes in the fatty acids of the diglyceride portions of the complex lipids in *S. aureus*.

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