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Fatty acid metabolism in *Serratia marcescens*: III. The constituent fatty acids of the cell

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SUMMARY

An analysis of the lipids of the bacterium *Serratia marcescens* has shown that palmitic acid and 9,10-methylenehexadecanoic acid constitute about 75% of the total fatty acids. Monounsaturated acids account for only about 10% of the total fatty acid and consist predominantly of 9,10-hexadecenoic acid (palmitoleic acid) and 11,12-octadecenoic acid (vaccenic acid). Three hydroxy acids that may function as intermediates in the biosynthesis of unsaturated fatty acids have also been isolated and characterized.

The nature and distribution of fatty acids as components of microorganisms have been recently reviewed (1, 2). Very little is known of the fatty acid metabolism of the genus *Serratia*, although studies of fatty acid oxidation by *Serratia marcescens* have been reported (3–7).

The ability of long-chain unsaturated fatty acids to stimulate growth and pigment production of *Serratia marcescens*, even inducing pigment formation at temperatures higher than normal, was first reported by Linnane and Still (8). A study of all aspects of the fatty acid metabolism of this organism has been undertaken in an attempt to elucidate the mechanism of this effect.

The ability of the organism to oxidize saturated fatty acids and the occurrence of hydroxy fatty acids have already been described (9, 10). This communication reports an analysis of the component fatty acids of the organism.

METHODS

Microorganisms. The strain of *S. marcescens* used in this investigation was obtained from the National Collection of Type Cultures, England (No. 1377). It produced abundant red pigment when grown at 30° and showed no tendency to form colorless mutants.

Culture. The glycerol-ammonium citrate medium of Bunting (11) was routinely employed. For analysis of the cellular lipid, the cells were grown in liquid medium 3 mm in depth in Roux bottles for 48 hr at 30°. The yield from 48 bottles was harvested by centrifugation and the cells were frozen and lyophilized. For the large-scale preparation of fatty acids, the cells were grown on Bunting's agar medium in glass dishes and harvested as previously described (12). Chloroform extracts of the media used did not contain any detectable amounts of fatty acids.

Reagents. All solvents employed were purified and redistilled before use. 3-Hydroxydecanoic acid was obtained from the Delta Chemical Co.

Extraction and Separation of Lipids. The extraction procedure employed is shown in Fig. 1, together with an actual set of figures obtained in a typical analytical experiment. In large-scale experiments, the same procedure was followed with greater amounts of solvent. All extractions were carried out at room temperature on a shaking machine. The use of ethanol and acetone ensured complete extraction of serratamic acid (13), which is only slightly soluble in ether.

Subfractionation of the "free" lipid was achieved by washing the extract into a separating funnel with 20 ml of a 1:1 mixture of ethanol and light petroleum (bp 40–60°) and rinsing the flask with a further 20 ml of the same solution. Twenty milliliters of water was added to the combined solutions and the light petroleum phase drawn off. The ethanol–water layer was re-extracted with a further 20 ml of light petroleum and the light petroleum extracts were combined.

No further material could be extracted from the cell residue after removal of the "bound" lipid by refluxing the residue in ethanol containing 10% HCl.

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The extracts were saponified in a ten-fold excess of 1 M KOH in methanol for 6 hr. After removal of nonsaponifiable material, the acids were extracted with ether and methylated with diazomethane. In large-scale preparations, boron trifluoride in methanol (14) was used as the methylating reagent.

Analytical Methods. The methyl esters of fatty acids were analyzed by gas-liquid chromatography (GLC) using a thermal conductivity cell as detector. The chromatographic columns (1 m long x 4 mm I.D.) were packed with 60–85 mesh acid–alkali washed Celite 545 impregnated with 20% (w/w) Apiezon L and operated at 197° with a carrier gas (helium) flow of 80 ml/min. The peaks were identified by comparison with known standards, and additional evidence of the identity of the components in the mixture was obtained by analyzing the mixture of esters or isolated components at 180° on a column of acid-washed Celite 545 impregnated with polyethylene–glycol adipate 8:2.5 (w/w).

Large scale separations were carried out at 220° using columns (1 m long x 12 mm I.D.) containing 44-60 mesh acid–alkali washed Celite 545 impregnated with 25% (w/w) Apiezon L.

Phosphate was estimated by Allen's procedure (15).

Infrared absorption spectra were measured with a Perkin-Elmer model 21 recording spectrophotometer with the sample in a film phase between two rocksalt plates.

RESULTS

The composition of the three lipid fractions obtained after extraction of lyophilized cells is shown in Table 1. No acid containing 20 or more carbon atoms could be detected. It was found that palmitic acid and an acid containing 17 carbon atoms and a cyclopropane ring accounted for about 75% of the total fatty acid. No acids with more than one double bond could be detected and the unsaturated acids containing 16 and 18 carbon atoms accounted for only 10% of the total fatty acids. Very little stearic acid is present; this fact has been observed in other microorganisms (16–20).

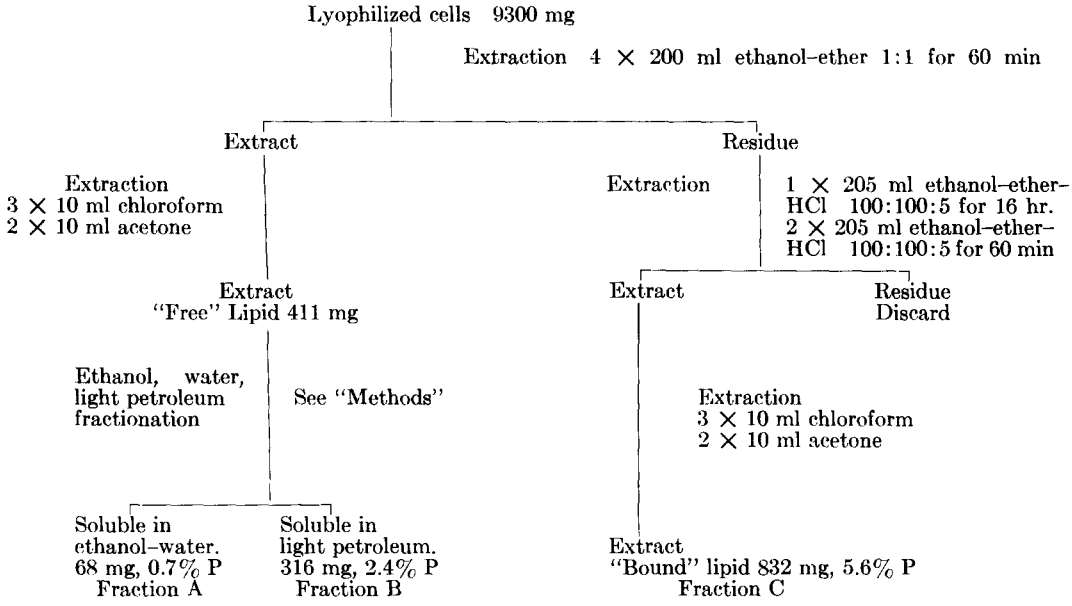

FIG. 1. Extraction scheme and weight of lipid fractions.

The occurrence of the three hydroxy acids has been previously reported (10). It can be seen that sub-fractionation of the "free" lipid results in almost complete separation of the hydroxy acids into the ethanol–water phase, together with small amounts of myristic, palmitic, and the C_{17} cyclopropane acids.

The form in which the hydroxy acids occur in the cell has not yet been elucidated, but the isolation of serratamic acid (*N*-3-hydroxydecanoyl-serine) (13) and serratamolide (a dilactone of serratamic acid) (21) from *Serratia* have previously been reported. Fukui and Axelrod (22) have isolated a similar amino acid–fatty acid compound, *N*-oleoyl-phenylalanine, from rat liver. At this stage, the only amino acid detected in the hydrolysate of the material in the ethanol–water phase

TABLE 1. COMPONENT FATTY ACIDS OF *Serratia marcescens* LIPID

	Fraction of "Free" Lipid Soluble in Ethanol–Water	Fraction of "Free" Lipid Soluble in Light Petroleum	"Bound" Lipid
Wt of fraction (mg)	68	316	832
Wt of acids isolated after saponification (mg)	24	243	318
Fatty Acid	Percentage Composition of Fatty Acids		
Saturated acids			
Decanoic	...	+*	+
Undecanoic	...	+	+
Dodecanoic	...	+	+
Tridecanoic	...	+	+
Tetradecanoic	4.0	6.0	6.0
Pentadecanoic	...	+	0.6
Hexadecanoic	15.6	43.8	46.0
Unknown I	...	+	+
Heptadecanoic	...	+	+
Unknown II	...	+	+
Octadecanoic	...	0.5	0.8
Unsaturated acids			
Hexadecenoic	...	2.3	1.6
Octadecenoic	...	10.5	7.5
Hydroxy acids			
3,OH-decanoic	44.7	0.7	1.8
3,OH-5-dodecenoic	9.6	+	0.2
3,OH-dodecanoic	14.5	+	0.4
Cyclopropane acids			
Methylenehexadecanoic	11.1	32.4	29.8
Methyleneoctadecanoic	...	3.1	4.3

* A + sign indicates that the component was detected and identified on the basis of retention time but that it did not occur to an extent greater than 0.2%.

has been serine. Serine was identified by paper chromatography in butanol–butanone–water 2:2:1 and ethanol–butanol–water–dicyclohexylamine 10:10:5:2 by comparison with standard amino acids.

Identification of Hydroxy Acids. The structures of the three hydroxy acids have been previously established (10) by means of their infrared spectra, retention volumes on Apiezon L and polyethylene-glycol adipate columns in GLC, hydrogen uptake, and the fragments isolated after oxidation by potassium permanganate in acetic acid (23). The infrared spectra of the methyl esters were examined together with an authentic sample of methyl 3-hydroxydecanoate. Each compound showed the characteristic hydroxyl absorption at about 2.9 μ. The absorption of methyl 3-hydroxy-5-dodecenoate at 10.36 μ gave no indication of the presence of any *trans* isomer (24).

TABLE 2. RETENTION VOLUMES OF FATTY ACID METHYL ESTERS RELATIVE TO THOSE OF METHYL MYRISTATE

	Liquid Phase	
	Nonpolar (Apiezon L at 197°)	Polar (PEGA at 180°)
n-Decanoate	0.17	0.26
3-Hydroxydecanoate	0.31	1.58
n-Dodecanoate	0.43	0.51
3-Hydroxy-5-dodecenoate	0.68	3.37
3-Hydroxydodecanoate	0.85	3.10
9,10-Methylenehexadecanoate	3.40	3.14
11,12-Methyleneoctadecanoate	7.92	5.68

Table 2 shows the retention volume of the methyl esters of the hydroxy acids relative to methyl myristate on two stationary phases. The presence of an hydroxyl group leads to a greatly increased retention volume, particularly on the polyester column, and is a useful criterion for identification. A grid system constructed on the basis of log retention volume on two columns, used by James (25) for the identification of the degree of unsaturation of fatty acids, can also be used to indicate the presence of an hydroxyl group.

The Isomeric Composition of the Monounsaturated Acids. The palmitoleic and oleic acid esters were collected from the effluent of the gas chromatogram. The esters were oxidized with potassium permanganate and the resulting mixtures of mono- and dicarboxylic acids methylated and analyzed by GLC on Apiezon L at 197° (helium flow, 20 ml/min) (23). The results are shown in Tables 3 and 4. Five isomers of hexadecenoic acid were identified, although the Δ^9 and Δ^8 isomers comprised 93% of the total. In a similar fashion, Δ^{11} (vaccenic) and Δ^{10} octadecenoic acids account for 90% of the five isomeric octadecenoic acids detected. Infrared spectra did not reveal the presence of any *trans* isomers in the unsaturated acids.

Degradation of authentic samples of methyl palmitoleate and methyl oleate under the same experimental conditions gives rise to only traces of acids arising from further degradation of the primary reaction products. The total area of the peaks corresponding to these acids accounted for less than 2% of the total peak area. It is not considered likely, therefore, that the isomers occurring in small amounts in the hexadecenoic and octadecenoic acids are artifacts of the oxidation reaction. For example, the tridecanoic acid occurring in the oxidation products of the hexadecenoic acid peak could not arise from further degradation of the oxidation products of the predominating isomers.

The Structure of the Cyclopropane Acids. The presence of a cyclopropane ring in two of the fatty acids was

TABLE 3. FRAGMENTS ISOLATED AFTER OXIDATIVE DEGRADATION OF THE HEXADECENOIC ACID PEAK

Monocarboxylic Acid	Dicarboxylic Acid	Parent Unsaturated Acid	Approx. Percentage of Total*
C_7	C_9	Δ^9-Hexadecenoate	69
C_8	C_8	Δ^8-Hexadecenoate	24
C_9	C_7	Δ^7-Hexadecenoate	5
...	C_5	Δ^5-Hexadecenoate	+†
C_{13}	C_3	Δ^3-Hexadecenoate	+

* The percentage composition of the mixture of the isomers is based on the relative areas of the peaks corresponding to the recovered dicarboxylic acids.

† See footnote, Table 1.

established by infrared spectroscopy of the methyl esters, the peak at 9.8 μ characteristic of cyclopropane compounds being readily detectable. On the basis of retention volumes on GLC, one acid was found to contain 17 carbon atoms, the other 19. Cyclopropane acids exhibit properties similar to monounsaturated acids in GLC, moving ahead of the corresponding saturated acid on nonpolar columns and behind on polar columns (Table 2).

The infrared spectra of the two compounds were found to be very similar to one another and to an authentic sample of methyl dihydrosterculate (methyl 9,10-methyleneoctadecanoate). Neither compound took up hydrogen in the presence of platinum oxide in methanol.

The position of the cyclopropane ring was established by catalytic hydrogenation of the free acids in acetic acid, followed by oxidation of the resulting branched chain acids with chromium trioxide (26). Ketones were recovered from the reaction mixture by steam distillation and dicarboxylic acids were recovered by ether extraction of the residue.

Analysis by GLC of the ketones formed by oxidation of the C_{17} acid showed only the presence of 2-octanone and 2-nonanone. The predominant dicarboxylic acids in the residue were octandioic and nonandioic acids. The structure of the C_{17} acid is therefore shown to be 9,10-methylenehexadecanoic acid, identical to that of the acid isolated from the lipids of *Escherichia coli* (26).

TABLE 4. FRAGMENTS ISOLATED FROM OXIDATIVE DEGRADATION OF THE OCTADECENOIC ACID PEAK

Monocarboxylic Acid	Dicarboxylic Acid	Parent Unsaturated Acid	Approx. Percentage of Total*
C_7	C_{11}	Δ^{11}-Octadecenoate	74
C_8	C_{10}	Δ^{10}-Octadecenoate	16
C_9	C_9	Δ^9-Octadecenoate	7
C_{10}	C_8	Δ^8-Octadecenoate	2
...	C_5	Δ^5-Octadecenoate	+†

* The percentage composition of the mixture of the isomers is based on the relative areas of the peaks corresponding to the recovered dicarboxylic acids.

† See footnote, Table 1.

The position of the cyclopropane ring in the C_{19} acid was determined in the same way. The only ketones detected in the reaction products were 2-octanone and 2-nonanone. The major dicarboxylic acids detected were decandioic and undecandioic acids. The structure of the C_{19} acid is therefore 11,12-methyleneoctadecanoic acid, identical to lactobacillic acid (27).

Unknown Acids. Two unidentified components (I and II) were regularly present in the fatty acids but always at levels less than 0.3% of the total fatty acid. On the basis of retention volumes, I would appear to contain 17 carbon atoms, and II, 18 carbon atoms. Neither acid took up hydrogen in the presence of platinum oxide in methanol. Infrared spectra of the methyl esters did not reveal the presence of hydroxyl or cyclopropane groups in either acid. Lack of sufficient quantities has so far prevented further investigation of these components.

DISCUSSION

The three predominant acids in the lipids of *S. marcescens* (palmitic, 9,10-methylenehexadecanoic, and the octadecenoic acid isomers) together comprise about 85% of the total fatty acid, palmitic acid being present to the extent of 43% of the total fatty acid. Palmitic acid has also been found to be a major component in a wide variety of bacteria, both aerobic and anaerobic (16, 17, 26, 28, 29). By contrast, in the same organisms stearic acid is responsible for only a very minor part of the total fatty acid.

The presence of 3-hydroxydecanoic acid in *S. marcescens* in bound forms (serratamic acid and serratamolide) has been previously reported (13, 21). It has not yet been established whether all the 3-hydroxydecanoic acid is present as serratamic acid or serratamolide, and whether 3-hydroxy-5-dodecenoic and 3-hydroxydodecanoic acids are also present in a bound form in the cell.

The characterization of 3-hydroxydodecanoic acid establishes yet another instance of the occurrence of saturated straight-chain 3-hydroxy acids in microorganisms. In addition to the widely occurring β-hydroxybutyric acid, 3-hydroxyhexanoic and 3-hydroxyoctanoic acids have been isolated from the maize smut *Ustilago zeae* (30). 3-Hydroxydecanoic acid has been found in several bound forms in bacteria.

It occurs as a rhamnoside in *Pseudomonas pyocyanea* (31) and *P. aeruginosa* (32) and as a component of the polypeptide antibiotic, viscosine, in *P. viscosa* (33, 34). Ikawa *et al.* (35) have isolated 3-hydroxytetradecanoic acid from a lipopolysaccharide of *Escherichia coli*.

The principal octadecenoic acid present is vaccenic acid, while oleic acid comprises only a minor part of the mixture. This result is in accord with determinations in other species of bacteria (16, 20, 26, 28, 36) where *cis*-vaccenic acid accounts for 70–100% of the isomers of octadecenoic acid.

It has been suggested (37, 38) that unsaturated fatty acids in some microorganisms are formed by the introduction of an hydroxyl group into a medium-chain fatty acid, followed by dehydration and chain elongation. This reaction does not require molecular oxygen. For example, Δ^9-hexadecenoic acid would be formed by dehydration of 3-hydroxydecanoic acid at the 3,4-position and the addition of three C_2 units to the C_{10} unsaturated acid so formed. But if the 3-hydroxydecanoic acid were dehydrated in the 2,3-position, the resulting hexadecenoic acid would be Δ^8-hexadecenoic acid. Oxidation of the hexadecenoic acid from *S. marcescens* (Table 3) showed that the Δ^9 and Δ^8 isomers predominate. It is conceivable, therefore, that these two isomers could be formed by dehydration of the same hydroxy intermediate on either side of the hydroxyl group. An identical reaction, involving the addition of four C_2 units after dehydration of 3-hydroxydecanoic acid would give rise to Δ^{10} and Δ^{11} octadecenoate, which have been shown (Table 4) to be the major components of the octadecenoic acid of *S. marcescens*.

A similar dehydration at the hydroxyl group of 3-hydroxydodecanoic acid followed by chain elongation would give rise to the Δ^7 and Δ^6 isomers of hexadecenoic acid and Δ^9 and Δ^8 octadecenoic acids. Of these, only Δ^6-hexadecenoic acid has not been detected in the fatty acids of *S. marcescens*, and 99% of both the hexadecenoic and octadecenoic acids consist of isomers that could be formed by this scheme of chain elongation after dehydration at the hydroxyl group of a 3-hydroxy acid. The two hydroxy acids essential to this scheme, 3-hydroxydecanoic and 3-hydroxydodecanoic, have been detected in the cell lipid.

No diunsaturated acid was found to occur in the fatty acids of *S. marcescens*, but Hofmann and Tausig (36) found that the "bound" lipids of a *Streptococcus* contained 2.8% dienoic acids. Other analyses of bacterial fatty acids have failed to show the presence of such acids (17, 19, 26, 28).

The existence of cyclopropane acids in bacteria has now been established in several species (16–19, 26, 28, 37, 39). The C_{17} cyclopropane acid isolated in this study appears identical with that isolated from *E. coli* (26) and the structure of the C_{19} acid is identical with that of lactobacillic acid (27). The concentration of the cyclopropane acids in the cellular fatty acids is in agreement with those recently reported by Zalkin and Law (39).

It has been shown (19, 40) that the methylene bridge carbon atom of lactobacillic acid and the C_{17} cyclopropane acid is derived from an S-methyl carbon atom of methionine and is inserted across the double bond of an unsaturated acid. It is interesting, then, that only one isomer of each cyclopropane acid was detected. But if the amount of 9,10-hexadecenoic acid used in the formation of the C_{17} cyclopropane acid is included in the hexadecenoic acid fraction, it is found that the 9,10-isomer accounts for more than 98% of the hexadecenoic acid. In the same way, after allowing for the quantity of 11,12-octadecenoic acid incorporated into lactobacillic acid, it is found that 82% of the octadecenoic acid existed as the 11,12-isomer. Thus, unless the enzyme(s) catalyzing the transmethylation reaction had a high affinity for acids other than 9,10-hexadecenoic acid and 11,12-octadecenoic acid, the cyclopropane acids derived from these compounds would be expected to predominate by virtue of the amount of substrate available.

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