J. Gen. Appl. Microbiol., **25**, 1–9 (1979)

LIPID PROFILE OF THE STRAINS OF *AGROBACTERIUM TUMEFACIENS* IN RELATION TO AGROCIN RESISTANCE

PIJUSH K. DAS, MITALI BASU, AND GORA C. CHATTERJEE

Department of Biochemistry, University of Calcutta,
35 Ballygunge Circular Road, Calcutta 700 019, India

(Received May 30, 1978)

Studies have been carried out in detail on the lipid make up of *Agrobacterium tumefaciens* using virulent agrocin-sensitive and avirulent agrocin-resistant strains. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and phosphatidylglycerol are the major phospholipids present in *A. tumefaciens*. Although the total lipid content is increased due to development of resistance towards agrocin, the proportion of phospholipids and neutral lipids in the total lipid fraction remains almost the same either in the normal or in the resistant strains. The relative proportion of most of the individual phospholipid and neutral lipid components also remains unaltered due to development of resistance towards agrocin. With the exception of the proportion of neutral lipid, those of total lipid and phospholipid decrease steadily with an increase in the age of the culture. It is further revealed that agrocin resistance brought about an increase in the amount of unsaturated fatty acids with concomitant decrease of saturated fatty acid content in *A. tumefaciens* as obtained by gas-liquid chromatographic analysis.

Lipid and fatty acid composition of several strains of *Agrobacterium tumefaciens* have been studied earlier by several workers (*1*, *2*). It is known that certain strains of *A. tumefaciens* which are sensitive to agrocin lose their plasmids and also the capability of inducing crown gall tumor in plants with the acquirement of resistance towards agrocin (*3*). Certain resistant strains are also developed which do not lose plasmids but are incapable of inducing crown gall tumor formation. It is also known that a change in the lipid fraction is associated with difference in virulence in certain strains of pathogenic bacteria and fungi (*4*, *5*). In order to know the molecular mechanism that might be related to the alteration of virulence of the resistant strains and also the role of plasmid in this regard, it is thought worthwhile to study the lipid composition of the agrocin-sensitive, agrocin-resistant plasmid-containing and agrocin-resistant plasmid-free strains of *A. tumefaciens*. The results of this study is presented in this communication.

MATERIALS AND METHODS

Test organism and growth medium. Strains used in this investigation are agrocin-sensitive *Agrobacterium tumefaciens* TIP (Kerr 14), agrocin-resistant *A. tumefaciens* TIP (Kerr 14) R, probably completely cured of plasmid, and agrocin-resistant *A. tumefaciens* S1005. Agrocin-resistant *A. tumefaciens* TIP (Kerr 14) R and *A. tumefaciens* S1005 strains are referred to as resistant and resistant (P), respectively, throughout this communication. Cultures of all these *A. tumefaciens* strains have been obtained from Prof. J. Schell, Director, Laboratorium voor Genetica, Gent, Belgium. For lipid analysis, cultures were grown in rich YT medium containing Bactotryptone 0.8%, Bactoyeast extract 0.5%, and NaCl 0.5%, under rotatory shaking conditions at 28°.

Chemicals. All the chemicals used were of analytical grade. Organic solvents were all distilled before use. Reference phospholipid compounds were obtained from Vallav Bhai Patel Chest Institute, University of Delhi, New Delhi, India.

Extraction and purification of phospholipids and neutral lipids. Cells of desired culture age were harvested, washed thoroughly with cold 0.9% NaCl solution at 4°, and blotted dry. The wet and dry weights of the cells were taken. Lipids were extracted successively with 2: 1 (v/v) $CHCl_3$: MeOH (20 vol., twice) and 1: 1 (v/v) $CHCl_3$: MeOH (10 vol.). Extracts were pooled, the $CHCl_3$: MeOH ratio was adjusted to 2: 1 (v/v), washed with 0.9% NaCl (0.2 vol.), and evaporated to dryness under reduced pressure at 30° (*6*). The semisolid residue was dissolved in 100 volumes of $CHCl_3$ and charged on a silicic acid column (1 × 10 cm), washed with 10 volumes of $CHCl_3$ to remove neutral lipids. Polar lipids were eluted with 10 volumes of MeOH: $CHCl_3$ (9: 1, v/v) mixture. The mixture was concentrated at 30° and rechromatographed on silicic acid column, washed with 40 volumes of acetone, 5 volumes of $CHCl_3$: MeOH (9: 1, v/v), and finally phospholipids were eluted with 10 volumes of MeOH (*7, 8*).

Separation and identification of different phospholipid and neutral lipid components. Solvents were removed from eluates under reduced pressure, residue was taken up in 100 volumes of hexane, and fractionated by thin-layer chromatography (TLC) on activated silica gel G layer (250 μm thick, 20 × 20 cm). The developing solvent used for one-dimensional chromatography was $CHCl_3$: MeOH: glacial AcOH: H_2O (65: 15: 10: 4, v/v) (*9*). For two-dimensional chromatography, the solvents used were $CHCl_3$: MeOH: 7 N NH_4OH (65: 25: 4, v/v), with $CHCl_3$: MeOH: glacial AcOH: H_2O (175: 25: 25: 4, v/v) as the second solvent. For separation of neutral lipid components the solvents were petroleum ether (bp 60–70°): ether: glacial AcOH (80: 20: 1, v/v) (*10*). The separated components of phospholipids and neutral lipids were identified by chromatographic spray reagents (Table 1) after the method of DAWSON (*11*) and also by comparison of their *Rf* values with authentic samples and rat liver lipids.

Chemical estimation. Phospholipids were scraped off from the TLC plate into digestion tubes and digested at 180° with 70% $HClO_4$ saturated with ammo-

Table 1. Spray reagents used for identification of different phospholipids and neutral lipids on chromatogram.

Sl. No.	Reagents	Reference	Responsive compounds
1	Iodine vapour	—	All lipids
2	Rhodamin B and 6G	—	All lipids
3	(a) Molybdate	*12*	All phospholipids
	(b) Molybdate: $HClO_3$	*11*	All phosphates and glycerophosphates
4	Ninhydrin	*16*	Amino acids and amino lipids
5	Dragendorff	*16*	Compounds containing choline
6	Periodate-Schiff	*16*	Compounds containing –C(OH)–C(OH)– or –$C(NH_2)$–C(OH) groups
7	Anisaldehyde-H_2SO_4	*16*	Compounds containing sugars
8	Ferric chloride-hydroxylamine (50% $FeCl_3$ in 36% HCl: ether = 1: 9)	*16*	Glycerides
9	Dichlorophenol-indophenol	*16*	Free fatty acids

nium molybdate. Inorganic phosphorus was measured colorimetrically (*12*). Phospholipids were estimated on the basis of the inorganic phosphorus content. Neutral lipids, glycerides, and free fatty acids were estimated by the chromic acid reduction method (*13*). Cultures were washed, blotted dry, and weighed. Total lipid content of the weighed culture was estimated gravimetrically (*7*).

Characterization of fatty acids. Fatty acids of the total lipid fraction were esterified by the method of MANGOLD (*10*). The pooled methyl esters of the fatty acids were analysed by gas chromatography. Nitrogen was used as the carrier gas with an outlet flow of 40 ml/min and a column inlet pressure of 40 lb/in^2. Two stainless steel columns (0.63 cm × 1.8 m) were packed with 15% LP–71 (diethylene glycol succinate on 60–80 mesh Diatoport ST–111). The effluent was monitored by a hydrogen flame ionization detector in an analytical gas chromatograph (Model 700–R, F and M Scientific Corp., U. S. A.). Isothermal determinations were made at 169 and 180°. Retention time of standard samples of saturated even-numbered as well as unsaturated acids was compared with retention time of fractions in the extracted samples. Relative amounts of fatty acids were calculated from areas under the recorded peaks.

RESULTS

Separation and identification of different phospholipid and neutral lipid components on chromatogram

A typical analysis of different phospholipid and neutral lipid components is shown in Tables 1 and 2. Individual phospholipid and neutral lipids were resolved by thin-layer chromatography (both one- and two-dimensional), stained with dif-

Table 2. Phospholipid components of *A. tumefaciens*.

Component No.	*Rf* × 100 in solvent system			Reactive spray reagent	Identification of components
	1	2	3		
1	15	17	25	1, 2, 3a, 3b, 6	Phosphatidylinositol
2	17	22	15	1, 2, 3a, 3b, 4, 6	Phosphatidylserine
3	28	40	40	1, 2, 3a, 3b, 5	Phosphatidylcholine
4	64	11	70	1, 2, 3a, 3b	Phosphatidic acid
5	46	50	41	1, 2, 3a, 3b, 4, 6	Phosphatidylethanolamine
6	44	54	39	1, 2, 3a, 3b, 4	Phosphatidylmonomethylethanolamine
7	47	51	59	1, 2, 3a, 3b, 6	Phosphatidylglycerol
8	51	56	61	1, 2, 3a, 3b, 6	Cardiolipin

Composition of solvents: 1. $CHCl_3$: CH_3OH: CH_3COOH: H_2O = 65: 15: 10: 4.
2. $CHCl_3$: CH_3OH: 7 N NH_4OH = 65: 25: 4.
3. $CHCl_3$: CH_3OH: CH_3COOH: H_2O = 170: 25: 25: 4.

Table 3. Amount of total lipid, phospholipid, and neutral lipid in normal and agrocin-resistant *A. tumefaciens*.

Strain	Growth phase	Total lipid (μg/mg cell dry wt.)	Total phospholipid (μg/mg total lipid)	Total neutral lipid (μg/mg total lipid)
Normal	Early log	131.8±3.1	670.1±9.6	115.6±8.1
	Mid log	123.4±3.7	569.5±6.1	104.9±7.7
	Stationary	119.0±2.7	535.7±8.5	107.6±5.7
Resistant	Early log	157.8±3.1	652.0±4.6	110.5±8.1
	Mid log	144.0±2.6	574.9±6.3	95.5±5.6
	Stationary	142.1±4.5	513.6±7.7	100.9±9.3
Resistant (P)	Early log	153.6±2.9	640.1±8.4	105.9±6.9
	Mid log	140.6±1.8	594.2±8.4	102.2±4.9
	Stationary	139.3±2.4	530.3±7.9	99.6±6.4

Calculated from 4 independent determinations, expressed as mean value ± S.D.

ferent spray reagents characteristic of a particular functional group and also their *Rf* values were compared in different solvent systems with that of the pure reference lipid compounds. Details of the composition of different solvent systems and the responses to different spray reagents are shown in Tables 1 and 2. Eight different phospholipid compounds were identified in *A. tumefaciens* and, of the neutral lipids, mainly triglycerides, diglycerides, and free fatty acids were identified.

Quantitative estimation of different lipids in A. tumefaciens

A comparison of total lipid, phospholipid, and neutral lipid extracted from the wild-type and mutant strains of *A. tumefaciens* in different growth phase is shown in Table 3. Total lipid was estimated gravimetrically (*7*), phospholipid and neutral lipids were separated by silicic acid column chromatography and estimated after the method of RANDERATH (*12*) and the chromic acid reduction method of AMENTA

Table 4. Relative distribution of phospholipid components in different growth phase.

Strain	Growth phase	PC	PE	PMME	PG	diPG	PA	PS	PI
Normal	Early log	38.6±2.5	21.9±3.3	11.1±0.6	12.2±1.1	5.3±0.4	6.4±0.8	1.7±0.3	1.8±0.2
	Mid log	27.2±2.7	30.2±3.5	13.3±1.6	14.0±1.3	4.2±0.6	6.8±0.5	1.6±0.4	1.8±0.2
	Stationary	24.3±2.8	22.4±1.9	16.2±1.0	14.8±1.6	12.7±1.5	4.6±0.5	1.7±0.3	1.8±0.2
Resistant	Early log	34.9±4.4	24.4±2.3	13.7±1.6	11.8±1.4	6.3±0.4	5.6±0.3	1.5±0.1	1.7±0.1
	Mid log	26.4±2.4	29.9±3.6	14.8±1.4	14.5±1.2	5.5±0.6	5.3±0.2	1.7±0.1	1.6±0.1
	Stationary	25.7±2.8	20.4±2.6	17.1±1.5	14.2±1.2	11.8±1.1	5.9±0.3	1.8±0.1	1.8±0.1
Resistant (P)	Early log	36.8±2.1	23.7±2.9	12.4±1.2	11.9±1.2	5.4±0.3	6.3±0.6	1.5±0.2	1.5±0.1
	Mid log	27.2±1.9	30.0±3.5	14.5±1.4	13.8±1.6	4.8±0.5	6.2±0.7	1.5±0.1	1.6±0.3
	Stationary	24.6±2.8	21.9±2.4	16.3±1.5	14.4±1.7	12.4±1.7	5.3±0.3	1.9±0.2	2.0±0.2

Calculated from 4 independent determinations, expressed as mean value ± S.D. of % phospholipid in total phospholipid.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PG, phosphatidylglycerol; diPG, diphosphatidylglycerol or cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine; and PI, phosphatidylinositol.

(*13*), respectively. It is notable that the amount of total lipid and phospholipid decreases with increasing age of culture in both normal and mutant strains, but the change is not so significant in case of neutral lipids. It is also evident that the total lipid is increased in the resistant strains irrespective of their plasmid content.

Relative distribution of different phospholipid and neutral lipid components in A. tumefaciens

Percentage content of different phospholipid and neutral lipid components was estimated during growth and also due to resistance of *A. tumefaciens* towards agrocin, and the results are given in Tables 4 and 5.

Table 5. Relative distribution of neutral lipid components in different growth phase.

Strain	Growth phase	Triglyceride	Diglyceride	Free fatty acid
Normal	Early log	29.8±2.7	16.0±1.3	17.7±1.5
	Mid log	30.4±2.0	15.6±1.6	14.8±1.3
	Stationary	32.9±2.5	14.9±1.2	11.1±1.4
Resistant	Early log	31.7±2.4	17.4±1.9	19.2±1.9
	Mid log	30.4±2.2	16.6±2.0	16.1±1.4
	Stationary	31.6±3.3	17.2±2.1	12.4±1.4
Resistant (P)	Early log	32.0±2.3	16.4±2.1	18.3±1.7
	Mid log	30.7±2.6	16.3±1.1	15.0±1.6
	Stationary	30.3±2.7	16.4±1.9	11.1±0.7

Calculated from 4 independent determinations, expressed as mean value±S.D. of % neutral lipid in total neutral lipid.

Gas-liquid chromatographic pattern of fatty acids in normal and agrocin-resistant A. tumefaciens

The fatty acids from the total lipids of normal and agrocin-resistant *A. tumefaciens* were converted to the methyl esters and analysed by gas-liquid chromatography (GLC) (Table 6). A typical GLC tracing is presented in Fig. 1. Compounds A and B were identified as methyl esters of myristic and palmitic acids by comparing retention volume with authentic compounds. Compounds C and D had retention volume corresponding to methyl esters of hexadecenoic and octadecenoic acids, respectively. Compound E had the same retention volume as methyl lactobacillate. It is noted from Table 6 that in the case of resistant strains the amount of unsaturated fatty acids is increased with simultaneous decrease in the amount of saturated fatty acids as compared to those in the normal strains.

DISCUSSION

From responses to different spray reagents (Table 1) and by comparison of *Rf* values in different solvent systems (Table 2) with those of pure reference lipid

Table 6. Fatty acid composition of total lipids of *A. tumefaciens*.

Fatty acid methyl ester	Weight percentage		
	Normal	Resistant	Resistant (P)
Myristic ($C_{14:0}$)	0.8	Trace	Trace
Palmitic ($C_{16:0}$)	22.4	13.7	11.8
Hexadecenoic ($C_{16:1}$)	6.3	9.5	10.6
Octadecenoic ($C_{18:1}$)	30.3	43.1	40.4
Lactobacillic	41.2	33.7	37.2

Calculated from 3 independent determinations, expressed as mean value.

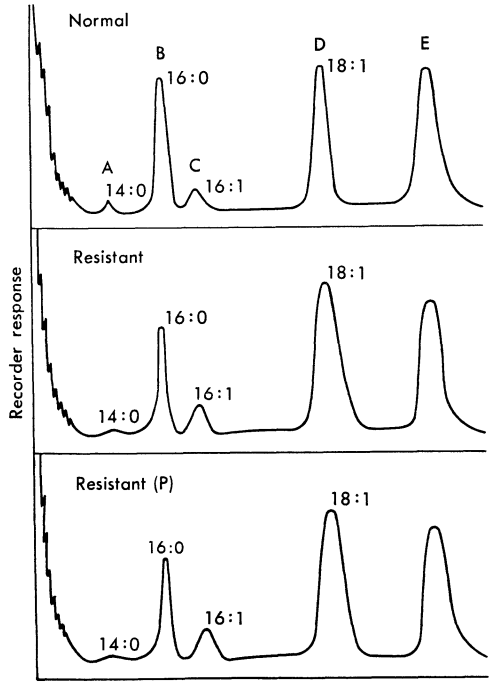

Fig. 1. Gas-liquid chromatogram of methyl esters from resistant *A. tumefaciens*.

compounds, the following phospholipids were identified in *A. tumefaciens*: Phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylethanol amine, phosphatidylmonomethylethanolamine, phosphatidic acid, and cardiolipin. Of the neutral lipids, triglycerides, diglycerides, and free fatty acids were identified in significant amounts.

It is revealed from Table 3 that the total lipid content is increased in the resistant strains as compared to that of the sensitive strains. The possibility that a higher lipid content may help to protect the microorganisms against attack by host defence mechanism was also suggested by *in vitro* experiments (*14*). It was

shown that exposure of a microorganism to antimicrobial agents led to production of much higher cellular lipid concentration. The tendency of the microorganisms to produce more lipid under conditions of stress implies that a high concentration of lipid is in some way protective. The proportion of phospholipid and neutral lipids in the total lipid fraction (Table 3) and also the relative proportion of most of the individual phospholipid components in the total phospholipid fraction (Table 4) remain almost the same in sensitive as well as in resistant organisms. Although some information is available regarding certain major phospholipid components and their relative amounts (*2*), detailed studies about the distribution of several phospholipids have been made using both the agrocin-sensitive and -resistant strains. Table 5 shows the relative proportion of triglycerides, diglycerides, and free fatty acids in the total neutral lipid fraction, which also reveals no change in the relative proportion of individual components in the strain due to development of resistance towards agrocin. Therefore it is revealed from this investigation that although the total lipid content is increased in the resistant strains, the proportion of phospholipid and neutral lipid remains almost the same in either the normal or the resistant mutant strains. This indicates that the rate of synthesis of phospholipid and neutral lipid components is also increased in the same proportion as that of the total lipid due to development of resistance towards agrocin. Plasmid has nothing to do with the synthesis of lipids as the extent of lipid formation is more or less the same in both plasmid-free and plasmid-containing *A. tumefaciens*.

It is further evident that although the proportion of neutral lipid remains almost the same (Table 3), those of total lipid and phospholipid decrease steadily with an increase in the age of the culture. This may be attributed to the decrease in the rate of synthesis of phospholipid during growth. The quantitative estimation of individual phospholipid components (Table 4) further reveals that the decrease in the extent of formation during growth mainly lies with phosphatidylcholine which comprises the major phospholipid component in *A. tumefaciens*.

The fatty acid composition of the gram-negative bacteria, *Agrobacterium tumefaciens*, conforms to a rather simple pattern common to many bacteria (*15*). Palmitic acid is the dominant straight-chain saturated fatty acid; myristic acid is the only other normal saturated fatty acid present in detectable amounts. Hexadecenoic and octadecenoic acids are the only unsaturated fatty acids. The amount of these unsaturated fatty acids are found to be increased in both the resistant strains with concomitant decrease in the amount of saturated fatty acids as compared to that of the normal strain. Therefore, it is revealed that the development of resistance towards agrocin is associated with a significant elevation in the unsaturated fatty acid content of the total lipid fraction from *A. tumefaciens*.

Thanks are due to the University Grants Commission, India, for financing the project. Thanks are also due to Dr. (Mrs.) P. Kar and Mr. J. Dutta, Bose Institute, Calcutta, for their help in connection with gas chromatographic analysis.

REFERENCES

1) T. KANESHIRO and A. G. MARR, *J. Lipid Res.*, **3**, 184 (1962).
2) C. L. RANDLE, P. W. ALBRO, and J. C. DITTMER, *Biochim. Biophys. Acta*, **197**, 214 (1969).
3) A. KERR and K. HTAY, *Physiol. Plant Pathol.*, **4**, 37 (1974).
4) L. S. NIELSEN, *J. Bacteriol.*, **91**, 273 (1966).
5) A. F. DISALVO and J. F. DENTON, *J. Bacteriol.*, **85**, 927 (1963).
6) J. FOLCH, M. LEES, and G. H. SLOAN-STANLEY, *J. Biol. Chem.*, **226**, 497 (1957).
7) G. ROUSER, G. KRITCHEVSKY, and A. YAMAMOTO, *In* Lipid Chromatographic Analysis, Vol. 1, ed. by G. V. MARINETTI, Marcel Dekker, Inc., New York (1967), p. 99.
8) J. HIRSCH and E. AHRENS, *J. Biol. Chem.*, **233**, 311 (1958).
9) B. W. NICHOLS, *In* New Biochemical Separations, ed. by A. T. JAMES and L. J. MORRIS, D. Van Nostrand, London (1964), p. 321.
10) H. K. MANGOLD, *In* Thin Layer Chromatography. A Laboratory Handbook, 2nd Ed., ed. by E. STAHL, George Allen & Unwin, Ltd., London (1969), p. 363.
11) R. M. C. DAWSON, *In* Lipid Chromatographic Analysis, Vol. I, ed. by G. V. MARINETTI, Marcel Dekker, Inc., New York (1967), p. 163.
12) K. RANDERATH, *In* Thin Layer Chromatography, 2nd Ed., Academic Press, New York (1966), p. 173.
13) J. S. AMENTA, *J. Lipid Res.*, **5**, 270 (1964).
14) E. A. ANDERES, W. B. SANDINE, and P. R. ELLIKER, *Can. J. Microbiol.*, **17**, 1357 (1971).
15) K. HOFMANN, C. Y. HSIAO, D. B. HENIS, and C. PANOS, *J. Biol. Chem.*, **217**, 49 (1955).
16) K. G. KREBS, D. HEUSSER, and H. WIMMER, *In* Thin Layer Chromatography. A Laboratory Handbook, 2nd Ed., ed. by E. Stahl, George Allen & Unwin, Ltd., London (1969), p. 854.