Effect of Independent Variations in Fatty Acid Structure and Chain Length on Lipid Polar Headgroup Composition in *Acholeplasma laidlawii* B Membranes: Regulation of Lamellar/Nonlamellar Phase Propensity†

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ABSTRACT: We have studied the biosynthetic regulation of the membrane lipid polar headgroup distribution in *Acholeplasma laidlawii* B cells made fatty acid auxotrophic by growth in the presence of the biotin-binding agent avidin to test whether this organism has the ability to coherently regulate the lamellar/nonlamellar phase propensity of its membrane lipids. The addition of various single normal growth-supporting exogenous fatty acids to such cell cultures produces fatty acid−homogeneous cells in which the hydrocarbon chain length and structure of the fatty acyl chains of the membrane lipids can be independently varied. Moreover, in analyzing our results, we consider the fact that the individual membrane lipid classes of this organism can form either normal micellar, lamellar, or reversed cubic or hexagonal phases in isolation (Lewis, R. N. A. H., and McElhaney, R. N. (1995) *Biochemistry 34,* 13818−13824). When *A. laidlawii* cells are highly enriched in one of a homologous series of methyl isobranched, methyl anteisobranched, or ω-cyclohexyl fatty acids, neither the ratio of normal micellar/lamellar nor of inverted cubic or hexagonal/lamellar phase-forming lipids are coherently regulated, and in fact in the former case, the changes in lipid polar headgroup composition observed are generally in a direction opposite to that required to maintain the overall lamellar/nonlamellar phase preference of the total membrane lipids constant when hydrocarbon chain length is varied. Similarly, when lipid hydrocarbon structure is varied at a constant effective chain length, a similar lack of coherent regulation of membrane lipid polar headgroup distribution is also observed, although in this case a weak overall trend in the expected direction occurs. We also confirm our previous finding (Foht, P. J., Tran, Q. M., Lewis, R. N. A. H., and McElhaney, R. N. (1995) *Biochemistry 34,* 13811−13817) that the ratio of inverted phase-forming monoglucosyl diacylglycerol to the lamellar phase-forming glycolipid diglucosyl diacylglycerol, previously used to estimate membrane lipid phase preference in *A. laidlawii* A and B, is not by itself a reliable indicator of the overall lamellar/nonlamellar phase propensity of the total membrane lipids of these organisms. Our results indicate that *A. laidlawii* B lacks a coherent mechanism to biosynthetically regulate the polar headgroup distribution of its membrane lipids to maintain the micellar/lamellar/inverted phase propensity constant in the face of induced variations in either the chain length or the structure of its lipid hydrocarbon chains. Finally, we suggest that the lack of a coherent regulatory mechanism to regulate the overall phase-forming propensity of the total membrane lipids of this organism under these circumstances may result in part from its inability to optimize all of the biologically relevant physical properties of its membrane lipid bilayer simultaneously.

The mixture of lipids present in all biological membranes studied to date appears to exist exclusively in the liquid−crystalline lamellar phase under physiologically relevant conditions of temperature and hydration. However, individual membrane lipids can potentially form a variety of liquid crystalline normal micellar, lamellar, or reversed cubic or hexagonal phases when dispersed in water, depending primarily on their effective molecular shapes. For these rodlike amphiphilic lipid molecules, the relative effective sizes of their polar and nonpolar regions are important elements in determining their preferred molecular shapes, in particular the relative cross-sectional areas occupied by their polar headgroups and nonpolar hydrocarbon chains. The effective cross-sectional area of a lipid polar headgroup appears to depend primarily on its effective headgroup volume (see below), while the effective cross-section area of the hydrocarbon chains depend primarily on their length and chemical structure. If the effective cross-sectional area of the polar headgroup exceeds that of the nonpolar hydrocarbon chains, then the lipid molecule will have an inverted conical shape and will tend to aggregate in water to form normal micelles or related structures. Conversely, if the relative cross-sectional of the polar headgroup is less than that of the hydrocarbon chains, the lipid will have a conical shape and will tend to aggregate in water to form a reversed

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cubic or hexagonal phase. If, however, the relative areas occupied by the polar headgroup and the hydrocarbon chains are roughly equal, the molecules will be cylindrical in shape and will tend to form a lamellar or bilayer phase. Since the effective area of the hydrocarbon chains in the liquid−crystalline state increases to a much greater extent with temperature than does that of the polar headgroup, increases in temperature favor the formation of lamellar over normal and reversed over lamellar phases (see refs *1*−*5*).

Acholeplasma laidlawii is a simple, cell wall-less prokaryotic microorganism that possesses a number of features that make it attractive for studies of the structural and functional roles of lipids in biological membranes (see refs *6* and *7*). Two particularly useful features of this organism for such studies are the ability to dramatically alter its membrane lipid fatty acid composition and cholesterol content. Utilizing the former feature, we have determined the relative phase preferences of all of the major lipids of fatty acid−homogeneous *A. laidlawii* B membranes by determining the effect of small amounts of each lipid on the lamellar/reversed hexagonal phase transition temperature of a PE[1] matrix of identical fatty acid composition using differential scanning calorimetry (*8*). Although the total membrane lipids from this organism form only lamellar phases under physiological conditions, the individual membrane lipids appear to exhibit a wide range of phase preferences. Phosphatidylglycerol (PG) and DGDG seem to have relatively strong and weak preferences for the lamellar liquid-crystalline phase, respectively, MGDG and especially APG strongly prefer the reversed hexagonal phase, while GPDGDG actually prefers the normal micellar phase in isolation (*9*). We also showed that the characteristic effect of the individual *A. laidlawii* B membrane lipids on the lamellar/reversed hexagonal phase transition temperature of the PE matrix is not well-correlated with their polar headgroup intrinsic volumes. This result indicates that the effective cross-sectional area of the polar headgroups of these lipid species must be strongly influenced by factors such as charge, hydration, orientation, and motional freedom, as well as by intrinsic headgroup size (see also ref *5*).

It has been shown that alterations in membrane lipid fatty acid composition, cholesterol content, and growth temperature induce marked changes in the quantitative distribution of the major glycolipids of the *A. laidlawii* A membrane (*10*−*14*). In particular, the MGDG to DGDG ratio was shown to increase with an increase in the degree of unsaturation of the membrane lipid hydrocarbon chains, with an increase in cholesterol incorporation, and with an increase in growth temperature. Since aqueous dispersions of MGDG prefer to form inverted nonlamellar phases at higher temperatures, particularly if its fatty acyl chains are unsaturated or cholesterol is present, while the DGDG exists exclusively in the lamellar state under these conditions (*13*−*17*), these characteristic shifts in the MGDG/DGDG ratio were interpreted as a compensatory mechanism to maintain an optimal balance of lamellar and reverse hexagonal phase-forming lipids. Specifically, these workers postulated that this organism maintains its membrane lipid in a lamellar liquid−crystalline state at any particular growth temperature, but in close proximity to a lamellar/reversed cubic or hexagonal phase transition, by appropriate biochemical alterations in the polar headgroup composition of its membrane lipids. Moreover, evidence for at least a partially efficacious regulation of the intrinsic monolayer curvature of the membrane lipids of *A. laidlawii* A with alterations in the 16:0/18:1_c[2] ratio have been reported (*18*). Although this hypothesis seems to be relatively well-supported in *A. laidlawii* A (refs *16*−*23*, but see discussion in refs *6*, *7*, and *24*), the results of similar studies in the closely related *A. laidlawii* B are not fully compatible with this hypothesis (*8*, *9*, *24*, *25*).

All of the previous studies of the regulation of membrane lipid polar headgroup composition in *A. laidlawii* A, and several such studies in *A. laidlawii* B, have utilized cells grown in the absence of avidin and which therefore contain a mixture of endogenously and exogenously derived fatty acids in their membrane lipids (see ref *26*). This results in the production of membrane lipids that are heterogeneous in both hydrocarbon chain length and chemical structure. As well, the fatty acid compositions of the various membrane lipid classes present are different under these circumstances since fatty acids of different chemical structure and chain length are preferentially utilized in their biosynthesis (see ref *26*). Moreover, in most cases, mixtures of exogenous saturated and unsaturated fatty acids of different chain lengths, particularly 16:0 and 18:1_c, have been utilized in such studies. However, since both fatty acid structure and chain length are potent independent determinants of lipid thermotropic phase behavior generally and of lipid lamellar/nonlamellar phase preference in particular (see refs *5* and *27*−*29*), it is not possible to effectively disentangle these two variables in most previous work. As well, in all previous studies of *A. laidlawii* A, and in several such studies in *A. laidlawii* B, the MGDG/DGDG ratio alone was used to estimate the lamellar/nonlamellar phase-forming propensity of the total membrane lipids. However, our previous work has shown that this ratio is not a reliable indicator of this parameter since *A. laidlawii* membranes with similar MGDG/DGDG ratios can have markedly different absolute levels of MGDG and vice versa (*8*, *9*). Moreover, the MGDG/DGDG ratio does not account for the fact that *A. laidlawii* membranes also contain variable quantities of the normal micellar phase-preferring lipid GPDGDG. Therefore, to overcome these problems, in the present study we have utilized *A. laidlawii* B cells grown in the presence of avidin that have been supplemented with only a single member of a homologous series of exogenous fatty acids of different hydrocarbon chain structure. These cells are thus not only devoid of heterogeneity in fatty acid structure and chain length, but the fatty acid compositions of all of the membrane lipid classes are identical (*29*). Moreover, the effects of variations in membrane lipid hydrocarbon chain length and structure can be independently determined in these fatty

[1] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DGDG, diglucosyl diacyglycerol; MGDG, monoglucosyl diacylglycerol; GPDGDG, glycerylphosphoryldiglucosyl diacylglycerol; APG, acyl polyprenyl glucoside.

[2] Fatty acids are designated by the number of carbon atoms followed by the number of double bonds, if any, present in the hydrocarbon chains; the subscripts c and t denote the *cis* and *trans* configurations, respectively, of any double bond present, while the subscripts i and ai represent methyl iso- and anteisobranched, e represents ethyl iso-branched, and ch represents omega-cyclohexyl fatty acids, respectively.

acid−homogeneous cells. Finally, in accessing whether this organism coherently regulates the lamellar/nonlamellar phase preference of its total membrane lipids, we consider the amounts and phase preferences of all of the major lipid components present.

MATERIALS AND METHODS

A. laidlawii B cells were grown at 37 °C in a lipid-poor growth medium and harvested at mid-log phase as described previously (*30*, *31*). Avidin, an inhibitor of de novo fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism (*30*), was added to the growth medium, as was a single exogenous fatty acid. All of the branched chain and ω-cyclohexyl fatty acids utilized in this study were chemically synthesized from appropriate precursors utilizing the procedures described in detail previously (*33*−*35*), while the palmitelaidic acid was purchased from Nu-Chek Prep (Elysian, MN). The total lipids were extracted from isolated membranes by a modified Bligh and Dyer (*32*) procedure and purified by silicic acid column chromatography, and the individual membrane polar lipids were separated by preparative thin-layer chromatography on silica gel G and quantitated by gas−liquid chromatography of their fatty acid methyl esters, all as previously described (*31*). The total neutral lipids, consisting primarily of free fatty acids, diacylglycerols, and carotenoid pigments, accounted for less than 3 wt % of the total membrane lipids and were not included in our analyses. However, as far as we could determine, the amount and composition of the neutral lipids did not vary significantly with variations in the fatty acid composition of the membrane polar lipids. The variations in fatty acid composition induced in this study also result in only small variations in the lipid/protein ratio of the *A. laidlawii* B membrane and no detectable variations in the overall membrane protein composition (*24*).

RESULTS

Influence of Variations in Fatty Acid Chain Length on Membrane Lipid Polar Headgroup Composition. To investigate the effects of variations in fatty acid chain length on membrane lipid polar headgroup distribution, *A. laidlawii* B cells were grown in the presence of avidin and supplemented with a single member of a homologous series of methyl isobranched, methyl anteisobranched, or ω-cyclohexyl fatty acids. These three fatty acid classes were chosen for study here because a wide range of hydrocarbon chain lengths within each class support good growth of this organism (*24*, *30*). The membrane lipid polar headgroup compositions of fatty-acid homogeneous *A. laidlawii* B cells grown with single methyl iso- or anteisobranched or ω-cyclohexyl fatty acids are presented in Table 1, and trends in the individual membrane lipids classes with variations in fatty acid hydrocarbon chain length are illustrated in Figure 1. Let us consider the results for each fatty acid class separately since the effects of variation in hydrocarbon chain length on membrane lipid polar headgroup composition vary considerably with fatty acid type (see below).

As hydrocarbon chain length increases in the methyl isobranched fatty acid homologous series, MGDG and PG levels remain relatively constant, DGDG levels increase markedly, GPDGDG levels decrease substantially, and APG is found in significant quantities only with the longest chain length fatty acid studied. As hydrocarbon chain length progressively increases in the homologous series of methyl anteisobranched fatty acids, MGDG and DGDG levels decrease, PG and GPDGDG levels increase, and modest quantities of APG are again found only with the longest chain length studied. Finally, as hydrocarbon chain length progressively increases in the ω-cyclohexyl fatty acid homologous series, MGDG levels decrease markedly, DGDG and PG levels increase substantially and plateau, GPDGDG remain low and relatively constant, and substantial amounts of APG are again present only with the longest chain length fatty acid tested.

Table 1: Membrane Lipid Polar Headgroup Compositions of Fatty Acid−Homogeneous *A. Laidlawii* B Cells Grown with Single Exogenous Methyl Isobranched, Methyl Anteisobranched, or Omega-Cyclohexyl Fatty Acids of Various Chain Lengths[a]

fatty acid	Membrane Lipid Polar Headgroup Composition (mol %)				
	MGDG	DGDG	PG	GPDGDG	APG
$14{:}0_{i}$	46.8 ± 4.7	2.8 ± 1.3	21.4 ± 3.8	29.1 ± 0.4	
$15{:}0_{i}$	47.1 ± 2.9	8.5 ± 3.1	17.2 ± 3.1	27.2 ± 2.6	
$16{:}0_{i}$	50.8 ± 2.0	9.0 ± 2.4	25.3 ± 0.1	14.9 ± 0.3	
$17{:}0_{i}$	42.1 ± 9.6	11.9 ± 1.7	14.7 ± 0.8	2.7 ± 0.3	28.6 ± 10.0
$14{:}0_{ai}$	53.2 ± 3.4	24.1 ± 2.1	17.6 ± 0.9	5.1 ± 0.6	
$15{:}0_{ai}$	50.4 ± 4.7	25.1 ± 1.4	19.4 ± 1.1	4.9 ± 1.1	
$16{:}0_{ai}$	48.0 ± 2.9	26.3 ± 3.1	19.9 ± 0.9	5.7 ± 0.9	
$17{:}0_{ai}$	45.1 ± 4.1	24.7 ± 2.7	20.3 ± 1.6	10.0 ± 0.7	
$18{:}0_{ai}$	40.3 ± 7.2	18.9 ± 1.9	21.8 ± 0.7	9.7 ± 0.6	9.3 ± 6.8
$16{:}0_{ch}$	60.3 ± 5.9	19.4 ± 1.8	15.4 ± 2.4	4.8 ± 1.6	
$17{:}0_{ch}$	49.9 ± 4.7	24.7 ± 2.3	20.9 ± 4.5	4.5 ± 2.0	
$18{:}0_{ch}$	40.1 ± 4.0	32.1 ± 4.4	22.9 ± 3.5	5.1 ± 1.8	
$19{:}0_{ch}$	32.9 ± 3.2	34.1 ± 1.2	27.6 ± 3.2	5.3 ± 0.7	
$20{:}0_{ch}$	25.4 ± 4.6	33.1 ± 8.5	20.8 ± 7.2	2.0 ± 0.3	18.8 ± 5.6

[a] Values presented are the mean, and average deviation from the mean, of at least three independent determinations of the polar headgroup composition for each fatty acid composition studied.

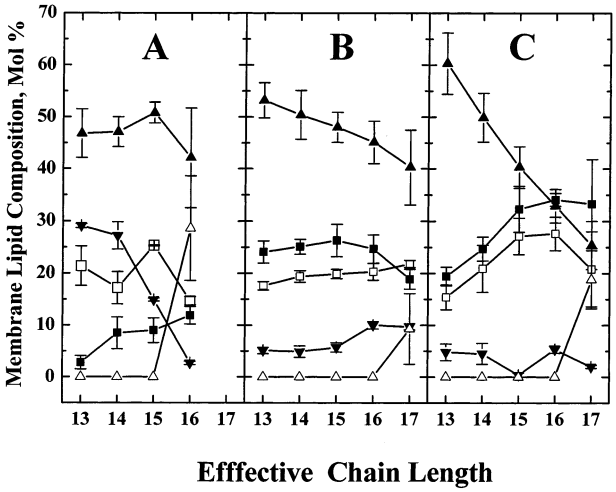

FIGURE 1: Plots of the lipid polar headgroup composition vs the effective hydrocarbon chain length in *A. laidlawii* cells made homogeneous in one of a homologous series of methyl isobranched (panel A), methyl anteisobranched (panel B), or ω-cyclohexyl (panel C) fatty acids. The inverted phase-preferring lipids MGDG and APG are denoted −▲− and −△−, respectively; the lamellar phase-preferring lipids DGDG and PG are denoted −■− and −□−, respectively; and the micellar phase-preferring lipid GPDGDG is denoted −▼−, with the symbols representing the effective shapes of these lipid molecules.

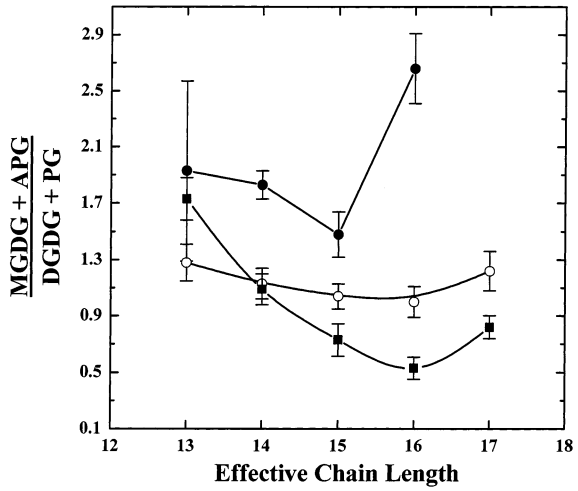

FIGURE 2: Plot illustrating the relationship between the molar ratios of the inverted phase-preferring lipids (MGDG plus APG, when present) to the lamellar phase-preferring lipids (DGDG plus PG) and the equivalent hydrocarbon chain length in fatty acid−homogeneous *A. laidlawii* membranes enriched in various methyl isobranched (●), methyl anteisobranched (○), or ω-cyclohexyl (■) fatty acids.

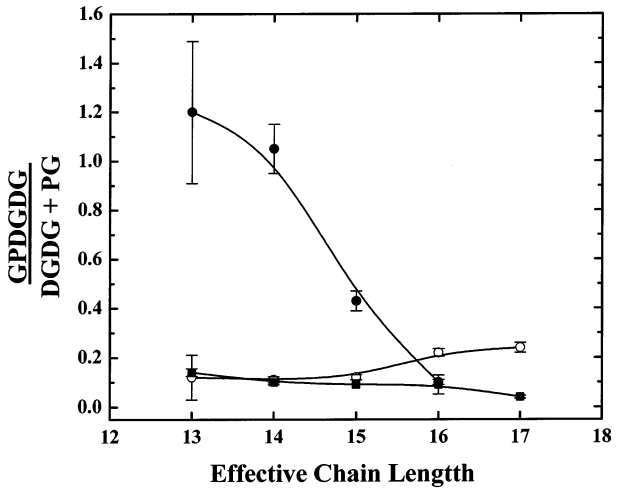

FIGURE 3: Plot illustrating the relationship between the molar ratios of the micellar phase-preferring lipid (GPDGDG) to the lamellar phase-preferring lipids (DGDG plus PG) and the equivalent hydrocarbon chain length in fatty acid−homogeneous *A. laidlawii* membranes enriched in various methyl isobranched (●), methyl anteisobranched (○), or ω-cyclohexyl (■) fatty acids.

As discussed in the introductory paragraphs, increases in phospholipid or glycolipid hydrocarbon chain length should favor the formation of lamellar over normal micellar phases and reversed cubic or hexagonal over lamellar phases at a constant growth temperature. Therefore, if *A. laidlawii* B does indeed possess a biochemical regulatory mechanism to maintain the lamellar/nonlamellar phase propensity of its membrane lipids constant at its optimal growth temperature of 37 °C, one predicts that the proportion of the reversed phase-preferring lipids MGDG and APG relative to those of the bilayer phase-preferring lipids DGDG and PG should decrease and that the proportion of the normal micellar phase-preferring lipid GPDGDG should increase, as the chain length of the biosynthetically incorporated exogenous fatty acid increases. However, in the present experiments, these two trends are not consistently observed. In fact, in the methyl isobranched fatty acid series, MGDG levels remain relatively constant, and APG levels abruptly increase with increases in hydrocarbon chain length, and GPDGDG levels markedly decrease. Thus, as illustrated in Figures 2 and 3, respectively, the (MGDG + APG)/(DGDG + PG) ratio actually increases after an initial small decrease, while the GPDGDG/(DGDG + PG) ratio markedly decreases with increases in membrane lipid hydrocarbon chain length, almost the exact opposite result to that predicted. In contrast, in the methyl anteisobranched fatty acid series, MGDG levels do decline, and GPDGDG levels do increase, as predicted by the lamellar/nonlamellar phase regulation hypothesis, although the abrupt appearance of the strongly inverted nonlamellar phase-preferring lipid APG and the abrupt decline in the bilayer phase-preferring lipid DGDG levels at the longest chain length studied would not be predicted. Thus, as illustrated in Figures 2 and 3, respectively, the (MGDG + APG)/(DGDG + PG) ratio changes little overall rather than declining significantly, although the GPDGDG/(DGDG + PG) ratio does increase significantly, as predicted. Finally, in the ω-cyclohexyl fatty acid series, MGDG levels do markedly decline with increases in hydrocarbon chain length, as predicted, but APG levels unexpectedly increase at the longest chain length and GPDGDG levels, while relatively low, seem not to change significantly, whereas a decrease in their levels with increasing hydrocarbon chain length would be predicted. Thus, as illustrated in Figures 2 and 3, respectively, the (MGDG + APG)/(DGDG + PG) ratio does indeed decrease markedly with increases in hydrocarbon chain length, as expected, although it increases at the longest chain length tested, but the GPDGDG/(DGDG + PG) ratio actually declines, which is the opposite change to that predicted. Thus, the shifts in the proportions of the reversed cubic or hexagonal phase and normal micelle phase-preferring to the bilayer phase-preferring membrane lipids required to maintain the lamellar/nonlamellar phase propensity of the lipid bilayer constant are not consistently observed in any of these experiments, suggesting that this organism may not be capable of efficaciously regulating this property of its membrane lipids when the hydrocarbon chain length of its fatty acids is varied.

The lipid composition data presented thus far demonstrates the changes in the (MGDG + APG)/(DGDG + PG) and the GPDGDG/(DGDG + PG) ratios with hydrocarbon chain length are not always correlated. Thus, to estimate the relative overall normal micellar/lamellar/inverted phase preference of the distribution of lipid classes present in the various fatty acid−homogeneous *A. laidlawii* B membranes, we adopt a phase preference index for the total lipid mixture. Since there exists at present no absolute scale for ranking the phase preference of various individual membrane lipids (see ref *5*), we arbitrarily assign a value of +1.0 to the micelle phase-preferring lipid GPDGDG, values of 0.0 to the bilayer phase-preferring lipids DGDG and PG, and values of −1.0 for the inverted phase-preferring lipids MGDG and APG. The values of +1.0, 0.0, and −1.0 were arbitrarily chosen to correspond qualitatively to the positive, zero, and negative intrinsic radii of monolayer curvature characteristic of normal micellar, lamellar, and inverted phase-forming membrane lipids (see refs *4* and *5*). We are aware of the fact that this scale is only semiquantitative because our previous studies have indicated that DGDG may have a stronger preference for forming the lamellar phase than does PG and that APG does have a

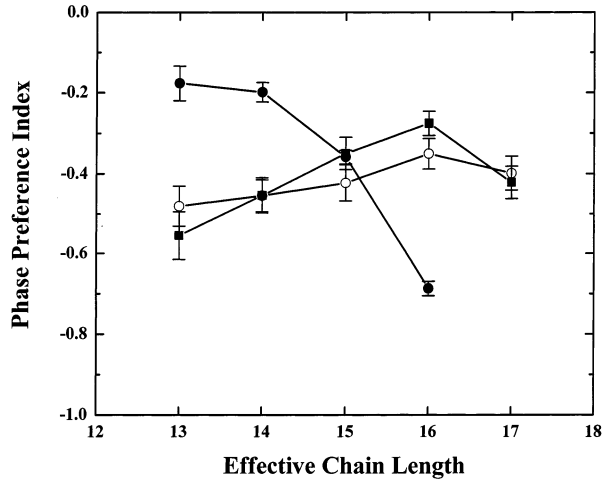

FIGURE 4: Plot illustrating the relationship between the phase preference index of the total lipid mixture and the equivalent hydrocarbon chain length in fatty acid−homogeneous *A. laidlawii* membranes enriched in various methyl isobranched (●), methyl anteisobranched (○), or ω-cyclohexyl (■) fatty acids.

stronger preference for forming the inverted cubic or hexagonal phase than does MGDG (*8*, *36*, *37*). Nevertheless, the present index should accurately reflect the relative overall phase preferences of the total membrane lipid mixture and allow any major trends in the regulation of this parameter with fatty acid chain length to be detected.

The relationships between the phase preference index of the total membrane lipid mixture and the effective hydrocarbon chain length of the homologous series of methyl isobranched, methyl anteisobranched and ω-cyclohexyl fatty acids present in the *A. laidlawii* membrane lipids is illustrated in Figure 4. Since an increase in hydrocarbon chain length favors the formation of lamellar over micellar phases and the formation of inverted nonlamellar phases over lamellar phases (see refs *1*−*5*), one would expect that *A. laidlawii* B should respond to an increase in the hydrocarbon chain length with an increase in the phase preference index if the phase propensity of the total membrane lipids is to remain constant (i.e., the phase preference index should become more positive). Indeed, in the methyl anteisobranched and ω-cyclohexyl fatty acid homologous series, a tendency for a modest increase in this index is observed for equivalent hydrocarbon chain lengths of 13−16, although in both cases the phase preference index declines at the longest chain length tested because of the appearance of APG. However, in the methyl isobranched homologous series, the phase preference index actually decreases with increasing fatty acid hydrocarbon chain length, a response opposite to that predicted. Overall, these results confirm that *A. laidlawii* B is not able to consistently regulate the balance of normal micellar-, lamellar-, and inverted phase-preferring lipid components in response to alterations in the hydrocarbon chain length of its membrane lipids.

Influence of Variations in Fatty Acid Structure on Membrane Lipid Polar Headgroup Composition. To investigate the effects of variation in fatty acid chemical structure on membrane lipid polar headgroup distribution, *A. laidlawii* B cells were again grown in the presence of avidin and supplemented with one member of six different fatty acid classes. The six different branched chain, *trans*-unsaturated, or ω-cyclohexyl exogenous fatty acids selected for study all support good growth of this organism (*24*, *30*), and all six exogenous fatty acids have the same equivalent main chain length of 16 carbon atoms, so that variations in fatty acid structure could be studied independently of variations in fatty acid chain length.

Table 2: Membrane Lipid Polar Headgroup Compositions of Fatty Acid−Homogeneous *A. laidlawii* B Cells Grown with Single Exogenous Fatty Acids of Variable Structure but Equivalent Effective Hydrocarbon Chain Length[a]

fatty acid	Membrane Lipid Polar Headgroup Composition (mol %)				
	MGDG	DGDG	PG	GPDGDG	APG
$17:0_i$	42.1 ± 9.6	11.9 ± 1.7	14.7 ± 0.8	2.7 ± 0.3	28.6 ± 10.0
$19:0_{ch}$	32.9 ± 3.2	34.1 ± 1.2	27.6 ± 3.2	5.3 ± 0.7	
$17:0_{ai}$	45.1 ± 4.1	24.7 ± 2.7	20.3 ± 1.6	10.0 ± 0.7	
$18:0_{dmi}$	50.8 ± 5.8	26.1 ± 4.6	18.6 ± 1.4	4.7 ± 0.1	
$16:1_t$	58.4 ± 1.9	9.4 ± 1.8	16.1 ± 1.6	15.9 ± 2.0	
$18:0_{eai}$	28.4 ± 2.5	26.6 ± 1.1	31.1 ± 1.2	13.8 ± 0.4	

[a] Values presented are the mean, and average deviation from the mean, of at least three independent determinations of the polar headgroup composition for each fatty acid composition studied.

The membrane lipid polar headgroup compositions of fatty acid homogeneous *A. laidlawii* B cells enriched in one of these six exogenous branched chain, *trans*-unsaturated, or ω-cyclohexyl fatty acids are presented in Table 2. These exogenous fatty acids are arranged in Table 2 in order of their decreasing lamellar/inverted hexagonal phase phase transition temperatures when present in synthetic MGDGs or PEs (*28*, *29*). Specifically, we demonstrated previously that the liquid−crystalline lamellar/inverted hexagonal phase transition temperatures of the MGDG present in *A. laidlawii* B membranes decrease in the following order: $17:0_i$ (67.9 °C) > 19.0_{ch} (60.8 °C) > $17:0_{ai}$ (56.6 °C) > $18:0_{dmi}$ (52.0 °C) > $16:1_t$ (38.0 °C) > $18:0_{eai}$ (33.1 °C), and a similar order is observed in synthetic PEs (*28*, *29*).

As discussed in the introductory paragraphs, if *A. laidlawii* B does indeed possess an efficient biochemical regulatory mechanism to maintain the normal micellar/lamellar/inverted phase preference of its membrane lipids constant in the face of variations in the structure of biosynthetically incorporated exogenous fatty acids, then we can make two predictions. First, as the lamellar/inverted hexagonal phase transition temperature of the MGDG and APG component of the total membrane lipid mixture increases, the ratio of inverted phase-preferring to lamellar phase-preferring lipids (the MGDG + APG/DGDG + PG ratio) should also increase to compensate for the weaker tendency of MGDG and APG to form an inverted cubic or hexagonal phase at the growth temperature of 37 °C. Second, as the lamellar/inverted hexagonal phase transition temperature of the MGDG and PG components increases, a decrease in the micellar phase-preferring to lamellar phase-preferring lipid components (the GPDGDG/DGDG + PG ratio) should also occur for the same reason. However, as indicated in Figure 5, although one might argue that an overall tendency for the (MGDG + APG)/(DGDG + PG) ratio to increase with an increasing lamellar/inverted hexagonal phase transition may exist, there is in fact no consistent relationship between these two variables. Specifically, although the (MGDG + APG)/(DGDG + PG) ratio is predicted to increase in the order $18:0_{eai}$ < $16:1_t$ < $18:0_{dmi}$ < $17:0_{ai}$ < $19:0_{ch}$ < $17:0_i$, the observed order of increase is $18:0_{eai}$ < $19:0_{ch}$ < $18:0_{dmi}$ < $17:0_{ai}$ < $16:1_t$ < $17:0_i$.

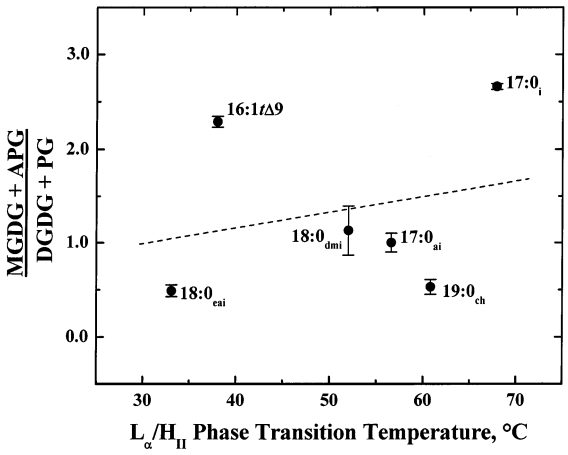

FIGURE 5: Plot illustrating the relationship between the molar ratios of the inverted phase-preferring lipids (MGDG + APG, when present) to the lamellar phase-preferring lipids (DGDG + PG) and the L_α/H_{11} phase transition temperature of the MGDG component of *A. laidlawii* B membranes made homogeneous in one of a series of exogenous fatty acids having the same effective hydrocarbon chain length but different structures. The dashed line represents the best linear fit to the experimental data. The MGDG L_α/H_{11} phase transition temperatures were taken from ref *29*.

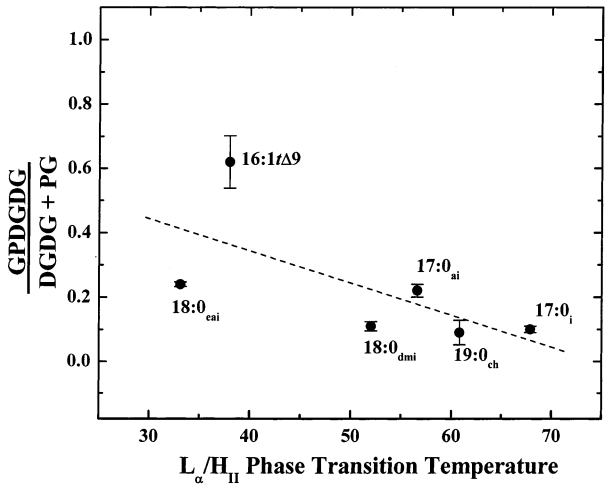

FIGURE 6: Plot illustrating the relationship between the molar ratio of the micellar phase-preferring lipid (GPDGDG) to the lamellar phase-preferring lipids (DGDG + PG) and the L_α/H_{11} phase transition temperature of the MGDG component of *A. laidlawii* B membranes made homogeneous in one of a series of exogenous fatty acids having the same effective hydrocarbon chain length but different structures. The dashed line represents the best linear fit to the experimental data. The MGDG L_α/H_{11} phase transition temperatures were taken from ref *29*.

Similarly, as illustrated in Figure 6, although an overall trend for the GPDGDG/(DGDG + PG) ratio to decrease with the increasing lamellar/inverted hexagonal phase transition temperature of the MGDG component appears to exist, the order of decreasing GPDGDG/(DGDG + PG) ratio observed is $16{:}1_t > 17{:}0_{ai} \cong 18{:}0_{eai} > 18{:}0_{dmi} \cong 17{:}0_i \cong 19{:}0_{ch}$, whereas the predicted order of decrease would be $18{:}0_{eai} > 16{:}1_t > 18{:}0_{dmi} > 17{:}0_{ai} > 19{:}0_{ch} > 17{:}0_{ai}$. These results indicate that this organism does not possess the ability to regulate either the ratio of inverted phase-preferring or micellar phase-preferring to lamellar phase-preferring lipids in a coherent manner.

Finally, to assess the overall ability of *A. laidlawii* B to regulate the normal micellar/lamellar/inverted phase-forming propensity of its total membrane lipid mixture, a plot of the phase preference index, as defined earlier, versus the lamellar/inverted hexagonal phase transition temperature of the MGDG component of the membrane lipids, was constructed, and this plot is illustrated in Figure 7. If an efficient biochemical mechanism to regulate the overall phase preference of the membrane lipid exists in this organism, the prediction would be that the phase preference index should decrease (become more negative) as the lamellar/inverted hexagonal phase transition temperature increases. Again, although it could be argued that such an overall trend is evident in Figure 7, in fact there is no consistent relationship between these two parameters. Specifically, although the phase preference index is predicted to decrease in the order $18{:}0_{eai} > 16{:}1_t > 18{:}0_{dmi} > 17{:}0_{ai} > 19{:}0_{ch} > 17{:}0_i$, the observed order of decrease is $18{:}0_{eai} > 19{:}0_{ch} > 17{:}0_{ai} > 16{:}1_t \cong 18{:}0_{dmi} > 17{:}0_i$. Thus, if *A. laidlawii* B does possess a biochemical regulatory mechanism to regulate the phase overall preference of its membrane lipids with variations in fatty acid structure, such a mechanism appears to be rather imprecise in its operation.

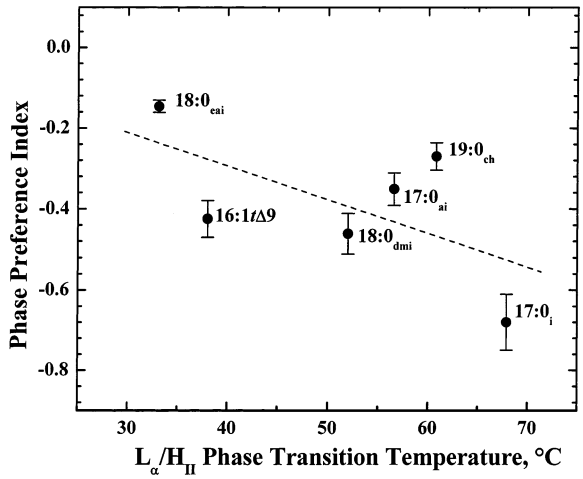

FIGURE 7: Plot illustrating the relationship between the phase preference index of the total membrane lipid mixture and the L_α/H_{11} phase transition temperature of the MGDG component of *A. laidlawii* B membranes made homogeneous in one of a series of exogenous fatty acids having the same effective hydrocarbon chain length but different structures. The dashed line represents the best linear fit to the experimental data. The MGDG L_α/H_{11} phase transition temperatures were taken from ref *29*.

DISCUSSION

As mentioned in the introductory paragraphs, the MGDG/DGDG ratio has been used in all previous studies of *A. laidlawii* A, and in many previous studies of *A. laidlawii* B, as an indicator of the overall lamellar/inverted nonlamellar phase preference of the total membrane lipids of this organism. However, the lipid polar headgroup compositional data presented in Tables 1 and 2 demonstrate that this ratio is not a reliable indicator of the relative amounts of inverted phase-preferring and lamellar phase-preferring lipid components present in the *A. laidlawii* membrane. For example, when the hydrocarbon chain length of the methyl isobranched fatty acids studied is increased in fatty acid−homogeneous *A. laidlawii* B cells (see Table 1), the MGDG/DGDG ratio progressively decreases from 16.7 ($14{:}0_i$) to 3.5 ($17{:}0_i$), which might appear to indicate that the ratio of inverted nonlamellar

phase-preferring to lamellar phase-preferring lipids is decreasing. However, a closer inspection of the data in Table 1 actually shows that the absolute amount of the reversed phase-preferring lipid MGDG present in *A. laidlawii* B membrane is increasing from $14{:}0_i$ through $16{:}0_i$ and only begins to decline at the longest chain length examined ($17{:}0_i$). Thus, the decrease in the MGDG/DGDG ratio with increasing chain length is actually due to a progressive increase in the amount of DGDG present and not to a decrease in MGDG levels. Moreover, since the amount of the other lamellar phase-preferring lipid PG decreases with increases in the chain length of the biosynthetically incorporated methyl isobranched fatty acid and because of the appearance of substantial amounts of the more strongly reversed phase-preferring lipid APG at the longest chain length tested, the ratio of lipids with a tendency to form inverted nonlamellar as compared to those with a tendency to form lamellar phases (the MGDG + APG/DGDG + PG ratio) actually increases with increasing hydrocarbon chain length (see Figure 2), in contrast to the behavior implied by the decrease in the MGDG/DGDG ratio. Moreover, although the MGDG/DGDG ratios do more accurately reflect the broad trends in the relative proportions of the reversed phase-preferring and lamellar phase-preferring lipids in the anteisobranched and ω-cyclohexyl fatty acid series, the quantitative correspondence between the individual MGDG/DGDG and (MGDG + APG)/(DGDG + PG) ratio values is not good, particularly for the longest chain members of these series, and the correspondence between the MGDG/DGDG values and the phase preference index is even poorer (data not presented). Similarly, the MGDG/DGDG ratios for the six exogenous fatty acids of different structure but similar chain length (see Table 2) are only roughly correlated with their (MGDG + APG)/(DGDG + PG) ratio values and are poorly correlated with the phase preference index values of these fatty acids. Therefore, as demonstrated previously for *A. laidlawii* B cells grown without fatty acid supplementation or supplemented with a mixture of palmitic and various unsaturated fatty acids (*9*), the MGDG/DGDG ratio is not a reliable indicator of the relative proportions of lamellar and inverted phase-forming lipid components present in the membrane of this organism. Moreover, because the MGDG/DGDG ratio also does not consider the presence of variable quantities of the normal micellar phase-forming lipid GPDGDG, it is an even poorer measure of the overall normal micellar/lamellar/inverted cubic or hexagonal phase propensity of the total membrane lipids of *A. laidlawii*.

The question arises as to whether *A. laidlawii* A and B differ significantly in their abilities to regulate the overall normal micellar/lamellar/reversed cubic or hexagonal phase-forming propensities of their membrane lipids and what physiological consequences derive from such differences, if such exist. Although it would seem that the A strain is more capable than the B strain in this regard, a direct comparison between these two strains is difficult because of the use of the MGDG/DGDG ratio as a proxy for the overall phase preference of the total membrane lipids in previous work on strain A (see ref *2*). Moreover, in the A strain, later work has shown that substantial amounts of strongly inverted phase-preferring glycolipids other than MGDG and APG may be present under certain circumstances (*36*, *37*), which of course are again not reflected in the MGDG/DGDG ratio. (These other glycolipid components are present in the B strain in only trace amounts.) Thus, additional work on *A. laidlawii* A, using an inclusive phase preference index approach, will be required for a direct comparison with the present results on *A. laidlawii* B. However, we note that the B strain seems to grow at least as well as the A strain when various exogenous fatty acids are added to the growth medium or when the growth temperature is varied. Therefore, the apparent lack of a coherent biochemical regulatory mechanism to finely control the balance of normal micellar/lamellar/inverted cubic or hexagonal lipid components in the membrane of this organism does not seem to have any discernible negative effect on membrane function, at least under laboratory conditions. This result in turn brings into question whether such a regulatory mechanism is really required by this organism, particularly in its natural environment in the lower intestinal tract of higher animals.

Prior to discussing the broader significance of the experimental results obtained in this study, it is important to understand the lipid biosynthetic capabilities of *A. laidlawii* B and how these biosynthetic capabilities are regulated or not by variations in growth temperature and by the fatty acid composition and cholesterol content of the growth medium. For a comprehensive review of previous studies in this area, the reader is referred to ref *6*.

Our previous work has clearly shown that this organism, unlike most conventional bacteria, lacks a fatty acid composition-based homeophasic or homeoviscous regulatory mechanism. Specifically, both the average chain length of de novo synthesized saturated fatty acids and the degree of the chain elongation of exogenous short chain or unsaturated fatty acids by *A. laidlawii* B cells grown in the absence of avidin does not depend on growth temperature and is not influenced by the presence or absence of cholesterol (*30*, *38*, *39*). Similarly, the uptake of single exogenous fatty acids or fatty acid combinations that are not substrates for the chain elongation system also does not depend on growth temperature or the degree of cholesterol incorporation, either in normal or avidin-cultured cells (*30*, *38*, *39*). Thus, in the absence of substantial changes in the polar headgroup distribution of the *A. laidlawii* membrane, which only occurs in special circumstances (see below), this organism is unable to alter the gel/liquid−crystalline phase transition temperature and therefore the phase state or fluidity of its membrane lipids, which in turn restricts its ability to exploit its full potential growth temperature range. However, the lack of an effective homeophasic or homeoviscous adaptation mechanism permits the facile experimental manipulation of lipid phase state and fluidity, which in turn makes this an excellent organism in which to study the correlation between lipid composition and physical properties and membrane structure and function (see refs *6*, *7*, and *26*).

In contrast, *A. laidlawii* B, and in particular the closely related *A. laidlawii* A, do have the ability to alter their membrane lipid polar headgroup distributions somewhat in response to changes in temperature and cholesterol incorporation and fairly markedly in response to variations in the chain length and degree of unsaturation of biosynthetically incorporated exogenous fatty acids (see refs *2*, *5*, *6*, and *26*). These lipid polar headgroup compositional alterations, which usually do not significantly alter membrane lipid phase state and fluidity, are of two types. The first, which involves

primarily changes in the MGDG/DGDG ratio and has already been discussed in the introductory paragraphs, has been rationalized as a mechanism for maintaining the lamellar/nonlamellar phase preference of the total membrane lipids relatively constant. The other lipid polar headgroup biosynthetic regulatory mechanism present in *A. laidlawii* A and B normally operates to maintain the negative surface charge density of the membrane lipid bilayer relatively constant in the face of variations in the salt content of the growth medium or in the cross-sectional areas of the lipids in the bilayer that are induced by alterations in fatty acid composition or cholesterol content (*40*, *41*). This surface charge density regulation is obtained by variations in the ratio of uncharged glycolipids to charged phospholipids and phosphoglycolipids in the membrane of this organism. Thus, for example, an increase in the salt concentration in the growth medium, in the degree of unsaturation of the membrane lipids, or in the incorporation of cholesterol will decrease the (MGDG + DGDG)/(PG + GPDGDG) ratio in the lipid bilayer. Again, although we have argued previously that at least in *A. laidlawii* B this regulatory mechanism either does not function under certain conditions or is not fully efficacious (*25*), it does seem to operate in a more consistent manner in *A. laidlawii* A.

A. laidlawii B cells made fatty acid auxotrophic by growth in the presence of the biotin-binding agent avidin grow increasingly poorly at 37 °C when supplemented with single exogenous linear saturated fatty acids of decreasing hydrocarbon chain length (*30*, *42*). Interestingly, this progressive decrease in growth yields with decreasing hydrocarbon chain length is not observed when cells are cultured in the presence of other classes of exogenous fatty acids. Moreover, normal growth is observed with other types of fatty acids with equivalent or shorter hydrocarbon chain lengths, indicating that poor growth in the presence of short chain linear saturated fatty acids cannot be due to a decrease in membrane lipid bilayer thickness per se. To understand the molecular basis of such growth inhibition, the growth yields, membrane lipid fatty acid and polar headgroups compositions, and phase state and fluidity of the membrane lipids were determined in cells progressively biosynthetically enriched in tridecanoic acid (13:0) or dodecanoic acid (12:0) (*43*). The growth of fatty acid auxotrophic *A. laidlawii* B cells grown in the presence of binary combinations of an exogenous fatty acid that supports normal growth on its own and 13:0 or 12:0 revealed that growth inhibition is not observed until 13:0 and 12:0 biosynthetic incorporation levels reach about 90 and 60 mol %, respectively, after which growth is markedly inhibited. Differential scanning calorimetric analyses of membranes from cells maximally enriched in 13:0 indicate that the lipid gel/liquid−crystalline phase transition temperature is unexpectedly high but that at the growth temperature of 37 °C, the membrane lipid bilayer is almost exclusively in the liquid−crystalline state but is certainly not excessively fluid. However, high levels of 13:0 incorporation produce a greatly elevated level of the high melting, reversed nonlamellar phase-preferring lipid component MGDG and greatly reduced levels of all other membrane lipid components. This marked elevation of MGDG levels can be rationalized as a regulatory response that maintains the lamellar/nonlamellar phase-forming propensity of the total membrane lipid mixture relatively constant in the face of the biosynthetic incorporation of increasing quantities of short chain saturated fatty acids, which favor the lamellar phase. However, this lipid biosynthetic response produces a marked decline in the levels of anionic PG and GPDGDG that are probably required to maintain the minimal negative surface charge density of the lipid bilayer, which we suggest is responsible for the observed growth inhibition. This work shows that the lipid biosynthetic regulatory mechanisms present in this organism may sometimes operate at cross purposes such that it is not possible to simultaneously optimize all of the biologically relevant physical properties of the membrane lipid bilayer (*43*).

It is interesting to note the *A. laidlawii* B is capable of coherently regulating membrane lipid phase state and fluidity, lamellar/nonlamellar phase-forming propensity, and negative surface charge density, at least qualitatively, under some other circumstances. For example, if this organism is grown in the presence of large quantities of long chain saturated fatty acids in the absence of avidin, cells can grow at 37 °C even though it would be predicted that the gel/liquid−crystalline phase transition temperature of the membrane lipids would be higher than the growth temperature; thus, cell growth would not be possible. Under these circumstances, *A. laidlawii* B synthesizes substantial quantities of APG, mainly at the expense of MGDG (*44*). Subsequent studies showed that this additional glycolipid has both a considerably lower gel/liquid−crystalline phase transition temperature and a considerably higher propensity to form reversed nonlamellar phases than MGDG, the lipid that it largely replaces (*8*, *45*). This is exactly what is required to compensate for the elevation of the lamellar gel/liquid−crystalline and lamellar liquid−crystalline/inverted nonlamellar phase transition temperatures of the total membrane lipids produced by the incorporation of long chain saturated fatty acids. As well, the modest increase in the total neutral glycolipid components relative to the anionic lipid components observed under these circumstances should also help compensate for the increased surface charge density that would otherwise result under these circumstances. Note, however, that under these conditions, total anionic lipid levels remain at about 20 mol % of the total membrane lipid, so that cell growth is not inhibited by an inadequate negative surface charge density.

In view of the apparent roles of APG in 16:0-enriched *A. laidlawii* B membrane discussed above, it is interesting to consider APG levels in the homologous series of methyl iso- or anteisobranchyed or ω-cyclohexyl fatty acid−homogeneous cells studied here. As illustrated in Table 1 and Figure 1, APG is absent from membranes at the shorter hydrocarbon chain length in each case and only appears in significant quantities in membranes from cells grown with the longest chain length member of each fatty acid class. Moreover, as the lamellar gel/liquid−crystalline phase transition temperatures of the membrane lipids decrease in the order $17{:}0_{i} > 20{:}0_{ch} > 17{:}0_{ai}$, the amount of APG present decreases from 28.6 to 18.8 to 9.3 mol % of the total lipids. Considering that APG may reach levels near 60 mol % in *A. laidlawii* B membranes highly enriched in 16:0, the progressively smaller amounts of APG present in the progressively more fluid membranes studied here can be rationalized by homeoviscous regulatory theory based on the lower lamellar gel/liquid−crystalline phase transition temperature of this component.

However, the appearance of any APG in cells enriched with these latter three exogenous fatty acids is in any case not required since at 37 °C the membrane lipids would remain exclusively in the lamellar liquid−crystalline state even without the appearance of this lipid component. Moreover, it could be argued that in the already quite fluid 17:0_{ai}-enriched membranes, the presence of APG is counterproductive. As well, the appearance of APG at these longer chain lengths also appears to be counterproductive from the standpoint of regulating the normal micellar/lamellar/inverted nonlamellar phase propensity of the total membrane lipids since it drives the phase preference index to lower (more negative) values (see Figure 4), when in fact they should continue toward higher (more positive) values to compensate for the increasing tendency of the total membrane lipids to form inverted nonlamellar phases at longer fatty acid hydrocarbon chain lengths. In fact, if account were taken of the greater intrinsic inverted phase-forming propensity of APG relative to MGDG in calculating the phase preference index, the magnitude of this apparently counter compensatory effect would be greater. These latter results taken together again highlight the fact that the lipid biosynthetic regulatory mechanisms present in this organism are sometimes unable to optimize all of the desirable physical properties of the membrane lipid bilayer at the same time.

In conclusion, the results of the present study strongly suggest that *A. laidlawii* B does not coherently regulate the normal micellar/lamellar/inverted cubic or hexagonal phase-forming propensity of its membrane lipids in response to independent variations in the hydrocarbon chain length and structure of its fatty acyl chains, perhaps at least in part because such regulation may compromise other biologically relevant physical properties of the membrane lipid bilayer, under these circumstances.

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