Total Lipids with Short and Long Acyl Chains from *Acholeplasma* Form Nonlamellar Phases

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ABSTRACT The cell-wall-less bacterium *Acholeplasma laidlawii* A-EF22 synthesizes eight glycerolipids. Some of them form lamellar phases, whereas others are able to form normal or reversed nonlamellar phases. In this study we examined the phase properties of total lipid extracts with limiting average acyl chain lengths of 15 and 19 carbon atoms. The temperature at which these extracts formed reversed hexagonal (H_{II}) phases differed by 5–10°C when the water contents were 20–30 wt%. Thus the cells adjust the ratio between lamellar-forming and nonlamellar-forming lipids to the acyl chain lengths. Because short acyl chains generally increase the potential of lipids to form bilayers, it was judged interesting to determine which of the *A. laidlawii* A lipids are able to form reversed nonlamellar phases with short acyl chains. The two candidates with this ability are monoacyldiglucosyldiacylglycerol (MADGlcDAG) and monoglucosyldiacylglycerol. The average acyl chain lengths were 14.7 and 15.1 carbon atoms, and the degrees of acyl chain unsaturation were 32 and 46 mol%, respectively. The only liquid crystalline phase formed by MADGlcDAG is an H_{II} phase. Monoglucosyldiacylglycerol forms reversed cubic (*Ia3d*) and H_{II} phases at high temperatures. Thus, even when the organism is grown with short fatty acids, it synthesizes two lipids that have the capacity to maintain the nonlamellar tendency of the lipid bilayer. MADGlcDAG in particular contributes very powerfully to this tendency.

INTRODUCTION

Currently there is great interest in the phase behavior of membrane lipids. One of our main interests concerns the presence and function of so-called nonlamellar-forming lipids in cell membranes. This issue can be conveniently studied using prokaryotic organisms, like *Acholeplasma laidlawii* and *Escherichia coli* (Morein et al., 1996; Rietveld et al., 1993; Rilfors et al., 1993). *A. laidlawii* in particular is suitable, because the organism lacks a cell wall and possesses only a cytoplasmic membrane. In addition, *A. laidlawii* can be grown in media that permit the acyl chain composition of its lipids to be manipulated. For the cells to cope with acyl chain variations, the polar headgroup composition in *A. laidlawii* A is regulated in a coherent way (Andersson et al., 1996; Rilfors et al., 1993). Generally, the fraction of the lipids forming reversed nonlamellar structures increases when the length and the unsaturation of the acyl chains are reduced. The regulation of the ratio between the lipids forming lamellar and nonlamellar phases is expected to yield phase transition temperatures from a lamellar to a nonlamellar phase (T_{NL}) within a rather narrow interval for total lipid extracts (Lindblom et al., 1986; Niemi et al., 1997; Osterberg et al., 1995; Rilfors et al., 1994). In those studies the average acyl chain lengths (C_n) of the total lipid extracts were in the range of 16–18 carbon atoms. However, *A. laidlawii* A can be forced to have lipids with C_n values between 14.5 and 20 carbon atoms (Wieslander et al., 1995). From studies of synthetic lipids it is well established that such a large difference in chain length has a dramatic impact on the T_{NL} values (Koynova and Caffrey, 1994; Mannock et al., 1990; Sen et al., 1990).

The questions we ask in this study are: 1) Are the cells able to maintain T_{NL} of the total lipids within a narrow range at the limiting C_n values? 2) Which lipids are responsible for the nonlamellar tendencies at these C_n values? For this purpose *A. laidlawii* A was grown in media supplemented with the shortest and longest fatty acids possible for growth, and the phase behavior of the extracted total lipids was investigated to answer the first question.

The lipids with the capacity to form reversed nonlamellar phases in *A. laidlawii* A are 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDAG), 1,2-diacyl-3-*O*-[6-*O*-acyl-(α-D-glucopyranosyl)]-*sn*-glycerol (MAMGlcDAG), and 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1 → 2)-*O*-(6-*O*-acyl-α-D-glucopyranosyl)]-*sn*-glycerol (MADGlcDAG) (Andersson et al., 1996; Lindblom et al., 1986, 1993; Niemi et al., 1995). It is well known that long acyl chains shift the phase equilibria toward nonlamellar phases, and therefore the important balance between lamellar-forming and nonlamellar-forming lipids is obviously maintained when *A. laidlawii* A is grown with long chain fatty acids. However, it is still unknown whether any lipid with short acyl chains in this organism is able to form nonlamellar phases close to physiological temperatures. It has been observed that MGlcDAG and MADGlcDAG are present in short-chain lipid extracts (Andersson et al., 1996), and the phase behavior of these lipids was studied in this work to answer the second question. We anticipate that MADGlcDAG will play an important role in this regulation process.

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MATERIALS AND METHODS

Cell growth

Strain A-EF22 of *A. laidlawii* was grown in a lipid-depleted bovine serum albumin/tryptose medium (Andersson et al., 1996). Twenty liters of the medium was supplemented with 75 μM α-deuterated myristic acid (14:0-d_2) and 75 μM palmitoleic acid (16:1c), and 5 l of the medium was supplemented with 120 μM α-deuterated arachidic acid (20:0-d_2) and 30 μM α-deuterated oleic acid (18:1c-d_2). α-Deuterated oleic acid (18:1c-d_2) was synthesized according to the method of Tulloch (1977), and 14:0-d_2 and 20:0-d_2 were obtained from Larodan Fine Chemicals (Malmö, Sweden). The cells were grown at 37°C and adapted to the two fatty acid pairs by at least five consecutive daily inoculations. The final two inoculations were 5% (v/v), and the time of growth was 20 $\pm$ 1 h. The cell cultures were harvested as described by Andersson et al. (1996).

Lipid extraction

The membrane lipids were extracted and purified as described previously (Andersson et al., 1996). Divalent cations were removed from the total lipid extracts and exchanged for sodium ions by a modified version (Rilfors et al., 1994) of the procedure described by Smaal and colleagues (1985). This procedure was only performed on 120 mg of the total lipid extract isolated from the cells supplemented with 14:0-d_2 and 16:1c (see next section).

Purification of MGlcDAG and MADGlcDAG

MGlcDAG and MADGlcDAG were purified from the lipid extracts isolated from the cell cultures supplemented with 14:0-d_2 and 16:1c. The remainder of the total lipid extract (see previous section) was applied to a silica gel (Silica gel S, 230–400 mesh; Riedel-de Häen, Seelze, Germany) column. A slight N_2 pressure was maintained over the column to prevent oxidation of the lipids. Pigments and neutral lipids were eluted with chloroform, and the glucolipids with acetone. The acetone fractions, which mainly contained MGlcDAG and MADGlcDAG, were selected and applied to preparative thin-layer chromatography (TLC) plates to separate the two lipids (Hauksson et al., 1995). The glucolipids were eluted from the gel as described previously (Lindblom et al., 1986). Divalent cations were removed from the purified lipids and exchanged for sodium as described in the previous section.

Determination of lipid composition

The acyl chain distributions in the two glucolipids and the total lipid extracts were determined by gas-liquid chromatography after converting the acyl chains to their methyl esters (Rilfors et al., 1978). The analyses of the methyl esters and the calculations of the molar percentages were performed as in Andersson et al. (1996).

The polar headgroup distribution in the total lipid extracts were analyzed with high-performance liquid chromatography (HPLC), by a modified version (Andersson et al., 1996) of the procedure described by Arnoldsson and Kaufmann (1994). A mixture of MGlcDAG, MAMGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDAG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and 1,2-diacyl-3-*O*-[glycerophosphoryl-6-*O*-(α-D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*-α-D-glucopyranosyl)]-*sn*-glycerol (GPDGlcDAG) was analyzed to determine their molar response factors. POPG was obtained from Avanti Polar Lipids (Birmingham, AL), and a preparation of MAMGlcDAG from Lindblom et al. (1993) was used. The preparations of DGlcDAG and GPDGlcDAG used were from Andersson et al. (1996). The molar response factors were determined for each of the five lipids, and by interpolation the response factors for MADGlcDAG and 1,2-diacyl-3-*O*-[glycerophosphoryl-6-*O*-(α-D-glucopyranosyl-(1 $\rightarrow$ 2)-monoacylglycerophosphoryl-6-*O*-α-D-glucopyranosyl)]-*sn*-glycerol (MABGPDGlcDAG) were determined from a plot of response factors versus retention time. The fraction designated 1,2-diacylglycerol (DAG) contains 75–95 mol% of DAG, the remainder being mainly free fatty acids and probably some pigments (Wieslander et al., 1995). Therefore, this fraction is presented as area % values in Table 2. To determine the approximate mol% values, the molar response factor for this fraction was estimated by extrapolation, using the plot of response factors versus retention time. The peaks in the chromatogram were assigned by comparing their retention times with those of the purified *A. laidlawii* lipids, and the molar percentages were calculated from the obtained molar response factors.

The purity of the MGlcDAG and MADGlcDAG preparations was determined by HPLC and TLC (Andersson et al., 1996). The purity was determined by TLC to be $\geq$99% for MGlcDAG and $\geq$90% for MADGlcDAG; the contaminant in the latter preparation was mainly MGlcDAG. HPLC could not detect any impurities in the MGlcDAG preparation, whereas the MADGlcDAG preparation contained 3.8 mol% MGlcDAG.

Preparation of lipid samples for NMR and x-ray studies

The lipids (20–30 mg) were dried to a film in an 8-mm outer diameter glass tube with N_2 and then dried to constant weight in vacuum. After the addition of 20, 30 or 40 wt% water, the tubes were centrifuged and flame-sealed. Deuterium-depleted water (^1H_2O) (Fluka, Buchs, Switzerland) or deuterium oxide (Cambridge Isotope Laboratories, Woburn, MA) was used for the ^2H NMR studies and the diffusion measurements, respectively. The samples were mixed by extended centrifugation and freeze-thawed for 10 cycles to ensure complete equilibration.

^{2}H-NMR measurements and data processing

^2H-NMR spectra were obtained for the lipid samples at a frequency of 76.77 MHz on a Bruker AMX2–500 spectrometer. A selective ^2H high-power probe with an 8-mm horizontal solenoid coil (500/8/X; Cryomagnetic Systems, Indianapolis, IN) was used. A phase-cycled quadrupole echo pulse sequence was used (Davis et al., 1976), with a $\pi/2$ pulse length of 6.4 μs and a 40-μs pulse separation. A total of 20,000–25,000 scans were collected for each temperature, with a recycle time of 0.15 s. The temperature was controlled with a Eurotherm B-VT 2000 unit and checked by a second thermocouple placed close to the sample. A temperature calibration was made on the standard settings, from which the desired temperatures were calculated. Each temperature increment was 2.5°C and was kept for 30 min, i.e., the sample had 30 min of equilibration time before the acquisition started. The data processing was performed according to the method of Andersson et al. (1996). To determine the fractions of the phases present in the MGlcDAG samples, simulations of the spectra were performed with the FTNMR program (Hare Research). No decomposition of the lipids was observed according to the TLC analyses performed after the measurements. The phase transitions can be conveniently followed from a measurement of the NMR quadrupole splittings as a function of temperature and composition (Lindblom, 1996).

NMR diffusion measurements

The self-diffusion coefficient of MGlcDAG in the cubic liquid crystalline phase was determined with the Fourier-transform pulsed magnetic field gradient spin-echo technique (Lindblom and Orädd, 1994; Stejksal and Tanner, 1965; Stilbs, 1987). A Hahn-echo sequence ($\pi/2$–τ–π–τ–acquisition) was used to refocus the magnetization.

The diffusion experiments were performed at 55°C on a Chemagnetics CMX-100 spectrometer equipped with a HP-90 proton diffusion goniometer probe (Cryomagnet Systems, Indianapolis, IN). The magnet gradient pulses were generated by a home-built gradient unit driven by a Kenwood PD35–20D power supply.

The gradient pulses of rectangular shape with duration δ and strength g were applied on each side of the 180° pulse with a separation of $\Delta = \tau$. In

TABLE 1 Acyl chain composition in the total lipid extracts and purified glucolipids from *A. laidlawii* A-EF22

Fatty acid supplement to growth medium*	Lipid	Acyl chain# composition (mol%)											
		12:0	13:0	14:0	15:0	16:0	16:1c	18:0	18:1c	20:0	ND§	C_n¶	UAC‖
20:0-d_2/18:1c–d_2 (4:1)	Total lipid extract**	0.3	0.6	1.9	0.8	2	0.3	0.3	30.6	63	—	19.1	30.9
14:0-d_2/16:1c (1:1)	Total lipid extract**	0.4	0.4	51	0.2	1.2	41.6	0.4	—	1.5	3.6	15.1	41.6
14:0-d_2/16:1c (1:1)	MGlcDAG	0.2	0.4	48	0.2	1.2	46.2	0.5	—	0.3	3.2	15.1	46.2
14:0-d_2/16:1c (1:1)	MADGlcDAG	1.4	0.9	62	0.2	1.3	31.7	0.1	—	—	2.3	14.7	31.7

*The total concentration of the fatty acids supplemented to the growth medium was 150 μM.
#Fatty acids and acyl chains are denoted as *n:k*, where *n* is the number of carbons and *k* is the number of *cis* double bonds.
§Not determined or acyl chains in minor amounts.
¶Average acyl chain length.
‖Unsaturated acyl chains (mol%).
**The degrees of incorporation of the exogenously supplied fatty acids into the membrane lipids were ≥92 mol%, the remainder being synthesized by the organism.

addition, the diffusion experiments were performed by varying δ while keeping the other parameters constant. The experimental parameters were $\tau = \Delta = 100$ ms, $\delta = 1$–20 ms, $g = 0.958$ T/m.

X-ray diffraction

The x-ray measurements of MADGlcDAG and MGlcDAG were performed at Station 8.2 at the Daresbury Laboratory (Cheshire, England) with a monochromatic beam of wavelength 1.5 Å. This station provides the possibility of simultaneously measuring small-angle (SAXS) and wide-angle (WAXS) x-ray scattering (Bras et al., 1993). The sample-to-detector distance for the SAXS experiment was 1.5 m. SAXS data were calibrated against a sample of wet rat tail collagen, and the WAXS data were calibrated using ice peaks from frozen samples. Immediately before the diffraction experiments were performed, the samples were placed between mica sheets held by copper spacers. The sample temperatures were thermostatically controlled by mounting the samples on a modified microscope cryostage (Linkam, England) and monitored with a thermocouple embedded in the sample adjacent to the beam.

Starting at 25°C, the temperature was decreased at a rate of 3°C/min to −25°C and then raised at the same rate up to ~60°C. At certain intervals the temperature was held at a constant value for several minutes to ensure sample equilibration. No change in the diffractograms was observed during these constant temperature periods, and it was concluded that the sample was close to thermal equilibrium at all times. The gel phase was recognized from the sharp WAXS reflection around 5 Å. The SAXS reflections were used to distinguish the liquid crystalline phases (Seddon, 1990). After the measurements, the lipids were removed from the mica sheets and checked with TLC to make sure that no decomposition of the lipids had occurred.

RESULTS

Composition of *A. laidlawii* lipids

A "growth window," defined by the length and the degree of *cis*-monounsaturation of the supplemented fatty acids, has been established for *A. laidlawii* strain A-EF22 (Wieslander et al., 1995). The phase behavior of total lipid extracts with acyl chain compositions near the chain length boundaries of this "growth window" has been determined in the present study (Table 1).

A. laidlawii A regulates its polar headgroup composition of the membrane lipids according to the prevailing growth conditions (Andersson et al., 1996; Rilfors et al., 1993; Wieslander et al., 1980). The polar headgroup compositions in the total lipid extracts are presented in Table 2. The relative amounts of each lipid are consistent with earlier studies (Andersson et al., 1996). An important point to make is that the fraction of the lipids with a potential to induce the formation of reversed nonlamellar phases (DAG, MGlcDAG, MAMGlcDAG, and MADGlcDAG) is larger in the short-chain total lipid extract. However, the difference in this fraction between the total lipid extracts is most probably even larger than that seen in Table 2, because the lipid fraction designated DAG is overestimated by the area % values. The DAG fraction has the shortest HPLC retention time (Andersson et al., 1996), yielding a very low value of the molar response factor. By using an extrapolated response factor (see Materials and Methods) the area % value for DAG in the long-chain total lipid extract is converted to 17 mol%. This value is reasonable, because it was found in a previous study that the DAG fraction constituted 15–20 mol% for total lipid extracts with a $C_n \approx 18$ and 30 mol% unsaturated acyl chains (Wieslander et al., 1995).

Phase equilibria of *A. laidlawii* total lipid extracts

The phase equilibria of total lipid extracts from *A. laidlawii* A with C_n values of ~15 and ~19 carbon atoms were

TABLE 2 Polar headgroup composition (mol%) in the total lipid extracts from *A. laidlawii* A-EF22

Fatty acid supplement to growth medium*	Lipid# (mol%)							
	DAG	MGlcDAG	MAMGlcDAG	DGlcDAG	MADGlcDAG	PG	GPDGlcDAG	MABGPDGlcDAG
20:0-d_2/18:1c–d_2 (4:1)	34.8§ (17)	8.3	0.3	32.5	0.9	11.5	10.9	0.8
14:0-d_2/16:1c (1:1)	0.4§	53.7	—	6.9	6.3	9.2	9.6	13.4

*The total concentration of the fatty acids supplemented to the growth medium was 150 μM.
#For abbreviations, see main text.
§This lipid is presented in area %; see Results.

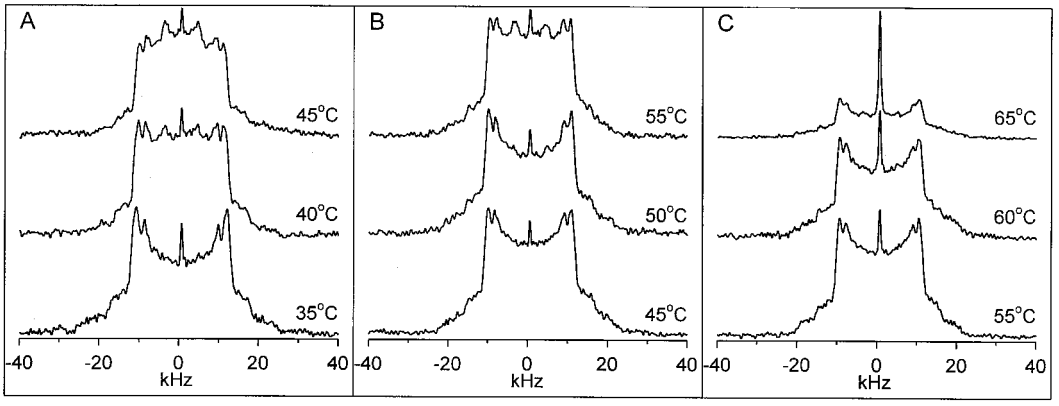

FIGURE 1 ^{2}H-NMR spectra of total membrane lipids extracted from *A. laidlawii* A-EF22 cells supplemented with 75/75 (μM/μM) 14:0-d_2/16:1c. The lipid extract contained the neutral lipids. (*A*) Sample 1 in Table 3, 20 wt% ^1H_2O. (*B*) Sample 3 in Table 3, 30 wt% ^1H_2O. (*C*) Sample 4 in Table 3, 40 wt% ^1H_2O.

examined by ^{2}H-NMR for three different water concentrations (20, 30, and 40 wt% ^1H_2O). Figs. 1 and 2 show the ^{2}H-NMR spectra of the total lipid extracts containing short and long acyl chains, respectively. The spectra recorded from the short-chain total lipid extract with 20 wt% water are presented in Fig. 1 *A*. At 35°C the magnitude of the quadrupole splittings ($\Delta\nu_Q$) indicates the presence of an L_α phase (dominating splitting with $\Delta\nu_Q \approx 22$ kHz). At 40°C additional splittings of less than half the magnitude compared to those originating from an L_α phase are observed (dominating splitting with $\Delta\nu_Q \approx 8$ kHz). The transition from an L_α phase to a reversed hexagonal liquid-crystalline (H_{II}) phase yields a reduction of the quadrupole splittings by a factor of $\sim$2 or more in a ^{2}H-NMR spectrum, as a result of an additional averaging by the translational diffusion around the symmetry axis of the water cylinders and more flexible chains in the H_{II} phase (Lindblom, 1996). Thus a transition from an L_α to an H_{II} phase occurred around 40°C, according to Fig. 1 *A*. In Fig. 1 *B* the spectra for the same total lipid extract with 30 wt% water are shown. Using the same reasoning as for Fig. 1 *A*, the magnitude of $\Delta\nu_Q$ indicates the presence of an L_α phase up to 45°C, whereas components corresponding to an H_{II} phase appear at 50°C. In Fig. 1 *C*, where the water content was 40 wt%, an L_α phase is present up to 60°C, whereas at 65°C a narrow signal is observed to be superimposed on the spectra originating from L_α and H_{II} phases. The narrow signal is an indication of a cubic phase in which fast isotropic motion occurs. The spectra recorded from the long-chain total lipid extract with 20 wt% water are presented in Fig. 2 *A*. At 40°C the magnitude of $\Delta\nu_Q$ in the spectrum indicates the presence of an L_α phase, whereas at 45°C components corresponding to an H_{II} phase have emerged. In Fig. 2 *B* spectra from the long-chain total lipid extract with 30 wt% water are shown. The spectrum recorded at 45°C indicates that an L_α phase is present, whereas at 50°C components arising from an H_{II} phase are observed. Finally, in Fig. 2 *C* the water content of the sample was 40 wt% and an L_α phase is present up 50°C, whereas at 55°C an H_{II} phase starts to form. The ^{2}H-NMR spectra recorded from the other samples were interpreted in an analogous way, and the temperatures at which an H_{II} and/or a reversed cubic phase

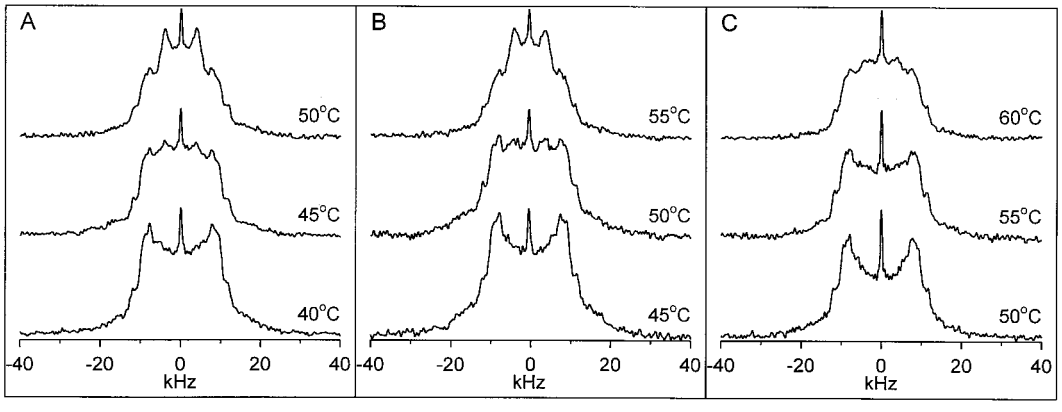

FIGURE 2 ^{2}H-NMR spectra of total membrane lipids extracted from *A. laidlawii* A-EF22 cells supplemented with 120/30 (μM/μM) 20:0-d_2/18:1c-d_2. The lipid extract contained the neutral lipids. (*A*) Sample 6 in Table 3, 20 wt% ^1H_2O. (*B*) Sample 7 in Table 3, 30 wt% ^1H_2O. (*C*) Sample 10 in Table 3, 40 wt% ^1H_2O.

first appeared in the ^{2}H-NMR spectra are summarized as T_{NL} values in Table 3. The reproducibility of the phase equilibria was checked by investigating duplicate samples and/or by remeasurements.

The T_{NL} value increases with increasing water concentration for both total lipid extracts (Figs. 1 and 2 and Table 3). The change from 20 to 40 wt% water resulted in an increase in the T_{NL} values of ~25°C and ~5–10°C for the lipids with short and long acyl chains, respectively. This entails that the T_{NL} value is slightly lower for the short-chain lipids than for the long-chain lipids when the water content is 20 wt%, but the value is higher for the former lipid extract when the water content is 40 wt%. Another difference between the two lipid extracts is that the long-chain lipids with 40 wt% water form L_α and H_{II} phases at high temperatures, whereas the short-chain lipids form an I_{II} phase in addition to the L_α and H_{II} phases under these conditions (Figs. 1 *C* and 2 *C*). The fraction of the cubic phase formed in the short-chain extract with 40 wt% water is estimated to be 10–15%. Finally, the values of the quadrupole splittings are larger for the short-chain lipids than for the long-chain lipids, which is in accordance with former studies (Monck et al., 1992; Thurmond et al., 1994).

Phase equilibria of MADGlcDAG and MGlcDAG

Short-chained MADGlcDAG (Table 1) with 20 wt% water, corresponding to 14.9 mol of ^1H_2O/mol of lipid, was investigated with ^{2}H-NMR and x-ray diffraction. It can be inferred from Fig. 3 that the ^{2}H-NMR spectra of MADGlcDAG exhibit a very broadened signal at temperatures up to 40–45°C, whereas well-resolved quadrupole splittings are observed at higher temperatures. The magnitude of the splittings is approximately half of the value of the splittings emanating from an L_α phase and is equal in magnitude to the splittings originating from an H_{II} phase (cf. Fig. 1). This strongly indicates that MADGlcDAG forms an H_{II} phase, and Fig. 3 illustrates that the H_{II} phase remains up to the highest temperature investigated (60°C). The ^{2}H-NMR results are in good agreement with the x-ray diffraction experiments. The latter showed that the last traces of a lamellar gel (L_β) phase disappear at 41°C, and only reflections originating from an H_{II} phase were detected up to 60°C (Fig. 4). Moreover, x-ray diffraction showed that the H_{II} phase is present together with the L_β phase at temperatures as low as −22°C. The lattice parameters for the phases formed by MADGlcDAG are presented in Table 4.

Fig. 5 presents some ^{2}H-NMR spectra recorded from short-chained MGlcDAG (Table 1). At 10 wt% water, corresponding to 4.3 mol of ^1H_2O/mol of lipid, an L_α phase is present at 25°C, and an isotropic component arises in the spectra at higher temperatures. This latter component is caused by an I_{II} phase (see x-ray diffraction results). The I_{II} phase is the only phase present at 50°C, but when the temperature has reached 55–60°C an H_{II} phase is in equi-

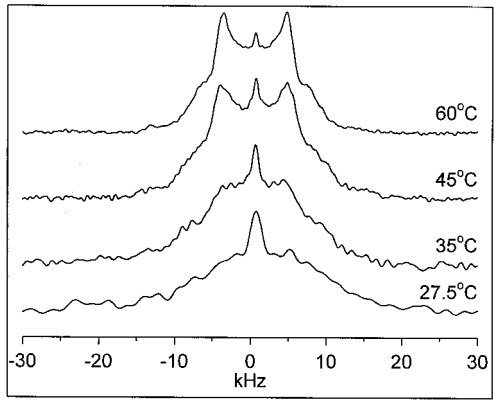

FIGURE 3 ^{2}H-NMR spectra of MADGlcDAG with an acyl chain composition given in Table 1. The water concentration of the sample was 20 wt%.

TABLE 3 The transition temperature (T_{NL}) from a lamellar liquid crystalline (L_α) to a reversed nonlamellar phase in total lipid extracts isolated from *A. laidlawii* A-EF22

	Total lipid extract with C_n* = 15.1 and UAC# = 41.6		Total lipid extract with C_n = 19.1 and UAC = 30.9	
Water content	Sample	T_{NL}	Sample	T_{NL}
20 wt% ^1H_2O	1§	37.5 ± 2.5°C	5	42.5 ± 2.5°C
	2	32.5 ± 2.5°C	6§	42.5 ± 2.5
30 wt% ^1H_2O	3§	47.5 ± 2.5°C	7§	47.5 ± 2.5°C
			8	52.5 ± 2.5°C
40 wt% ^1H_2O	4§	62.5 ± 2.5°C	9	47.5 ± 2.5°C
			10§	52.5 ± 2.5°C

The lipid extracts contained the neutral lipids.
*Average acyl chain length.
#Unsaturated acyl chains (mol%).
§The ^{2}H-NMR spectra of samples 1, 3, and 4 and of samples 6, 7, and 10 are presented in Figs. 1 and 2, respectively.

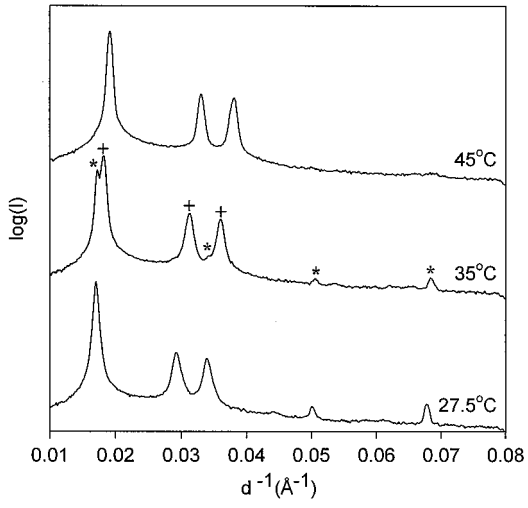

FIGURE 4 X-ray powder diffraction patterns obtained from MADGlcDAG with 20 wt% water. The acyl chain composition of MADGlcDAG is given in Table 1. At 35°C, the reflections arising from the gel phase are marked by a star and those from the H_{II} phase by a plus sign.

TABLE 4 Lattice parameters in the H_{II} (*a*) and the L_{β} (*d*) phases of the short-chain MADGlcDAG sample containing 20 wt% water at three temperatures

T (°C)	27.5	35	45
*a** (Å)	67.9	63.5	60
$d^{\#}$ (Å)	58.7	58.1	—

The acyl chain composition of MADGlcDAG is given in Table 1.
*The distance between the cylinder axes in the H_{II} phase.
#The lamellar repeat distance in the L_{β} phase.

librium with the I_{II} phase. At 20 wt% water, corresponding to 9.8 mol of ^1H_2O/mol of lipid, MGlcDAG forms an L_{α} phase in equilibrium with an I_{II} phase at temperatures between 25 and 55°C, whereas a further increase in the temperature to 60°C results in the formation of only an I_{II} phase.

X-ray diffraction experiments on short-chained MGlcDAG with 20 wt% water shed further light on the phase equilibria exhibited by this lipid. In the cooling mode starting at 24°C, reflections from three phases were observed, namely, I_{II}, L_{α}, and crystalline/gel phases. However, only one reflection from the cubic phase was observed. Below −16°C the reflections from the L_{α} phase were absent, and only the gel phase could be detected. In the subsequent heating mode the L_{α} phase appeared at −7°C, and a weak reflection from the I_{II} phase appeared at ~32°C. When the temperature was raised, more reflections belonging to the cubic phase appeared, and the gel phase disappeared completely at 40°C. At the highest temperature investigated with x-ray diffraction (57°C), traces of the L_{α} phase could still be detected (Fig. 6). The reflections from the cubic phase were indexed according to the body-centered space group *Ia3d*, and the validity of the indexing can be judged from the straight line passing through the origin of coordinates in the plot of $1/d$ versus $(h^2 + k^2 + l^2)^{1/2}$ (Fig. 7). From the slope, the unit cell dimension can be calculated to be equal to 115 Å (Table 5).

The translational diffusion coefficient of the lipids in a cubic phase can be used to distinguish between a bicontinuous one from a cubic phase composed of closed aggregates (Lindblom and Orädd, 1994). The translational diffusion coefficient for MGlcDAG in the I_{II} phase with 20 wt% water at 55°C was determined in a pulsed field gradient NMR experiment to be 3.4×10^{-12} m^2/s.

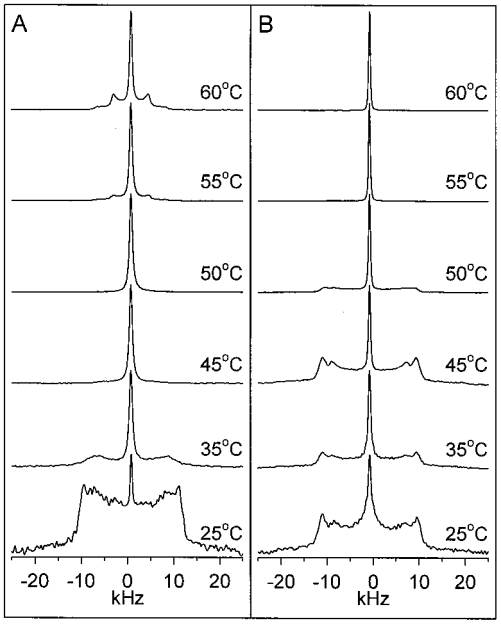

FIGURE 5 ^2H-NMR spectra of MGlcDAG with an acyl chain composition given in Table 1. (*A*) 10 wt% ^1H_2O. The fractions of the two phases at 55 and 60°C were obtained from simulations of the spectra and were estimated to be 32% H_{II} and 68% I_{II} at 55°C, and 58% H_{II} and 42% I_{II} at 60°C. (*B*) 20 wt% ^1H_2O. The fractions of the two phases at 45°C were estimated to be 85% L_{α} and 15% I_{II}.

DISCUSSION

Our major purpose in this study is to determine which lipids in a short-chain total lipid extract are able to form reversed nonlamellar phases and if they have the potential to induce such phases in total lipid extracts. Therefore, the discussion will first deal with the phase behavior of MGlcDAG and MADGlcDAG having short acyl chains. Subsequently, we will discuss the phase behavior of short- and long-chain total lipid extracts and relate these results to our model for the regulation of the lipid composition in the membrane of *A. laidlawii* A.

Phase properties of MADGlcDAG

In the previous phase studies of MADGlcDAG from *A. laidlawii* A, the acyl chains were ~1.6 carbon atoms longer and ~30 mol% more unsaturated (Andersson et al., 1996) than the chains of the corresponding lipid investigated in this work. In accordance with the earlier study, only one liquid crystalline phase, namely the H_{II} phase, was obtained above the chain melting temperature. Because MADGlcDAG with very short and more saturated acyl chains also

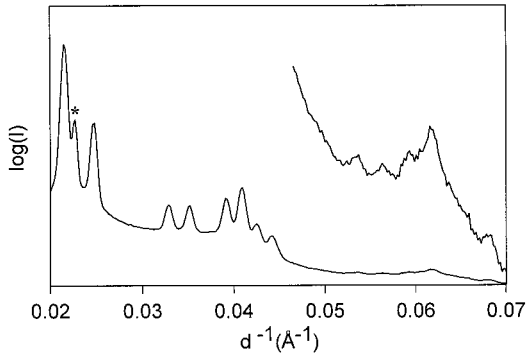

FIGURE 6 X-ray powder diffraction pattern obtained from the cubic phase formed by MGlcDAG with 20 wt% water at 57°C. The acyl chain composition is presented in Table 1. The reflection marked by a star originates from a small amount of L_{α} phase. The magnification shown is 10 times the original diffraction pattern.

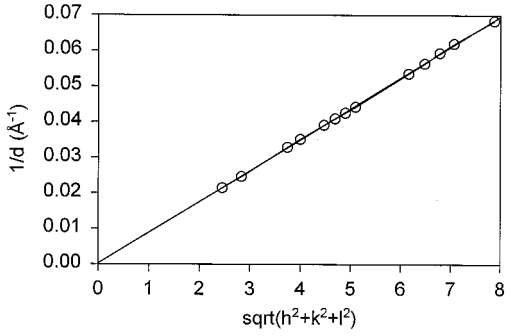

FIGURE 7 Plot of $1/d$ versus $(h^2 + k^2 + l^2)^{1/2}$ of the reflections obtained in the x-ray diffractogram (Fig. 6) recorded from the cubic phase formed by MGlcDAG. The water concentration of the sample was 20 wt%, and the temperature was 57°C.

forms an H_{II} phase, this convincingly shows that it has a large potential to form nonlamellar phases.

The distance between the cylinder axes (a) for MADGlcDAG in the H_{II} phase was found to be considerably larger for the short-chain MADGlcDAG ($a = 70$ Å) than for MADGlcDAG with longer acyl chains ($a = 57$ Å) (Andersson et al., 1996). However, the opposite difference in a would be expected, considering the effect of the chain length only, because it has been observed for the saturated species of synthetic MGlcDAG, 1,2-*O*-diacyl-3-*O*-β-D-galactosyl-*sn*-glycerol (β-MGalDAG), and PE that a increases by ~2–8 Å in the H_{II} phase with an increment of the acyl chain length of two carbons (Mannock and McElhaney, 1991; Seddon et al., 1984; Sen et al., 1990). The larger value of a for the short-chain MADGlcDAG is therefore most probably owing to its higher degree of acyl chain saturation compared to the previously studied MADGlcDAG. Support for this assertion comes from a comparison of a for two MGlcDAG preparations with a C_n value of ~17, but with different unsaturation; $a \approx 67$–68 Å for the saturated preparation, whereas $a = 49$ Å for a preparation with 58 mol% unsaturated acyl chains (Andersson et al., 1996; Sen et al., 1990). A reduction in a of a similar magnitude has been observed for β-MGalDAG when saturated acyl chains are exchanged for mainly polyunsaturated acyl chains of the same chain length (Mannock and McElhaney, 1991; Sen et al., 1981; Shipley et al., 1973).

Phase properties of MGlcDAG

The phase equilibria of some MGlcDAG preparations with medium chain lengths isolated from *A. laidlawii* strain A have been determined in earlier studies (Andersson et al., 1996; Lindblom et al., 1986). Dioleoyl-MGlcDAG (DOMGlcDAG) forms only I_{II} and H_{II} phases above 10°C at water concentrations between ~1 and 15 wt%, and above ~15 wt% only the H_{II} phase is formed (Lindblom et al., 1986). The MGlcDAG prepared by Andersson et al. (1996) had $C_n = 16.9$ and 58 mol% unsaturated acyl chains. This preparation formed mainly I_{II} and H_{II} phases above 30°C.

The present study of MGlcDAG with $C_n = 15.1$ and 46 mol% unsaturated acyl chains shows that this lipid forms mainly an L_α phase at lower temperatures, whereas an I_{II} phase is formed with increasing temperature. An H_{II} phase is formed at the highest temperatures and at a low water content (10 wt%). This is in accordance with former studies (Andersson et al., 1996), where it was found that the I_{II} phase remains for MGlcDAG at higher temperatures when the water concentration was raised.

The results obtained for MGlcDAG of natural origin are in agreement with studies of the phase behavior of synthetic MGlcDAG with a homologous series of saturated acyl chains with $C_n = 11$–20 (Mannock et al., 1990; Sen et al., 1990). It was found that di-14:0-MGlcDAG transforms from an L_α phase to an I_{II} phase at 105°C, and the phase transition temperature was reduced to 82.0°C and 79.1°C for the di-15:0- and di-16:0-MGlcDAG species, respectively. An H_{II} phase was formed instead of an I_{II} phase when $C_n > 16$. The transition temperature was found to be 76.6°C for di-17:0-MGlcDAG and was only slightly reduced for the longer chains. Thus a lipid with short acyl chains is less wedge-shaped than a lipid with long acyl chains, and because of packing restrictions short-chain lipids form aggregates with less curvature than long-chain lipids, i.e., a cubic phase is favored by the short-chain lipids (see also Lewis et al., 1997).

The reflections obtained by x-ray measurements on the MGlcDAG with short acyl chains, at 20% water, showed that the I_{II} phase belongs to the body-centered space group *Ia3d*. In the study by Lindblom et al. (1986), the cubic phase formed by DOMGlcDAG was also assigned to this space group. Sen et al. (1990) found that the cubic phase formed by synthetic MGlcDAG with short acyl chains belongs to the *Pn3m* or *Pn3* space group. The water content in the latter MGlcDAG samples was significantly higher than in the samples investigated in this work. These results are in line with the experimental and theoretical observations that the sequence of formation of different cubic phases with increasing water content is *Ia3d*→*Pn3m*→*Im3m* (Lindblom and Rilfors, 1989). The lipid translational diffusion coefficient obtained in this study is of a magnitude (10^{-12} m^2/s) similar to that measured for DOMGlcDAG (Lindblom et al., 1986), and it is of a magnitude similar to that observed, for example, for dioleoylphosphatidylcholine in a bicontinuous cubic phase (Lindblom, 1996). It is therefore concluded that the cubic phase of MGlcDAG investigated here is also bicontinuous (Lindblom, 1996; Lindblom and Orädd, 1994; Lindblom and Rilfors, 1989; Rilfors et al., 1986).

Nonlamellar tendencies in total lipid extracts

Theories for the self-assembly of lipid molecules (Gruner, 1985; Helfrich, 1973; Israelachvili, 1991) form the basis for the model that we have presented concerning the regulation of the membrane lipid composition in *A. laidlawii* A and *E. coli* (Andersson et al., 1996; Morein et al., 1996; Rilfors et

TABLE 5 Observed reflections in the cubic phase of the short-chain MGlcDAG sample containing 20 wt% water, together with the assigned *hkl* values according to the cubic space group *Ia3d* at 57°C

d (Å)	46.5	40.5	30.4	28.4	25.6	23.5	22.6	18.7	17.7	16.9	16.1	14.6
$h^2 + k^2 + l^2$	6	8	14	16	20	24	26	38	42	46	50	62

The acyl chain composition is given in Table 1.

al., 1993). Eight different polar headgroups occur in the membrane lipids of *A. laidlawii* A; four of these lipids are able to form, or to induce the formation of, reversed nonlamellar phases; three lipids form only lamellar phases; and one lipid can form a diluted solution phase of normal micelles (Andersson et al., 1996; Danino et al., 1997; Hauksson et al., 1994a,b, 1995; Lindblom et al., 1993; Rilfors et al., 1993). The molar fractions of all of these lipids are metabolically varied in relation to the structure of the fatty acids that are either synthesized endogenously or taken up by the cells from the growth medium and covalently incorporated into the lipids. From studies of *A. laidlawii* A lipids containing acyl chains of medium lengths, it has been concluded that the fractions of the eight lipids are balanced in such a way that the T_{NL} values for total lipid extracts are maintained within rather narrow limits (Lindblom et al., 1986; Niemi et al., 1997; Osterberg et al., 1995; Rilfors et al., 1994). From this conclusion we stated our model for the balance between lamellar-forming and nonlamellar-forming lipids, predicting a regulation of the membrane lipid composition in biological membranes. Because the acyl chain length has a large impact on the T_{NL} values for synthetic membrane lipids, one question we ask in the present study is whether our model also holds for total lipid extracts with very short and long acyl chains.

The T_{NL} values for the total lipid extracts are presented in Table 3 for different water contents, and it is obvious that nonlamellar phases can be formed by total lipid extracts with both $C_n = 15.0$ and $C_n = 19.1$. The T_{NL} values for the two lower water contents differ by 5–10°C. At 40 wt% water the difference is somewhat larger (10–15°C), and a small fraction of a cubic phase is formed, in addition to the H_{II} phase, in the short-chain lipid extract. The shift in phase equilibria toward an L_α phase with an increasing degree of hydration is in accordance with earlier reports on various lipid-water systems (Gulik et al., 1985, 1988; Gulik-Krzywicki et al., 1967; Luzzati and Husson, 1962; Rilfors et al., 1984; Rivas and Luzzati, 1969; Seddon, 1990).

Can the differences in the T_{NL} values (for example, ~10–15°C for 40 wt% water) for the total lipid extracts with long and short acyl chains be judged as large or small? One way to consider this question is to compare the variations in these T_{NL} values with the variations that would result if the polar headgroup composition were kept constant when the acyl chain length is altered. Unfortunately, with the available limited data on T_{NL} values for lipids and lipid mixtures from *A. laidlawii*, this question is difficult to answer. Indeed, T_{NL} values for saturated MGlcDAG species with different chain lengths have been determined (Mannock et al., 1990; Sen et al., 1990), but such data are not available for MAMGlcDAG and MADGlcDAG. Moreover, even if T_{NL} data from all single lipids were available, they would not be sufficient, of course, for a prediction of T_{NL} values for fictitious total lipid mixtures, because these values are not weighted averages.

The second question we ask in this study is, which lipids are responsible for the nonlamellar tendencies in total lipid extracts with short and long acyl chains? The different lipid classes in *A. laidlawii* A have been shown to be preferentially synthesized at different acyl chain compositions (Andersson, 1998; Andersson et al., 1996). MGlcDAG and MADGlcDAG are the major nonlamellar-forming lipids when the organism is grown with fatty acids having $C_n \leq 16$. With short acyl chains MGlcDAG forms predominantly L_α and I_{II} phases, whereas MADGlcDAG forms only an H_{II} phase above the chain melting temperature. Thus the triacylglucolipid MADGlcDAG is a much more potent nonlamellar-forming lipid than MGlcDAG under these conditions. Both glucolipids are responsible for the maintenance of the nonlamellar tendencies when the organism incorporates short-chain fatty acids, but the potency of MGlcDAG is probably too weak and it therefore has to be assisted by MADGlcDAG to maintain the nonlamellar tendencies according to our model. The difference in nonlamellar-forming potency between the two lipids is reflected by the fact that MGlcDAG constitutes a considerably larger fraction in the cell membrane (Table 2). MADGlcDAG also reduces the average area per acyl chain and thus causes a tighter packing of the lipid molecules (Andersson et al., 1998). This effect may be advantageous when the membrane lipids contain short acyl chains, because the permeability across the membrane probably increases with such chains (McElhaney, 1992a).

When *A. laidlawii* A is grown with medium-chain and long-chain fatty acids, the nonlamellar tendencies in the membrane are principally maintained by MGlcDAG, DAG, and MAMGlcDAG. The latter two lipids are mainly synthesized when saturated acyl chains constitute more than 50 mol% (Andersson et al., 1996; Wieslander et al., 1995). It is less straightforward to find a rationale for this combination of nonlamellar-forming and nonlamellar-inducing lipids than for the combination of MGlcDAG and MADGlcDAG with short acyl chains. DAGs do not form any liquid crystalline phase (Di and Small, 1993), and the existing literature data reporting the effects of different DAGs on phosphatidylcholine and phosphatidylethanolamine bilayers only permit speculations to be made about the roles of DAG in *A. laidlawii* A. The DAGs synthesized by the organism can be anticipated to decrease the T_{NL} value of lipids in a bilayer with a tendency to form reversed nonlamellar phases

(Epand, 1985; Siegel et al., 1989). MGlcDAG with long and predominantly saturated acyl chains has rather high T_{NL} values (Mannock et al., 1990; Sen et al., 1990). It may therefore be advantageous to the cells to exchange a fraction of MGlcDAG for DAG, because DAGs can drastically decrease the T_{NL} values (Epand, 1985; Siegel et al., 1989). When synthesized, MAMGlcDAG constitutes only a small fraction in *A. laidlawii* A membranes (Andersson, 1998; Andersson et al., 1996). However, it is a very potent nonlamellar-forming lipid (Lindblom et al., 1993), and it possibly contributes to keeping the balance between lamellar-forming and nonlamellar-forming lipids. Neither DAG nor MAMGlcDAG is synthesized when *A. laidlawii* A incorporates long, unsaturated acyl chains into the membrane lipids. The T_{NL} value of MGlcDAG is then decreased (Lindblom et al., 1986), and the other two lipids are probably not required.

Finally, the question can be asked if regulation of the polar headgroup composition in *A. laidlawii* A membranes can have the aim of keeping other physicochemical parameters constant, such as the surface charge density, the gel/L_α phase transition temperature (T_m), the order parameter of the acyl chains, or the spontaneous curvature of the membrane lipids. The anionic lipid fraction in *A. laidlawii* A generally increases with the degree of acyl chain unsaturation (Andersson, 1998; Andersson et al., 1996; Wieslander et al., 1995); however, the conclusion drawn by Christiansson et al. (1985), that the surface charge density of the lipid bilayer is kept constant, was not corroborated by a recent study by Andersson et al. (1998). It has been observed in several studies that *A. laidlawii* can tolerate large variations in the T_m values of its membrane lipids, even when it is grown at the same temperature, and a regulation of this parameter can be excluded in many cases (McElhaney, 1992b, 1994; Rilfors et al., 1993). The order parameter of the acyl chains has been determined in both strains A and B of *A. laidlawii* when the cells were grown with different fatty acids. The average order parameter in intact membranes varied over the range 0.14–0.19 and 0.14–0.18 in strain A and B, respectively (Monck et al., 1992; Thurmond et al., 1994). It can be noted that the highest values of the order parameter in strain A were obtained when the membrane lipids contained short acyl chains. The nonlamellar tendencies of a lipid bilayer can be expressed by the spontaneous radius of curvature (R_0) of the monolayers (Gruner, 1985). R_0 values for various total lipid extracts from *A. laidlawii* A fall within a narrow range (58–73 Å) compared to the range represented by pure MGlcDAG and DGlcDAG species (17–123 Å) (Osterberg et al., 1995). Thus the spontaneous radius of curvature is maintained within a narrow interval for *A. laidlawii* A membrane lipids.

From the previously described arguments we conclude that probably only two physicochemical parameters are consistently regulated in *A. laidlawii* A membranes, namely the balance between lamellar-forming and nonlamellar-forming lipids, and the spontaneous radius of curvature of the lipid monolayers. What is more, these two parameters represent two different ways to express the phase behavior of the membrane lipids (Osterberg et al., 1995). The regulation of the lipid composition is not strict enough to maintain these parameters at a well-defined value, which is probably to be expected, because of variations that emerge when lipids of natural origin are studied. However, the regulation is effective enough to keep the values within a limited range. Our present results, obtained from lipids with limiting acyl chain lengths, further support the conclusion that *A. laidlawii* A regulates the polar headgroup composition of its lipids, so that it has the capacity to maintain the nonlamellar tendency of its lipids irrespective of the structure of the acyl chains. The results also substantiate our revised model for the membrane lipid regulation (Andersson et al., 1996), stating that the regulation mechanism is more complex and sophisticated than initially proposed, and that several nonlamellar-forming lipids are involved.

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