The Biosynthetic Incorporation of Short-Chain Linear Saturated Fatty Acids by *Acholeplasma laidlawii* B May Suppress Cell Growth by Perturbing Membrane Lipid Polar Headgroup Distribution†

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ABSTRACT: *Acholeplasma 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. 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 is 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, we determined the growth yields, membrane lipid fatty acid and polar headgroups compositions, and phase state and fluidity of the membrane lipids in cells progressively biosynthetically enriched in tridecanoic acid (13:0) or dodecanoic acid (12:0). The growth of fatty acid auxotrophic *A. laidlawii* B cells grown in the presence of binary combinations of an exogenous fatty acid which 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 monoglucosyl diacylglycerol, and greatly reduced levels of all other membrane lipid components. This marked elevation of monoglucosyl diacylglycerol levels can be rationalized as a regulatory response which 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 phospholipid and phosphoglycolipid which 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.

Mycoplasmas such as *Acholeplasma laidlawii* B are particularly valuable for studies of the structure and function of cell membranes. Being nonphotosynthetic prokaryotes as well as lacking a cell wall or "outer membrane", mycoplasma cells possess only a single limiting or plasma membrane. This membrane contains essentially all the cellular lipid and, because these cells are small, a substantial fraction of the total cellular protein as well. Due to the absence of a cell wall, substantial quantities of highly pure membranes can be easily prepared by gentle osmotic lysis followed by differential centrifugation, a practical advantage not offered by other prokaryotic microorganisms. Moreover, relatively large quantities of a number of exogenous saturated, unsaturated, branched-chain, or alicyclic fatty acids can be biosynthetically incorporated into the membrane phospho- and glycolipids of these organisms. In cases where de novo fatty acid biosynthesis is either inhibited or absent, fatty-acid-homogeneous membranes (membranes whose glycerolipids contain only a single species of fatty acyl chain) can be produced. Moreover, by growing mycoplasmas in the presence or absence or various quantities of cholesterol or other sterols, the amount of these compounds present in the membrane can be dramatically altered. The ability to manipulate membrane lipid fatty acid composition and cholesterol content, and thus to alter the phase state and

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fluidity of the membrane lipid bilayer, makes these organisms ideal for studying the roles of lipids in biological membranes (*1*−*4*).

Many years ago, we studied the effect of the incorporation of various exogenous fatty acids on the growth of *A. laidlawii* B rendered totally auxotrophic for fatty acids by growth in the presence of avidin, a biotin-complexing protein, which results in the inhibition of de novo fatty acid biosynthesis and the hydrocarbon chain elongation of exogenous fatty acids (*5*, *6*). We demonstrated that *A. laidlawii* B could grow relatively normally when various single exogenous linear saturated, methyl iso- and anteiso-branched, ω-cyclohexyl or *trans*-monounsaturated or -cyclopropane fatty acids made up 97−99 mol % of the total membrane lipid fatty acyl groups. However, high-melting exogenous fatty acids such as long-chain linear saturates would not support growth at 37 °C, nor would the low-melting *cis*-cyclopropane and *cis*-unsaturated fatty acids tested. We subsequently showed by DTA[1] that the gel to liquid−crystalline membrane lipid phase transition midpoint temperatures of the normally growing, fatty-acid-homogeneous cells could range from −15 to +37 °C (*6*, *7*). Since the relative fluidities of the lipids from these various membranes are appreciably different at 37 °C, these results indicate that a broad range of lipid fluidities are compatible with at least relatively normal membrane function in this organism. We ascribed the inability of the higher-melting exogenous fatty acids to support cell growth at 37 °C to the fact that their quantitative incorporation into the membrane lipids would produce a predominantly gel state bilayer, which we had demonstrated previously inhibits cell growth and various membrane functions (*8*−*10*). Similarly, the inability of *cis*-cyclopropane and *cis*-unsaturated fatty acids to support growth could be explained by postulating that exogenous fatty acids giving rise to membrane lipids having phase transitions centered below about −15 to −20 °C would produce a hyperfluid liquid crystalline state at 37 °C. In this regard, it is significant that in *A. laidlawii* B cells retaining the ability to biosynthesize endogenous saturated fatty acids (*8*, *9*) and in a totally fatty acid-auxotrophic mutant of *Echerichia coli* (*12*), the minimum phase transition midpoint temperatures which can be achieved by decreasing the saturated/unsaturated fatty acid ratio of the membrane lipids without inhibiting cell growth are about −19 and −13 °C, respectively. Although these and other studies strongly suggest that an upper limit for membrane lipid fluidity does exist, quantitative physical measurements of this maximum fluidity limit have only recently been attempted (*13*).

We found in these previous studies that fatty acid chain length per se was also a factor in determining the growth-promoting activity of exogenous fatty acids. For most of the series of exogenous fatty acid classes tested, compounds having effective chain lengths of fewer than 13 carbon atoms or more than 17 or 18 carbon atoms do not support good growth, even if the incorporation of such fatty acids would not produce gel state or hyperfluid lipid bilayers (*5*, *6*). This result indicates that there are both lower and upper limits on lipid bilayer thickness compatible with normal membrane function. However, for the members of the methyl iso-branched, methyl anteisobranched, *trans*-monounsaturated, and ω-cyclohexyl fatty acid series, growth rates and growth yields are independent of chain length within these limits, indicating that cell growth is sensitive to large but not to moderate changes in lipid bilayer thickness. Interestingly, this was not found to be the case for the linear saturated fatty acid series, where growth progressively decreased from good (15:0) to fair (14:0) to poor (13:0) to negligible (12:0). This progressive decline in growth-promoting ability with decreasing hydrocarbon chain length observed only with linear saturated fatty acids could not be explained by the melting properties of these fatty acids, since the biosynthetic incorporation of even the shortest-chain member of this series should produce lipid bilayers with a gel to liquid−crystalline phase transition temperature above 0 °C and with thicknesses comparable to those produced by the incorporation of branched-chain and ω-cyclohexyl fatty acids of comparable effective chain lengths, which nevertheless support good growth. Moreover, our earlier work also showed that these short-chain-saturated fatty acids are not intrinsically cytotoxic and are well metabolized by the fatty acid chain elongation and membrane lipid biosynthetic pathways of this organism (*5*, *6*, *14*, *15*). In the present work, we investigate this problem further by monitoring the membrane lipid fatty acid and polar headgroup composition and thermotropic phase behavior of membranes from *A. laidlawii* cells incorporating high amounts of 13:0 or 12:0 into their membrane lipids. The results of these experiments suggest that the induction of a physiologically inappropriate distribution of membrane lipid classes by the incorporation of large amounts of these short-chain saturated exogenous fatty acids may be responsible for their growth-suppressive properties.

MATERIALS AND METHODS

A. laidlawii was grown at 37 °C in chloroform-extracted, avidin-containing media supplemented with various exogenous fatty acids, and growth yields were measured turbidimetrically, as previously described (*5*), except that double the previous avidin concentrations were utilized in order to completely bind all of the biotin in the growth media, ensuring the almost complete inhibition of de novo fatty acid biosynthesis and the chain elongation of exogenous fatty acids. Procedures for harvesting midlog phase *A. laidlawii* cells, and for isolating membranes and determining the polar headgroup and fatty acid composition of the membranes lipids, were essentially the same as those described previously (*7*, *13*). The lipid thermotropic phase behavior of isolated membranes was studied by DSC using a MicroCal MC-2 (MicroCal LLC, Northampton, MA) high-sensitivity calorimeter operating in the heating mode at a scan rate of 10 °C per hour.

RESULTS

As discussed in the Introduction, fatty acid auxotrophic *A. laidl*awii cells supplemented with exogenous 13:0 and 12:0 grow poorly or not at all at 37 °C. To determine the

[1] Abbreviations: DTA, differential thermal analysis; DSC, differential scanning calorimetry; MGDG, monoglucosyl diacylglycerol; DGDG, diglucosyl diacyglycerol; GPDGDG, glycerylphosphoryl diglucosyl diacylglycerol; PG, phosphatidylglycerol. Fatty acids are designated by the total number of carbon atoms, followed by the number and position of any double bonds present in the molecule, with the subscripts c and t denoting the cis and trans configurations of the double bond, respectively. The subscript *ch* indicates the presence of a terminal cyclohexane ring (an ω-cyclohexyl fatty acid), and the subscripts *i* and a represent methyl iso- and anteisobranched fatty acids, respectively.

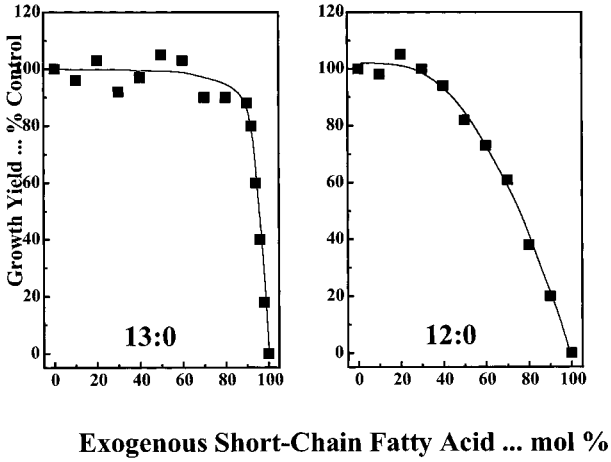

FIGURE 1: Growth yields of fatty acid auxotrophic *Acholeplasma laidlawii* B cultured in media supplemented with binary mixtures composed of C17:0_{ch} and 13:0 (left panel) and of C17:0_{ch} and 12:0 (right panel). Growth yields are plotted as a function of the mol % of the short-chain fatty acid and have been normalized with respect to the mean growth yields obtained in media supplemented with 17:0_{ch} alone. The data points represent typical data sets, and the curves represent mean values obtained from several data sets.

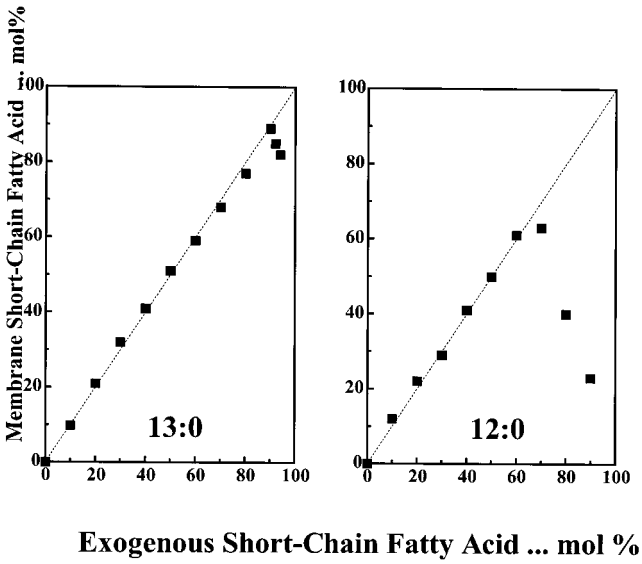

FIGURE 2: Fatty acid compositions of the total membranes of fatty acid auxotrophic *Acholeplasma laidlawii* B cells grown with binary mixtures of 17:0_{ch} and 13:0 (left panel) and 17:0_{ch} and 12:0 (right panel). To simplify data presentation, we plotted the mol % of short-chain fatty acid found in the membrane as a function of the mol % of that fatty acid supplied in the growth medium. The short-chain fatty acid and 11-cyclohexhy undecanoic acids together account for at least 98 mol % membrane fatty acids. The data presented were obtained from the same cultures presented in Figure 1.

maximum quantities of these short-chain linear saturated fatty acids which could be incorporated in the membrane lipids of this organism without inhibiting cell growth, we grew cells in a medium containing binary mixtures of 13:0 or 12:0 and one of a series of exogenous fatty acids which does support normal growth on its own. We chose 17:0_{ch} as the growth-supporting fatty acid component of these mixtures for a number of reasons. First, 17:0_{ch} supports good growth of fatty acid auxotyrophic *A. laidlawii* cells on its own or in the presence of substantial amounts of exogenous short-chain saturated fatty acids (*7*). Second, the effective main chain length of 17:0_{ch} (14 carbon atoms) is close enough to that of 13:0 and 12:0 to minimize potential problems of lateral phase separation due to hydrocarbon chain length mismatch (see refs *10* and *17*). Third, the gel/liquid−crystalline phase transition temperatures of synthetic phospholipids (*18*, *19*) or *A. laidlawii* membranes (*6*, *7*) containing 17:0_{ch} and 13:0 or 12:0 are reasonably similar, thus ensuring good lateral miscibility between the various lipid molecular species present in the *A. laidlawii* membrane (see 16, 17).

The results of an experiment, in which the growth yields of *A. laidlawii* cells grown at 37 °C in the presence of various quantities of exogenous 13:0 or 12:0 and 17:0_{ch} were determined, are presented in Figure 1. In the case of 13:0, cell growth is relatively normal in the presence of low to moderate quantities of the exogenous short-chain saturated fatty acid but decreases rapidly as the mol % of 13:0 reaches about 90 mol %, with growth falling essentially to zero at a mol % of exogenous 13:0 of 100. Qualitatively similar results are observed for binary mixtures of 12:0 and 17:0_{ch}, except that cell growth is progressively inhibited at much lower short-chain saturated fatty acid levels (40 mol %). These results indicate that the presence of substantial quantities 13:0 and 12:0 in the growth media inhibit cell growth and that such growth inhibition is greater with 12:0 than 13:0, in agreement with our previous findings (*5*). Qualitatively very similar results are obtained if growth in the presence of various binary mixtures of 13:0 or 12:0 with other exogenous fatty acids such as 18:1_t Δ9 or 16:0_i, which can also support the growth of fatty acid auxotrophic *A. laidlawii* cells on their own, is examined (data not presented).

Our previous work indicated that although in general the fatty acid composition of the *A. laidlawii* membrane lipids reflects the fatty acid composition of the growth medium (*1*−*4*), particularly in cells made fatty auxotrophic by growth in the presence of avidin (*5*−*7*), some fatty acid structural specificity can be manifest in *A. laidlawii* cells grown on binary mixtures of fatty acids (*7*, *13*). To determine if this is the case here, we determined the fatty acid composition of the total membrane lipids as a function of the amounts of 13:0 or 12:0 present in the 17:0_{ch}-containing growth medium and present the results in Figure 2. In both cases, the amount of 13:0 and 12:0 biosynthetically incorporated into membrane lipids is a linear function of the amount present in the growth medium at low to moderate exogenous short-chain saturated fatty acid levels, but decreases at higher levels. Moreover, this decrease in the biosynthetic incorporation takes place at much lower exogenous fatty acid levels in 12:0 as compared to in 13:0, just as observed for the relationship between exogenous fatty acid levels in the growth medium and cell growth illustrated in Figure 1. These results therefore indicate that cell growth is inhibited more by the biosynthetic incorporation of 12:0 as compared to by 13:0 and that the biosynthetic incorporation of both of these short-chain saturated fatty acids decreases when cell growth begins to be inhibited.

The lipid thermotropic phase behavior of *A. laidlawii* membranes, obtained from cells grown in the presence of mixtures of 13:0 and 17:0_{ch} (mole ratio 9:1), was studied by DSC in order to determine whether the presence of gel state or hyper fluid liquid−crystalline lipid domains is responsible for the growth inhibition which commences at this high level of 13:0 biosynthetic incorporation. As illustrated in Figure 3 (top trace), initial DSC heating thermograms of isolated

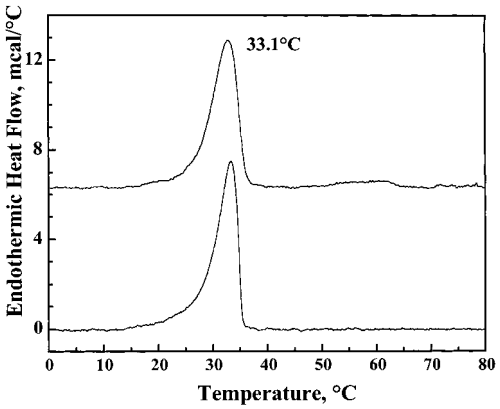

FIGURE 3: DSC thermograms of membranes isolated from fatty acid auxotrophic *Acholeplasma laidlawii* B cultures grown in media supplemented with 13:0 (90 mol %) and 17:0_{ch} (10 mol %). Heating thermograms obtained before (top) and after (bottom) denaturation of the membrane proteins are shown.

membranes exhibit a highly enthalpic, fairly cooperative endotherm centered near 33 °C and a series overlapping lower enthalpy, less cooperative endotherms occurring between about 50 and 85 °C. On subsequent cooling and reheating (Figure 3, bottom trace), the lower-temperature endotherm centered near 33 °C remains essentially unchanged, while the overlapping higher temperature endotherms disappear. These calorimetric results, along with our FTIR spectroscopic studies of these membranes (data not presented), indicate that the lower-temperature endotherm arises from the reversible gel/liquid−crystalline phase transition of the membrane lipids and the higher-temperature endotherms for an irreversible thermal denaturation of the membrane proteins (see also 20). Since the lipid gel/liquid−crystalline phase transition of these membranes takes place at a relatively high temperature but the membrane lipids exist almost exclusively in the fluid state at the growth temperature of 37 °C, it is clear that neither the existence of gel state or hyperfluid liquid−crystalline state lipid can be limiting membrane function and cell growth in this instance.

The relatively high gel/liquid−crystalline phase transition temperature of these isolated membranes was quite unexpected, given that the short-chain saturated fatty acid 13:0 made up about 90 mol % of the membrane lipid hydrocarbon chains in these cells (see Figure 2). In fact, from the previously established relationship between the phase transition temperatures of a series of synthetic phosphatidylcholines and fatty acid-homogeneous *A. laidlawii* membrane lipids containing the same fatty acids (*6*, *7*), one can estimate that the phase transition temperature of *A. laidlawii* membrane lipids this highly enriched in 13:0 should be about 18 °C, assuming a "typical" membrane lipid polar headgroup distribution. The fact that the observed lipid phase transition temperature of 33 °C significantly exceeds the predicted value in turn suggests that the normal distribution of lipid classes may be altered in membranes highly biosynthetically enriched in 13:0, with one or more lipid components characterized by high chain-melting phase transition temperatures being relatively enriched in this instance. In fact, the membrane lipid compositional analyses presented below indicate that this is indeed the case.

An analysis of the polar headgroup distributions of the total membrane lipids from fatty acid auxotrophic *A. laidlawii* cells grown in the presence of 17:0_{ch} alone, or in the presence of a binary mixture of 13:0 plus 17:0_{ch} (9:1 mol ratio) or 16:0 plus 18:1_t Δ9 (1:1 mol ratio), is presented in Table 1. In cells grown in 17:0_{ch} alone or in an equimolar mixture of 16:0 plus 18:1_tΔ9, the total neutral glycolipid content (MGDG plus DGDG) accounts for between 60 and 70 mol % of the total membrane lipid, although the MGDG/DGDG mol ratio varies form about 3:1 to 1:1. Similarly, the total anionic lipid content (PG plus GPDGDG) varies between about 30 and 40 mol %, although the PG/GPDGDG mol ratio varies fairly widely. Indeed, these membrane lipid polar headgroup distributions are quite representative of fatty acid auxotropic *A. laidlawii* cells grown with a wide variety of exogenous fatty acids or fatty acid combinations (*6*, *7*). In contrast, the membrane lipids from *A. laidlawii* cells grown in the presence of 13:0 and 17:0_{ch} (9:1 mol ratio) are very highly enriched in MGDG, very markedly depleted in DGDG and GPDGDG, and considerably reduced in PG. Thus, the net result of the shift in lipid polar headgroup distribution induced by the biosynthetic incorporation of very high amounts of 13:0 is to markedly increase both the quantities of MGDG and the ratio of uncharged glycolipids to anionic phospholipids present in the membrane of this organism. Moreover, since the gel/liquid−crystalline phase transition temperature of synthetic di-13:0-MGDG, which is by far the predominant lipid molecular species present in highly 13:0-enriched *A. laidlawii* membranes, is 32.9 °C (*21*), the unexpectedly high lipid-phase transition temperature of such membranes can now be rationalized. A similar marked increase in MGDG levels is also observed when the biosynthetic incorporation of 12:0 into the membrane lipids reach levels that begin to inhibit cell growth (data not presented).

Table 1: Polar Headgroup Compositions (Mol %) of the Total Membrane Lipids Isolated from Fatty Acid Auxotrophic *A. l*aidlawii B Cells Grown in Medium Supplemented with Various Exogenous Fatty Acids

	exogenous fatty acid(s)		
membrane lipid component	17:0_{ch} (alone)	13:0 + 17:0_{ch} (9:1 mol ratio)	16:0 + 18:1_tΔ9 (1:1 mol ratio)
MGDG	45.6	84.7	33.5
DGDG	15.1	2.2	34.2
PG[a]	20.5	11.8	26.1
GP-DGDG	17.6	1.3	6.2

[a] Trace amounts (~2 mol %) of O-aminoacyl phosphatidyl glycerol are sometimes present.

DISCUSSION

Prior to discussing the 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 *3*.

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 do not depend on growth temperature and are not influenced by the presence or absence of cholesterol. Similarly, the uptake of single exogenous fatty acids or fatty acid combinations which 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. 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 (*8*, *9*). 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 1−4).

In contrast, *A. laidlawii* B, and in particular the closely related *A. laidlawii* A, does 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 *3* and *22*). 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, has been rationalized as a mechanism for maintaining the lamellar/nonlamellar phase preference of the total membrane lipids relatively constant. Thus, for example, when the growth temperature is increased or when exogenous cholesterol or unsaturated fatty are biosynthetically incorporated into the membrane lipids, the amount of the reversed nonlamellar phase-preferring lipid MGDG is decreased relative to the lamellar phase-preferring lipid DGDG, thus preventing the actual formation of nonlamellar lipid domains in the membranes of these organisms which would otherwise occur under these conditions. Although we have argued elsewhere that the MGDG/DGDG ratio is not an adequate measure of the lamellar/nonlamellar phase preference of the total membrane lipids and that changes in the polar headgroup distribution of *A. laidlawii* A and particularly *A. laidlawii* B are not fully efficacious in this regard (*3*, *23*−*25*), the existence of such a lipid phase preference regulatory mechanism in these organisms nevertheless seems well established.

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 (*26*, *27*). 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 (*23*), it does seem to operate in a more consistent manner in *A. laidlawii* A.

We can now discuss the lipid compositional and DSC results obtained in the present study in light of the lipid biosynthetic potential and regulatory mechanisms summarized above. The major finding of this study, the marked elevation of MGDG levels at the expense of other lipid classes in highly 13:0-enriched membranes, could in principle be rationalized in two ways. First, since MGDG is the lipid component of the *A. laidlawii* membrane with the highest intrinsic gel/liquid−crystalline phase transition temperature (*7*) and since the phase transition temperature of membrane lipids decreases with decreasing chain length, one could argue that the observed elevation of MGDG levels represents a homeoviscous adaptation response designed to prevent the formation of hyperfluid lipid domains. Although this is a plausible suggestion, it is one which we do not favor for several reasons. First, without this marked elevation in MGDG levels, the estimated membrane lipid phase transition temperature would still be in the temperature range of 15−20 °C, well above the very low phase transition temperatures (<0 °C) required to form hyperfluid liquid−crystalline lipid bilayers and inhibit cell growth at 37 °C. In fact, the quite high lipid-phase transition temperature of highly 13:0-enriched membranes (33 °C) would represent, if anything, an overcompensation in terms of homeoviscous or homeophasic adaptation, since in this case the upper boundary of the gel/liquid−crystalline phase coexistence region is now near the growth temperature of 37 °C and the presence of a significant amount of gel-state lipid in the *A. laidlawii* B membrane would inhibit cell growth (*8*−*10*). Second, as discussed above, this organism does not possess a homeoviscous or homeophasic response to other environmental or lipid compositional variables.

The second way in which these high MGDG levels could be rationalized is by invoking the lipid lamellar/nonlamellar phase propensity regulatory mechanism which is known to exist in this organism (see refs *3* and *22*). Since the propensity of lipids which can potentially form inverted nonlamellar cubic and/or reversed hexagonal phases is well known to decrease with decreasing chain length, an elevation in the levels of a reversed phase-forming lipid like MGDG as a response to the biosynthetic incorporation of large quantities of short fatty acids would be expected, particularly if these short-acid fatty acids are linear saturated fatty acids, which intrinsically have the lowest tendency to promote reversed nonlamellar phase formation. In fact, Wieslander and co-workers (*27*) and Lindblom and co-workers (*28*) have previously reported a similar increase in the relative proportions of MGDG and other reversed nonlamellar phase-preferring glycolipids in *A. laidlawii* A grown with shorter-chain saturated fatty acids. Moreover, despite the high level of MGDG present, the lipids in the *A. laidlawii* B membrane would appear to remain exclusively in the lamellar phase at the growth temperature, since the lamellar/nonlamellar phase

transition temperature of di-13:0-MGDG is at least 100 °C (*21*). The question then arises, if the lipid bilayer in the membrane of *A. laidlawii* B cells highly enriched in 13:0 exists exclusively in the preferred lamellar liquid−crystalline phase state and has an acceptable degree of fluidity and an acceptable balance of lamellar and nonlamellar phase-preferring lipid classes, why is growth inhibited by the presence of additional 13:0?

We believe that the answer to this question lies in the fact that the extremely high MGDG levels induced by the biosynthetic incorporation of short-chain saturated fatty acids alters the surface charge density by driving the levels of the anionic lipids to unacceptably low levels. As noted previously (*7*, *18*, *26*, *27*) and as confirmed in this study, the total PG plus GPDGDG levels in this organism are normally maintained such that total anionic lipids levels are near one-third of the total membrane lipids although, as discussed above, these levels do vary somewhat with temperature and with the fatty acid composition and cholesterol of the membrane. Interestingly, in the closely related *A. laidlawii* A, the lowest level of anionic lipid which could be achieved by manipulating the salt concentration of the growth medium was about 20 mol % (*16*) and by manipulating the fatty acid composition about 20−25 mol % (*26*, *27*). However, in this study, total PG plus GPDGDG levels decrease to about 13 mol % at the point of maximum tolerable 13:0 incorporation levels. We thus suggest that the resultant decrease in the negative surface charge density below acceptable minimal levels results in the inhibition of membrane function and cell growth observed in this study. This suggestion is compatible with the fact that the lipid lamellar/nonlamellar phase propensity regulatory mechanism appears better developed than the lipid surface charge density regulatory mechanism in this organism. It is possible, however, that these anionic phospholipids also perform other functions in the membrane lipid bilayer of this organism which are adversely affected by their reduction to these low levels. Interestingly, and we believe significantly, total anionic lipid levels do not fall below about 25 mol % when fatty acid auxotropic *A. laidlawii* B cells are grown in the presence of short-chain members of other fatty acid classes whose biosynthetic incorporation into the membrane lipids of this organism in even higher quantities does not inhibit cell growth (*6*, *7*). In fact, the levels of anionic phospholipids are essentially independent of hydrocarbon chain length in *A. laidlawii* B cells supplemented with other classes of fatty acids (*5*−*7*).

A possible alternative interpretation of our experimental results is that the greatly reduced quantity of the neutral glycolipid DGDG, rather than the decreased amounts of the anionic phospholipids PG and GPDGDG, is responsible for the inhibition of the growth of *A. laidlawii* when high quantities of 13:0 are biosynthetically incorporated into the membrane lipids of this organism. Although this possibility is difficult to rule out entirely, we believe that the results of previous studies of the variations of the membrane lipid polar headgroup composition as a function of the structure and chain length of biosynthetically incorporated exogenous fatty acids make this possibility unlikely. For example, when *A. laidlawii* B is grown in the presence of large amounts of long-chain saturated fatty acids in the absence of avidin (see below), DGDG levels can fall to 8.5 mol % of the total membrane lipids (*6*, *7*). Similarly, when *A. laidlawii* A is grown on a binary mixture of short-chain saturated and unsaturated fatty acids, DGDG levels are reduced to 6.9 mol % (*28*). Finally, if *A. laidlawii* B is grown on $14:0_i$ in the presence of avidin, DGDG levels are reduced to only 0.8 mol % (*25*). However, in all of these cases the growth of these organisms is normal, indicating that marked reductions in the "normal" quantities of DGDG per se are not growth-inhibiting. Nevertheless, we do concede that in none of these previous studies were DGDG levels reduced as dramatically as in the present study (to 2.2 mol %), so the possibility remains that low DGDG levels could be responsible for, or at least contribute to, the growth inhibition observed when high levels of short-chain saturated fatty acids are biosynthetically incorporated into the *A. laidlawii* B membrane lipids.

If our suggestion that low levels of anionic phospholipids are responsible for the inhibition of the growth of *A. lai*dlawii B cells cultured in the presence of large amounts of short-chain saturated fatty acids is correct, an important general point that emerges is that organisms such as *A. laidlawii* may not always be able to optimize all the physical properties of their membrane lipid bilayers simultaneously, at least under certain sets of environmental conditions. For example, when the growth temperature is increased, *A. laidlawii* A and B both respond by lowering the MGDG/DGDG and the MGDG + DGDG/PG + GPDGDG levels. These changes in polar headgroup composition have the desirable effects of maintaining both the lamellar/nonlamellar phase propensity and the surface charge density of the membrane lipids relatively constant. However, because the phase transition temperature (*6*, *7*) and intrinsic order and tightness of packing (*29*, *30*) of MGDG are higher than that of DGDG and because the phase transition temperatures of both neutral glycolipids are higher than that of PG (*6*, *7*), the major anionic lipid present, these decreases in MGDG/DGDG and MGDG + DGDG/PG + GPDGDG ratios are actually in the opposite direction to that required for effective homeophasic or homeoviscous adaptation. Of course in other microorganisms such as *Escherichia coli* which can biosynthetically alter the fatty acid composition of their membrane lipids with variations in growth temperature, homeophasic and homeoviscous adaptation can in principle still be achieved even in the face of unfavorable alterations in membrane lipid polar headgroup composition (see ref *10*). However, whether all biologically relevant physical parameters can be optimized simultaneously by any organism under all conditions remains to be determined.

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 and thus cell growth would not be possible. Under these circumstances, *A. laidlawii* B synthesizes substantial quantities of an additional glycolipid (2−0-acyl, polyprenyl-α-D-glucopyranoside), mainly at the expense of MGDG (*31*). 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 which it largely replaces (*32*). 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 which 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, in contrast to the substantially lower levels reached in the present study in which enrichment with short-chain saturated fatty acids was studied.

In conclusion, our major finding here is that different membrane lipid biochemical regulatory mechanisms may operate at cross purposes under certain conditions, and thus that all of the biologically relevant physical properties of the lipid bilayer may not be optimized simultaneously. This finding should be borne in mind when studying changes in membrane lipid composition induced by variations in the environment, since such changes are often interpreted as if the optimization of only a single lipid physical property were important.

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