Fatty Acid and Sterol Composition of Three *Phytomonas* Species

Celso Vataru Nakamura+, Luciana Waldow, Sandra Regina Pelegrinello, Tânia Ueda-Nakamura, Benício Alves de Abreu Filho, Benedito Prado Dias Filho

Laboratório de Microbiologia, Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Estadual de Maringá, Campus Universitário, Av. Colombo 5790, 87020-900 Maringá, PR, Brasil

Fatty acid and sterol analysis were performed on Phytomonas serpens *and* Phytomonas *sp. grown in chemically defined and complex medium, and* P. françai *cultivated in complex medium. The three species of the genus* Phytomonas *had qualitatively identical fatty acid patterns. Oleic, linoleic, and linolenic were the major unsaturated fatty acids. Miristic and stearic were the major saturated fatty acids. Ergosterol was the only sterol isolated from* Phytmonas *sp. and* P. serpens *grown in a sterol-free medium, indicating that it was synthesized* de novo*. When* P. françai *that does not grow in defined medium was cultivated in a complex medium, cholesterol was the only sterol detected. The fatty acids and sterol isolated from* Phytomonas *sp. and* P. serpens *grown in a chemically defined lipid-free medium indicated that they were able to biosynthesize fatty acids and ergosterol from acetate or from acetate precursors such as glucose or threonine.*

Key words: *Phytomonas* - fatty acids - sterols - trypanosomatids

Protozoan parasites must interact with the host at a variety of levels: the acquisition of nutrients, evasion or confusion of the host's response, establishment and maintenance of the infected state. This interface and the interactions are, by necessity, membrane mediated (Fish 1995).

Lipids, which are essential structural components of biological membranes, also affect cell surface recognition, cell interactions, and the expression of antigenic determinants (Yamakawa & Nagai 1978, Cullis & Kruijiff 1979, MacMurchie & Raison 1979, Elbein 1979).

The cell membranes of a variety of biological systems are altered in response to temperature changes, a process for which the term homeoviscous adaptation has been proposed (Sinensky 1974). In general, the lipid composition is characterized by an increase in unsaturated fatty acid with the decrease in environmental temperature (Roy et al. 1991, Imhoff & Thiemann 1991, Buzzi et al. 1993). Moreover in protozoa, the lipid content and metabolism are often influenced by environmental factors including the composition of the growth medium (Pinto et al. 1982, Racagni et al. 1995, Ellis et al. 1996) and temperature (Fagundes et al. 1980, Jones et al. 1993, Avery et al. 1995, Florin-Christensen et al. 1997).

Flagellate trypanosomatids of the genus *Phytomonas* are etiologic agents of diseases affecting fruits and plants of great economical importance including tomato, cashew, coffee, cassava, coconut and oil palms (Lopez et al. 1975, Dollet & Lopez 1978, Vainstein & Roitman 1985, Conchon et al. 1989) although they also act as parasites of lactiferous without any apparent pathogenicity (Attias & De Souza 1986). Insects have been suspected as a vector of plant flagellates. Jankevicius et al. (1989) showed in controlled laboratory cage experiments that *P. serpens*, the tomato parasite, is transmitted by the bite of coreid insect *Phthia picta*. The presence of trypanosomatids in plants of economic interest has attracted the attention of several research groups. A study on the fatty acid and sterol composition of three *Phytomonas* strains was undertaken in the present work.

MATERIALS AND METHODS

Microorganisms - P. françai isolated from cassava (Vainstein & Roitman 1985), *P. serpens* isolated from the salivary glands of the phytophagous insect *P. picta* (Brasil et al. 1990), and *Phytomonas* sp. isolated from the latex of *Euphorbia hyssopifolia* (Attias & De Souza 1986), were maintained by weekly transfer in a complex medium with the following composition (g/l): sucrose 20,

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+Corresponding author. Fax: +55-44 -261.4490. E-mail: dac@dac.uem.br
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trypticase 5, yeast extract 5, folic acid 0.002, hemin dissolved in quadrol 25% 0.02; pH 7.0 (Roitman et al. 1972). Cells were grown at 28ºC for 48 hr and thereafter were kept at 4 to 6ºC. For the experiments, cells were grown in 1 l flasks containing 500 ml of complex medium. The medium was autoclaved at 121ºC for 20 min. However, in some experiments *Phytomonas* sp. and *P. serpens* were also cultivated in a chemically defined medium (Silva & Roitman 1989) (Table I). The inoculum consisted of 50 ml of a 48 hr culture, corresponding to approximately 2 x 10^8 cells. After 48 hr of incubation the cells were collected by centrifugation (2,000 g for 10 min at 4ºC) and washed four times in cold phosphate-buffered saline (PBS), pH 7.2, 0.01 M.

Extraction of lipids and identification of the fatty acid and sterols - Lipids were extracted from washed protozoan cells with 10 vols each of chloroform-methanol-water mixture (4:8:3 v/v) and chloroform-methanol mixture (1:1 v/v). Combined extracts were evaporated to dryness. Absolute methanol-diethyl ether (3:1 v/v) was added to the lipid extract followed by saponification with 1 ml of 5N NaOH. Fatty acids were then extracted in n-hexane after adding water and lowering the pH to 1.0. Fatty acids were converted to their corresponding methyl esters by treatment with ether-diazomethane and methanol-diethyl ether (1:9, v/v) (Pörschmann 1982). Methyl esters were analyzed by gas-liquid chromatography (GLC) with a temperature programmed and coupled to a mass spectrometer (MS) Hewlett Packard 5992 AGC/MS System with an ionizing energy of 70 eV. Methyl esters were identified by their retention time relative to methyl esters of known fatty acid standards. The chain lengths of unsaturated fatty acids were also identified by GLC of the products of catalytic hydrogenation of methyl esters carried out at room temperature for 1 hr in ethyl acetate, with 10% palladium on charcoal under a hydrogen pressure of 40 psi.

TABLE I

Chemically defined medium for *Phytomonas serpens* (Silva & Roitman 1989)

Compound	g/l	Compound	mg/l
β-Na glycerophosphate	10	Nicotinamide	2
Glucose	20	Ca pantothenate	2
Inositol	0.04	Na Riboflavine $PO_4.2H_2O$	1
Glutamic acid	0.1	Pyridoxamine.2HCl	0.6
L-Serine	0.2	Thiamine HCl	0.6
$MgSO_4$	0.05	Biotin	0.008
KCl	10	Folic acid	2
K_2HPO_4	1		
$K_3Citrate.H_2O$	1		
$Citric\ acid.H_2O$	0.5		
Malic acid	0.2		
Succinic acid	1		
$MgCO_3$	1		
$CaCO_3$	0.02		
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.01		
L-arginine HCl	0.4		
L-histidine (free base)	0.3		
L-isoleucine	0.2		
L-leucine	0.2		
L-lysine HCl	0.2		
L-methionine	0.1		
L-phenylalanine	0.2		
L-threonine	0.2		
L-tryptophan	0.1		
L-tyrosine ethyl ester	0.2		
L-valine	0.2		
Adenine	0.02		
Trace elements[a]	0.2		
Hemin[b]	0.01		

pH: 7.0 adjusted HCl; *a*: to yield the following final concentration (mg/ml) - Fe 6.0 as $Fe(NH_4)_2(SO_4)_2.6H_2O$, Mn 5.0 as $MnSO_4$, Cu 0.4 as $CuSO_4$ (anhyd.), Zn 5.0 as $ZnSO_4.7H_2O$, Mo 2.0 as $(NH_4)_6.Mo_7.4H_2O$, V 4.0 as NH_4VO_3, B 0.1 as H_3BO_3, Co 0.1 as $CoSO_4.7H_2O$; *b:* supplied from a 10 mg/ml solution in 25% aqueous Quadrol (ethylene-di-nitrolotetra-2-isopropanol).

Sterols of cells were extracted from total lipids by saponification with 1 ml of 5N NaOH for 5 hr. They were fractionated by thin-layer chromatography (TLC) on 0.25 mm layers of silica gel GF_{254} (Merck), using hexane-ethyl acetate (65:35, v/v) as solvent and the spots visualized by u.v. or by spraying with sulfuric acid-ceric acetate (Sthal 1969). After being visualized, the sterols were scraped from the TLC plates, dissolved in methanol and analyzed by u.v. spectroscopy (200-400 nm) in a Varian 1E/UV Visible Spectrophotometer. Cholesterol and ergosterol (Sigma Chemical Co.) were used as internal standards.

RESULTS

The fatty acid compositions of *P. françai, P. serpens* and *Phytomonas* sp. grown in the complex medium are shown in Table II. Lauric ($C_{12:0}$), miristic ($C_{14:0}$), palmitoleic ($_{C16:1}$), palmitic ($C_{16:0}$), linolenic ($C_{18:3}$), linoleic ($C_{18:2}$), oleic ($C_{18:1}$), stearic ($C_{18:0}$), eicosanoic ($C_{20:0}$), erucic ($C_{22:1}$), and docosanoic ($C_{22:0}$) acids were detected as components of the total lipid fraction from all parasites. Linolenic, linoleic, oleic, and stearic acids were the major fatty acids of the three *Phytomonas* sp. and accounted for more than 60% of the total fatty acids. *P. françai* and *P. serpens* contained higher levels of linolenic acid (33.5% and 49.9%, respectively) than that observed with *Phytomonas* sp. cells (10.2%). In *Phytomonas* sp. eicosanoic, euric, and docosanoid acids were absent, oleic (21.1%) and stearic acids (18.1%) were the prominent components and the degree of unsaturation (47.5%) was lower than those recorded for *P. françai* (59.4%) and *P. serpens* (71.6%).

When *Phytomonas* sp. and *P. serpens* were grown in chemically defined medium the fatty acids composition showed an increase in the degree of unsaturation. The proportion of the total unsaturated fatty acids of *Phytomonas* sp. grown in chemically defined medium increased by approximately 61%. Changes in the degree of unsaturation were due to variations in the proportion of the unsaturated fatty acids linolenic and oleic. The presence of unsaturated fatty acids was confirmed by catalytic hydrogenation; the characteristic peaks for palmitoleic, oleic, linoleic, and linolenic acids were completely abolished with a corresponding increase in the size or the peaks for palmitic and stearic acids (data not shown).

Only one sterol type with a Rf similar to ergosterol could be detected by TLC of the nonsaponifiable component of the lipid extract of *Phytmonas* sp. and *P. serpens* grown in both complex (Fig. 1A) and chemically defined medium (Fig 1B). Its ultraviolet absorption spectrum showed maximum absorption at 293, 283, 271, and 261 nm, which was in good agreement with authentic ergosterol (Fig. 2).

Cholesterol was the only sterol detected in *P. françai* grown in complex medium as showed in Fig. 1. This compound was also identified by GLC by comparison with the retention time of the cholesterol standard.

TABLE II

Fatty acid composition (%) of three *Phytomonas* species[a]

Fatty acids	Samples			
	P. françai	*P. serpens*	*Phytomonas* sp.	
			CM[b]	DM[c]
$C_{12:0}$	1.8	3.5	8.7	4.8
$C_{14:0}$	3.8	2.6	13.9	7.3
$C_{16:1}$	4.6	4.6	5.7	2.5
$C_{16:0}$	11.3	5.7	11.8	8.2
$C_{18:3}$	33.5	49.9	10.2	24.3
$C_{18:2}$	9.4	12.9	10.5	5.7
$C_{18:1}$	11.0	3.5	21.1	45.0
$C_{18:0}$	19.6	13.6	18.1	2.2
$C_{20:0}$	3.9	2.0	0	0
$C_{22:1}$	0.9	0.7	0	0
$C_{22:0}$	0.2	1.0	0	0
Total unsaturated (TU)	59.4	71.6	47.5	77.5
Total saturated (TS)	40.6	28.4	52.5	22.5
TU/TS	1.46	2.52	0.9	3.44

a: average of three experiments; *b*: complex medium; *c*: defined medium.

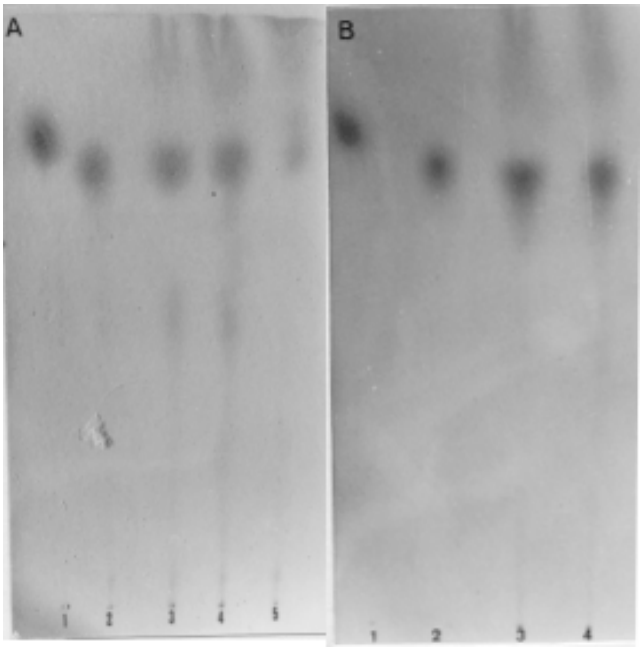

Fig. 1: thin-layer chromatography of sterols isolated from three *Phytomonas* strains cultivated in complex medium (A) and chemically defined medium (B). Sterols were fractionated by TLC in silica gel GF_{254}, using hexane-ethyl acetate (65:35, v/v) as solvent and the spots visualized by u.v. or by spraying with sulfuric acid-ceric acetate. (1) cholesterol, (2) ergosterol, (3) *Phytomonas serpens*, (4) *Phytomonas* sp., and (5) *P. françai.*

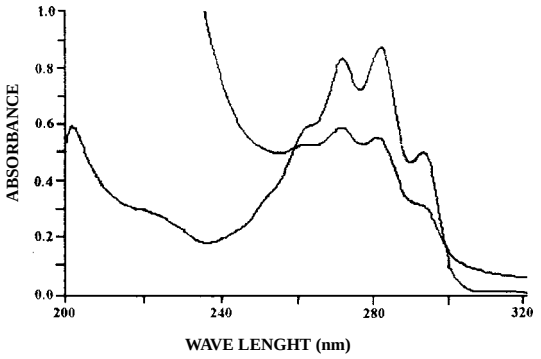

Fig. 2: U.V. spectrum of the sterol isolated from *Phytomonas* sp. (A) and ergosterol standard (B).

DISCUSSION

Even- and odd-numbered, saturated, monoenoic and polyenoic types of fatty acids ranging from C_{12} to C_{22} were characterized as components of the total lipid fraction of *P. françai*, *P. serpens*, and *Phytomonas* sp. In general, this fatty acids pattern resembles that observed for *Herpetomonas* (Pinto et al. 1982), *Leishmania donovani* (Glew et al. 1988), and *Trypanosoma cruzi* (Racagni et al. 1992). The major fatty acids of the three *Phytomonas* sp. consisted generally of C_{18} carbon chain lengths. Linolenic, linoleic, oleic, and stearic acids were the major fatty acids of three *Phytomonas* sp. and accounted for more than 60% of the total fatty acids. The three species of the genus *Phytomonas* had qualitatively identical fatty acid patterns. However, differences in the fatty acids content of lipid fraction was observed in this study. For example, *P. françai* and *P. serpens* contained higher levels of linolenic acid than observed with cells of *Phytomonas* sp. In *Phytomonas* sp. eicosanoic, euric, and docosanoid acids were absent, oleic and stearic acids were the prominent components and the degree of unsaturation was lower than those from *P. françai* and *P. serpens.*

In the chemically defined medium the lipid composition of *Phytomonas* sp. and *P. serpens* showed a increase in unsaturation. *P. françai* does not grow in chemically defined medium. Compared to cells grown in a complex medium, the proportion of the total unsaturated fatty acids of *Phytomonas* sp. grown in chemically defined medium increased by approximately 61% with a concomitant decrease in the proportion of saturated fatty acids. Changes in the degree of unsaturation were due to the variations in the proportion of the unsaturated fatty acids linolenic and oleic. In the protozoa *H. samuelpessoai* changes in the degree of unsaturation were accompanied by a variation on the amount of oleic and linoleic acids (Pinto et al. 1982).

The increase in the degree of unsaturation of fatty acids as result of lowering the environmental temperature has been described in *T. cruzi* (Florin-Christensen et al. 1997*)* and in several of other microorganisms including bacteria (Sinensky 1974, Roy et al. 1991, Imhoff & Thiemann 1991, Buzzi et al. 1993, Vigh et al. 1993, Avery et al. 1995). Most cells under environmental stress restore the suboptimal physical state of their membranes to a more functional condition by altering the lipid composition of their membranes. Changes in membrane composition by increasing unsaturated fatty acids would prevent "freezing" of membrane and inhibition of various cellular membranes functions (Ellis et al. 1996). The life cycle of phytomonads include stage in different environments such as the digestive tract and salivary glands of insects, the latex and the sap of plants, and the fruit and seeds of various species (Jankevicius et al. 1991). Increased membrane fluidity helps maintain vital membrane functions of plant parasite at these environmental conditions with very difference in terms of osmolarity, pH, food resources, and temperature.

The fatty acids isolated from *Phytomonas* sp. and *P. serpens* (data not shown) grown on a chemically defined lipid-free medium indicates that they were able to biosynthesize fatty acids from acetate or from acetate precursors such as glucose or threonine. The ability to use both sugars and amino acids as a source of energy is a feature of many trypanosomatids and has probably been invaluable in their adaptative radiation to colonise differents hosts. Sugars are present in blood and plant saps but soon disappear from the vector´s meal. Amino acids will become abundant as the blood meal is digested (Vickerman 1994). Nectar can be a source of both amino acids and suggar, even lipids in some species (Baker & Baker 1975) Studies in trypanosomatids, such as *Leishmania tarentolae* and *T. lewisi* indicated that these species are able to synthesize and elongate precursor short chain fatty acids (Korn et al. 1965). Changes in the structures of the fatty acid could be attributed to conversion (e.g., chain elongation, desaturation) or retroconversion (chain shortening), or to the introduction of branches or ring.

Ergosterol was the only sterol isolated from *Phytmonas* sp. and *P. serpens* grown in a sterol-free medium, indicating that it was synthesized *de novo*. The possible synthesis of other sterol is excluded by the fact that ergosterol was the only sterol present in cells of these parasites cultivated in both chemically defined and complex medium. However, when *P. françai* was cultivated in a complex medium cholesterol was the only sterol detected. *P. françai,* that is unable to grow in defined medium, has been maintained by monthly transfers in a biphasic medium containing blood agar in the solid phase and overlay of complex medium (Attias et al. 1988). The parasites die after three or four subcultures in complex medium. Thus, after two subcultures the parasites grown in complex medium must be harvested to obtain cells for lipid analysis.

It is well known that cholesterol added to grown medium becomes stably associated with cells. This association could be due to internalization or binding to the cell surface. Keenan and Zierdt (1994) showed that most of the cell-associated cholesterol can not be removed by washing, by incubation with serum albumin, or by brief exposure to hexane. Whether the cholesterol is synthesized by the organism, or is accumulated from growth medium remains to be determined. It is interesting that the bloodstream forms of *T. brucei* contain cholesterol that is provided from an exogenous source. In contrast, ergosterol is the major sterol that can be synthesized by the insect procyclic forms of *T. brucei* (Coppens & Courtoy 1995). *Tritrichomonas foetus* also take up preformed cholesterol and fatty acids from the medium to form cellular lipid components suggesting that the flagellates may be unable to synthesize the majority of their lipids (Dias Filho et al. 1985). Replacement of tetrahymanol by cholesterol in *Tetrahymena pyriformis* led to a decrease in cell size and an increase in the proportion of fatty acids that arise from the palmitoleic acid pathway (Conner et al. 1982). Avery et al. (1995) showed the relationship between temperature-dependent changes in phagocytotic activity of *Acanthamoeba castellanii* and the fatty acid composition and physical properties of plasma membranes. In this context, Ellis et al. (1996) observed that changes in *Giardia lamblia* lipids, increased fatty acid unsaturation and storage lipids, are consistent with parasite differentiation into a cyst stage that is able to survive outside the host at reduced temperature and reduced levels of available nutrient sources. Thus, although difference in the lipid composition of *Phytomonas* strains has been demonstrated in this work it is not clear, at this stage of knowledge, whether it may induce significant physiological cellular changes.

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