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REGULATION OF MEMBRANE LIPID FLUIDITY IN *ACHOLEPLASMA LAIDLAWII:* EFFECT OF CAROTENOID PIGMENT CONTENT

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SUMMARY

Acholeplasma laidlawii (oral strain) cells were grown in a lipid-extracted medium supplemented with arachidic acid ($C_{20:0}$). The carotenoid content of the plasma membrane was appreciably dependent upon the levels of acetate or propionate present in the growth medium.

1. Membranes isolated from cells grown in propionate (5 g/l) contained a low level of carotenoids, approximately 11 wt % of the total lipids. Neither the fatty acyl composition nor the membrane lipid/protein ratio was altered. However, spin-labelling experiments demonstrated a greater lipid fluidity in those membranes, as compared to those from cells grown in an acetate containing growth medium. Membranes isolated from cells grown in acetate (5 g/l) were characterized by a higher buoyant density, higher osmotic fragility and lower glycerol permeability.

2. Growing cells in a medium containing 20 g/l of propionate instead of acetate, the membrane carotenoid content decreased by 57-fold. For cells grown in an acetate medium (20 g/l), high levels of carotenoid pigments (about 38 wt % of the total lipids) were obtained; fewer arachidoyl and more unsaturated acyl groups were found in the membrane lipids. Spin-labelling experiments showed only a very slight difference in lipid fluidity between the two types of membrane.

3. These results suggest that carotenoid pigments rigidify the *Acholeplasma* membrane and the organism maintains its membrane fluidity within a narrow range by modifying the fatty acyl composition of the membrane lipids.

INTRODUCTION

Acholeplasma laidlawii cells contain carotenoid pigments in their limiting membranes [1]. The content of these pigments can be increased by growing cells in an acetate-containing medium or decreased by growing them in a medium containing

Abbreviation: 12-nitroxide stearate, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyl-oxyl.

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propionate, diphenylamine, or thallium acetate [2, 3]. The structure and function of these pigments in *Acholeplasma* cells has been the subject of several studies. These pigments may function as a transport carrier for glucose and acetate, since some carotenoid pigments isolated from the cell are covalently linked to glucose and/or acetate [1]. This hypothesis was later refuted because *A. laidlawii* cells could be grown under such conditions that neither carotenoids nor cholesterol were accumulated in the membrane [3]. It has also been reported that the carotenoid pigment in these cells can protect the membranous ATPase against photodynamic inactivation [4].

The membranes of all sterol-requiring Mycoplasmas examined to date contain large amounts of sterol, but no carotenoids; and those of sterol-nonrequiring species contain carotenoids instead of sterol. This fact suggests a common function of carotenoids and sterols such as cholesterol [5]. Since cholesterol reduces the lipid fluidity by interacting with membrane phospholipids [6–8], it is probable that carotenoids in *Acholeplasma* cells function similarly. For cells other than *Acholeplasma*, it has been suggested that carotenoids are perhaps involved in stabilizing membranes [9]. In this article, we present direct proof that carotenoids indeed play a role in controlling the membrane lipid fluidity. Furthermore, since the membrane lipid fluidity controls crucial functions, it is interesting to ask whether and how the membrane fluidity of *Acholeplasma* cells is regulated when the membrane is enriched with an extreme amount of carotenoid pigments.

METHODS

Organism and growth

A. laidlawii (oral strain) was a gift from Dr S. Rottem (The Hebrew University, Jerusalem, Israel). The growth medium consisted of 20 g/l lipid extracted tryptose [10], 5 g/l of D-glucose, 5 g/l of tris(hydroxymethyl)aminomethane, 4 g/l of fatty acid free bovine serum albumin, 500 units/ml of pencillin G, and 5 mg/l of arachidic acid [11]. Various amounts of sodium acetate or propionate were added to the medium in order to vary the carotenoid pigment content of the cells. The complete culture medium was inoculated (1 %, v/v) with a 24-h-old cell culture which has already been adapted to arachidic acid supplementation. The culture was grown statically in the dark at 37 °C, and harvested at late log phase [11]. Within 24 h the titer of the culture was about 10^9 cells/ml when the medium contained 5 g/l acetate or propionate; the titer was somewhat lower when the growth medium contained 20 g/l of acetate or propionate.

Plasma membrane preparation

The plasma membranes of *A. laidlawii* cells were prepared according to Razin et al. [12]. The membranes were washed twice in deionized distilled water and freshly used or stored at −20 °C in the dark for at most 24 h.

Determination of fatty acyl composition and the carotenoid content in the membrane lipids

Lipids were extracted from the isolated membranes by the methods of Folch et al. [13]. The absorbance of the "Folch lower phase" was measured at 450 nm against chloroform–methanol (2 : 1, v/v). The amounts of carotenoid pigments in

the membrane lipids are expressed as $A_{450\ nm}$/mg membrane protein. The *trans*-methylation of membrane lipids, the extraction of the methyl esters of fatty acids, and the subsequent analysis of these esters by gas chromatography was described recently [11]. The amount of lipids was determined colorimetrically [14], using cholesterol as standards.

Buoyant density of the membrane

The procedure of the isopycnic centrifugation was recently described [11], except that both the membrane suspension and the sucrose density gradient were prepared in deionized water instead of 1 : 20 β-buffer [15]. The membrane buoyant density was defined as that of the peak fraction collected from the centrifuge tube. Membrane proteins were assayed by the method of Lowry et al. [16].

Spin labelling of the membranes

A. laidlawii membranes were labelled in vitro with a fatty acid spin label, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (12-nitroxide stearate), as described [11]. However, deionized water was employed to wash the membranes and to disperse the spin label. The spin-labelled membrane suspension contained about 10 mg protein per ml and 0.1-0.2 μmole 12-nitroxide stearate per ml. Electron paramagnetic resonance (EPR) spectra were recorded with a Varian EPR spectrometer, model 4502-15, equipped with a variable temperature controller, model 4540.

Osmotic swelling, relative glycerol permeability and osmotic fragility of the cells

All procedures used have been described [11]. Swelling of the cells in hypotonic sucrose solutions was monitored by the absorbance at 600 nm. The passive glycerol permeability was estimated by measuring the initial swelling rate of cells in the isotonic glycerol solution at various temperatures. This method only yields the relative permeability [17]. The osmotic fragility was recorded kinetically by comparing the turbidity loss of the cell suspension in water or 0.25 M NaCl. The fraction of turbidity ($A_{600\ nm}$) left was defined as the ratio: ($A_{600\ nm}$ of cells in water)/($A_{600\ nm}$ of cells in 0.25 M NaCl). These ratios were normalized with respect to the zero time value. The error in zero time value was greatly reduced when the sample was rapidly injected. Our data are comparable to those reported [12].

RESULTS

Carotenoid content in A. laidlawii membranes

Membranes from cells grown in a medium with various amounts of sodium acetate contained high levels of carotenoid pigments and were bright yellow. In contrast, when grown in sodium propionate, cells contained practically no carotenoid pigments and were rather pale (Table I). Increasing the sodium acetate concentration from 5 g/l to 20 g/l, the carotenoid pigment content ($A_{450\ nm}$/mg membrane protein) rose from a value of 0.661 to 2.290. Since the exact chemical structure of the carotenoids in *Acholeplasma* is unknown, an average extinction coefficient (1 %, 1 cm optical path, 450 nm, in chloroform–methanol (2 : 1, v/v)) of about 250 was estimated from published data [18]. The membrane carotenoid pigment content was therefore about 11 and 38 wt % of total lipids for cells grown in 5 g/l and

TABLE I

TOTAL LIPID FATTY ACYL COMPOSITION, CAROTENOID CONTENT, AND LIPID/PROTEIN RATIO OF *A. LAIDLAWII* MEMBRANE

Cells were grown in medium supplemented with arachidic acid (5 mg/l).

Medium ingredient		Fatty acyl composition (% w/w):															Carotenoid content ($A_{450\ nm}$/mg membrane protein)	Lipid/protein ratio (mg/mg)
		$C_{10:0}$	$C_{12:0}$	$C_{12:1}$	$C_{13:0}$	$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	UI*		
Sodium propionate	5 g/l	–	5.5	–	–	2.7	–	0.5	4.4	0.9	2.0	4.8	1.1	72.3	–	2.9	0.065	0.49
	20 g/l	–	3.5	–	0.7	2.5	–	–	7.9	0.8	2.4	5.8	1.7	73.9	0.7	–	0.040	0.51
Sodium acetate	5 g/l	–	6.1	0.5	–	2.4	0.7	–	4.9	2.0	3.0	5.0	2.1	70.9	–	2.4	0.661	0.51
	20 g/l	1.7	8.8	–	–	4.2	–	–	13.6	3.5	5.5	9.2	1.9	37.5	4.2	10.1	2.290	0.47

* Unidentified, mostly unsaturated groups.

20 g/l of acetate, respectively. The propionate grown cells contained less than 1 wt %. Our results are in agreement with those reported earlier [4, 12]. Sodium acetate is known to be a precursor in the biosynthesis of carotenoids [19]. Sodium propionate probably inhibits the formation of these pigments by interfering with the transport across the cell membrane [20].

Lipid fatty acyl composition and membrane lipid/protein ratio

When low amounts (5 g/l) of acetate or propionate were present in the growth medium, the overall fatty acyl composition of membrane lipids remained practically unchanged (Table I). High concentrations of propionate (20 g/l) also did not alter appreciably the fatty acyl composition. In every case the membrane lipids were equally enriched with the arachidoyl group (about 70 wt %). However, high concentrations (20 g/l) of acetate caused a pronounced decrease in the arachidoyl group enrichment concomitant with more unsaturated acyl groups among the membrane lipids. As a result the overall lipid chain length became shorter, and the degree of unsaturation enhanced. The membrane lipid/protein ratio remained constant (Table I).

Membrane lipid fluidity

Membrane lipid fluidities of all types of membranes were detected by the spin-labelling method. The fatty acid spin label was introduced into the isolated membranes and intercalated into the hydrophobic region [21]. A typical EPR spectrum of these spin labelled membranes is shown in Fig. 1. The hyperfine splitting $2T_{//}$ in the spectrum is related to the rotational mobility of the spin label and therefore reports the local fluidity of membrane lipids [21]. A high value of $2T_{//}$ reflects a low fluidity and vice versa. At high temperatures, the high-field "dip" in the spectrum

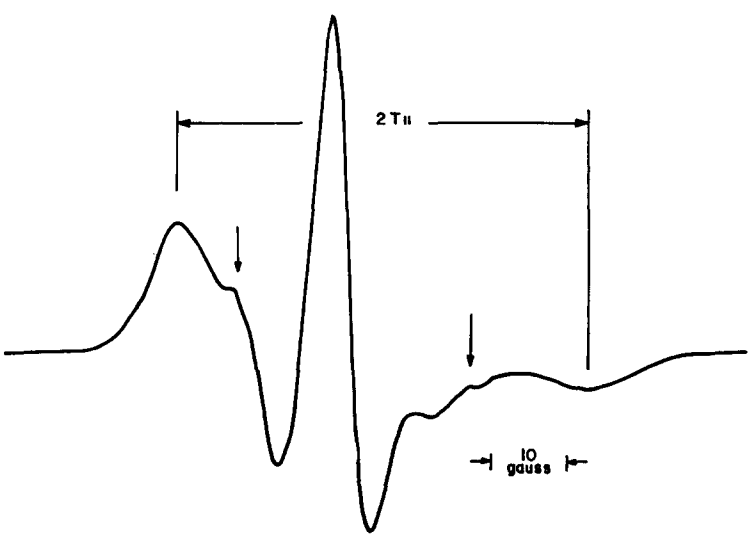

Fig. 1. Typical EPR spectrum of the spin label 5NS incorporated into *A. laidlawii* membranes. The spectrum was recorded at 8 °C from a membrane sample isolated from cells grown in a medium containing sodium propionate (5 g/l). Arrows indicate signals from unincorporated spin labels. Magnetic field strength increases from left to right.

became so shallow that an accurate measurement of $2T_{//}$ was not possible. In these cases an empirical parameter, the rotational correlation time τ_c, was calculated according to

$$\tau_c = 6.5 \cdot 10^{-10} \, W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right] \text{ s}$$

where W_0 is the width of the central peak; h_0 and h_{-1} are the heights of the central and high field peaks, respectively [22]. A large value of τ_c indicates a rigid microenvironment around the spin label. Table II lists the values of these motion parameters of all types of membrane at different temperatures. Generally, membranes from cells grown in a propionate containing medium had lower values of motion parameters at the temperatures tested (Table II). This implied that the hydrophobic regions of the carotenoid-poor membranes were more fluid as compared to those of the carotenoid-rich ones.

TABLE II

MOTION PARAMETERS OF THE SPIN LABEL 12-NITROXIDE STEARATE INCORPORATED INTO MEMBRANES OF *A. LAIDLAWII*

Cells were grown in medium supplemented with arachidic acid (5 mg/l). The hyperfine splitting $2T_{//}$ was measured to within ±0.4 G; and the rotational correlation time τ_c to within ±0.2 ns. n.d. means not determined.

Medium ingredient		$2T_{//}$ (G)								τ (ns) (37 °C)
		0 °C	5 °C	8 °C	10 °C	15 °C	20 °C	22 °C	25 °C	
5 g/l	propionate	n.d.	n.d.	56.2	n.d.	n.d.	n.d.	46.6	n.d.	2.6
	acetate	n.d.	n.d.	57.2	n.d.	n.d.	n.d.	49.0	n.d.	3.1
20 g/l	propionate	57.6	57.2	n.d.	56.2	51.7	49.9	n.d.	47.2	2.8
	acetate	58.6	57.6	n.d.	56.2	53.4	51.4	n.d.	48.3	3.1

Membrane buoyant density and relative glycerol permeability

The buoyant densities of membrane preparations from cells grown in a medium containing 5 g/l acetate or propionate are shown in Table III. Those membranes with greater amounts of carotenoid pigments were denser. Since these types of mem-

TABLE III

BUOYANT DENSITIES AND RELATIVE GLYCEROL PERMEABILITIES OF *A. LAIDLAWII* MEMBRANES

Cells were grown in medium supplemented with arachidic acid (5 g/l).

Medium ingredient (5 g/l)	Buoyant density (g/l at 20 °C)*	Initial swelling rate in glycerol solution (arbitrary units)**		
		15 °C	22 °C	38 °C
Sodium propionate	1.160±0.005	0.21±0.005	0.51±0.03	1.76±0.31
Sodium acetate	1.173±0.005	0.16±0.02	0.43±0.02	1.55±0.20

* Results from three different experiments. Data expressed as mean ±S.D.

** Results from four different experiments. Data expressed as ±S.D.

brane had nearly identical lipid/protein ratios and fatty acyl compositions, the difference in density likely resulted from a modification in lipid packing. The results of the spin-labelling experiment directly support this assumption. Since *A. laidlawii* cells behaved like an ideal osmometer, the initial swelling rate in the isotonic glycerol solution was proportional to the glycerol permeability. Cells grown in propionate medium were more permeable to glycerol than cells grown in an acetate containing medium at all temperatures tested (Table III). The initial swelling rate increased with temperature. At temperatures higher than 45 °C, the cells swelled so rapidly that the initial swelling rate became difficult to measure. When a non-electrolyte molecule such as glycerol permeates a lipid barrier, the rate of permeation depends on the packing of lipids. Therefore, the slower permeation in the carotenoid-rich cells indicated a more viscous lipid region in the membrane. This conclusion is again consistent with results from the spin-labelling experiments.

Osmotic fragility

The resistance of the cell towards osmotic lysis was determined kinetically for cells grown at a low level of acetate or propionate (5 g/l). The results showed that the propionate grown cells were more resistant to osmotic lysis than those grown in an acetate containing medium (Fig. 2). After 1 h at room temperature the propionate

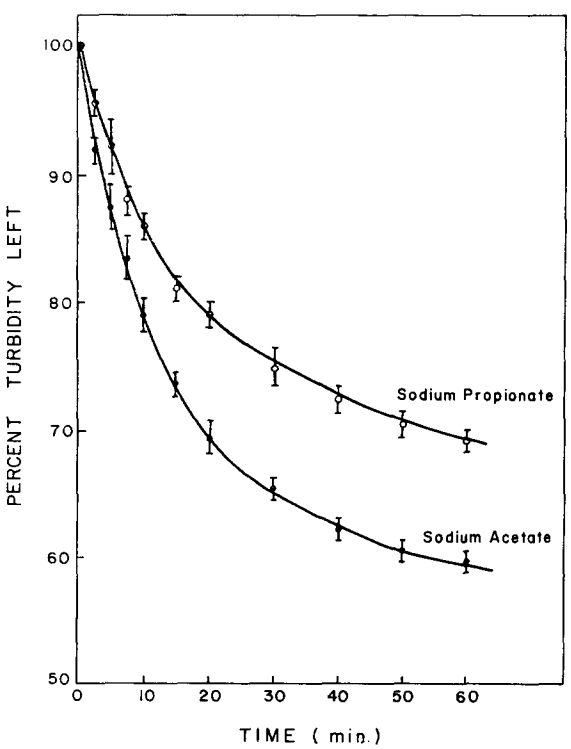

Fig. 2. Osmotic fragility of *A. laidlawii* cells. Cells were grown in an arachidic acid-supplemented medium containing sodium propionate or acetate (5 g/l).

grown cells had about 10 % more turbidity left than those grown in a medium with acetate. Thus, cells having more fluid membranes are tougher to lyse. This result agrees with the hypothesis that lipid fluidity enhances the membrane tensile strength against osmotic shock [12, 23].

DISCUSSION

Comparing membrane properties of cells grown in 5 g/l of acetate or propionate, it seems evident that the hydrophobic regions of the carotenoid-rich membrane are less fluid than the corresponding regions of the carotenoid-poor ones. Consequently, carotenoid-rich membranes are characterized by higher osmotic fragility, lower glycerol permeability, and higher buoyant density. However, if the membranes contain extreme amounts of carotenoids, the lipid fluidity does not decrease accordingly. This "saturation effect" can be interpreted as a result of cellular regulation. The organism can apparently tolerate a decrease in fluidity caused by a moderate enrichment in carotenoids. When the level of enrichment increases, the cell is forced to modify its fatty acyl composition in order to compensate for the otherwise drastic decrease in membrane fluidity. This is achieved by a decrease of average acyl change length and by a simultaneous enhancement in the amount of unsaturated acyl groups. As a net result, the membrane lipid fluidity is maintained within a narrow range.

Numerous reports demonstrated that cholesterol and a number of its derivatives condense the packing of phospholipids in various biological membranes [24, 25], and in model lipid membranes [6–8]. Our experiments showed that the carotenoid molecules may have similar functions. These findings are consistent with the hypothesis that carotenoids and sterols play a similar role in the membrane of Acholeplasma. However, it is presently unclear how these two types of lipid perform a similar function with entirely different chemical structures.

Growing *A. laidlawii* B in a medium containing cholesterol (25 mg/l), 8 % of the total membrane lipids was cholesterol without appreciable change in fatty acyl composition [26]. Our studies demonstrated that about 11 % of the total membrane lipids are carotenoids when the cells are grown in an acetate containing nutrient medium; the change in fatty acyl composition was insignificant too.

Upon incorporation of 8 % cholesterol [26], the glycerol permeability was reduced by a factor of about four. However, a 10-fold increase in carotenoid content yielded only a small change (about 20 %) in permeability, when cells grown in an acetate or propionate medium are compared.

Adapting Mycoplasma strains to a low cholesterol diet [27], the membrane cholesterol content decreased from about 24 % in the native strain to 3 % in the adapted strain. For these two types of membrane, spin labelling experiments showed no difference in microviscosity below 25 °C. Around 37 °C the hyperfine splitting of the fatty acid spin label employed changed by 3–4 G. Our findings demonstrated that a 10-fold change in carotenoid content was accompanied by a large change in membrane lipid fluidity, e.g. at 22 °C the hyperfine splitting of the 5NS spin label differed by approximately 2 G, and at 37 °C the correlation time varied by about 0.5 ns.

No significant change in membrane osmotic fragility was observed when the carotenoid content of strain B cells is increased 10-fold [12]. Also, protoplasts from *Sarcina lutea* prepared from a colorless mutant or from a wild type strain grown in

diphenylamine do not exhibit different membrane osmotic fragility as compared to those from wild type cells [28]. However, both reports did not present data about the membrane lipid fluidity and the fatty acyl composition. It is possible that the fatty acyl composition is modified upon a drastic alteration in carotenoid content such that the cell can maintain a proper membrane lipid fluidity necessary for normal growth. Consequently, one expects no significant change in osmotic fragility as long as the membrane lipid fluidity remains essentially constant. This argument is obviously supported by our results for cells grown in a medium with high amounts of acetate (20 g/l). Cells indeed modify their membrane fatty acyl composition and maintain the lipid fluidity within a narrow range when the carotenoid content is increased drastically.

The membrane lipid fluidity plays a crucial role in membrane functions, such as permeability [25], membrane-bound enzyme activity [29–31], transport of certain nutrients [32], and osmotic stability [23]. The ability of maintaining the proper lipid fluidity is one factor to enable *A. laidlawii* cells to survive in various kinds of environment. Preliminary results from this laboratory indicate that this organism is also capable to maintain the membrane fluidity when the growth temperature is changed or the exogenous fatty acid supplementation.

The nature of the interaction between carotenoids and membrane lipids is unknown. If the forces are mainly hydrophobic, one expects that the interaction would depend on the characteristics of hydrocarbon chains of adjacent lipids, such as the degree of unsaturation, branching and steric configuration. Experiments elucidating such aspects may be carried out by enriching membranes with suitable fatty acyl groups other than the arachidoyl one.

There are at least four major types of carotenoids in *A. laidlawii* membranes [18]. Whether one or several of these pigments is responsible for the control of membrane fluidity remains to be investigated.

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