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Biochimica et Biophysica Acta 1191 (1994) 331–342

BBA Biochimica et Biophysica Acta

Similar regulatory mechanisms despite differences in membrane lipid composition in *Acholeplasma laidlawii* strains A-EF22 and B-PG9. A multivariate data analysis

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(Received 19 August 1993; revised manuscript received 7 December 1993)

Abstract

Mycoplasmas are small, cell wall-deficient bacteria. The metabolic regulation of the lipid composition in the membrane of the species *Acholeplasma laidlawii*, strains A-EF22 and B-JU, is governed mainly by the balance between the potential formation of lamellar and nonlamellar phase structures. However, the regulatory features have not been consistently observed in the B-PG9 strain. A comparison has been performed between the membrane lipid composition for strains A-EF22 and B-PG9, simultaneously changing eight experimental conditions known to affect the regulation and packing properties of the A-EF22 lipids. Multiple regression and partial least-square discriminant analyses of many variables showed: (i) quantitative differences in membrane lipid and protein composition, and in membrane protein molecular masses of the two strains; (ii) different molar fractions of the major polar lipids monoglucosyldiacylglycerol (nonlamellar) and diglucosyldiacylglycerol (lamellar), which were caused by differences in lipid acyl chain length and unsaturation inherent in the strains and by the type of growth medium used; and (iii) similar regulatory mechanisms for changes in the lipid composition under most conditions, responding to the experimentally varied bilayer and nonbilayer properties of the lipid matrix. These regulatory principles are probably valid in other bacteria as well.

Key words: Membrane; Lipid; Regulation; Mycoplasma; Multivariate analysis; (*A. laidlawii*)

1. Introduction

Mycoplasmas are simple, cell wall-deficient bacteria with minimal genome and cell sizes, usually found as surface parasites on various eukaryotic cells [1]. Membranes of these cells are more sensitive to osmotic changes and lytic agents due to the absence of the physical support normally given by the cage-like peptidoglycan mesh in other bacteria. The species *Acholeplasma laidlawii* has contributed important knowledge about the basic molecular organization of biological membranes in general (e.g., [2–4]), and it probably has the most well-characterized membrane of all organisms with respect to its physico-chemical properties [5]. The membrane lipid polar headgroup composition in *A. laidlawii* strains A-EF22 and B-JU is regulated as a response to changes in several factors such as the growth temperature, the structure of endogenous or exogenous fatty acids incorporated as lipid acyl chains, and the presence of foreign additives like hydrocarbons, alcohols, detergents and steroids in the membrane (reviewed in [6]). One aim of this regulation seems to be to keep certain packing properties, visualized as the balance between bilayer and nonbilayer structures in the lipids, at a fixed level [6,7]. In addition, a constant lipid surface potential is maintained by the regulation of the anionic lipid fraction [8]. These features may be beneficial for adaptation upon host changes since *A. laidlawii* cannot synthesize the essential unsaturated fatty acids but has to incorporate these from the surroundings. However, these regulatory prin-

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SSDI 0005-2736(93)E0399-U

ciples have not been consistently observed in strain B-PG9 of *A. laidlawii* [9]. Furthermore, in contrast to strains A-EF22 and B-JU, strain B-PG9 is not able to grow with membrane lipids having only *cis*-mono- or diunsaturated acyl chains [10,11]. Possible reasons for these features can be: (1) The three strains, although supposedly of the same species, are truly different since *A. laidlawii* is known to be a cluster of various strains with different extents of relatedness [12]; (2) some crucial growth conditions differ between laboratories; and (3) all main factors involved in the lipid regulation mechanisms are not known yet. Hitherto only a few models, explaining the biochemical regulation of polar lipid composition in biological membranes on a physical basis, have been proposed [7,13,14].

In order to elucidate the three proposals above, we have investigated similarities and differences in growth responses and membrane composition between the A-EF22 and B-PG9 strains of *A. laidlawii* under similar conditions. For this purpose a multivariate analysis with a fractional factorial design (FFD) was used, which enabled many experimental conditions, and combinations thereof, to be conveniently examined. The differences between the strains were also investigated with a partial least-squares (PLS) discriminant analysis [15,16]. Multivariate techniques have previously been successfully used for, e.g., the grouping of microorganisms [17], the classification of *Escherichia coli* signal peptides [18], and the optimization of DNA promoter sequences from *E. coli* [19].

2. Materials and methods

Organisms. Two strains of *Acholeplasma laidlawii* were used; strain A-EF22, typed at the former WHO Mycoplasma Reference Laboratory, Aarhus, Denmark [20], and strain B-PG9 from Dr. R.N. McElhaney, Edmonton, Alberta, Canada [21]. Both strains have been used for a long time in a number of investigations.

Growth conditions. Two common basal growth media for mycoplasmas have been used routinely for these strains (Table 1); a tryptose (T) medium and a heart infusion broth (HIB) medium, cf. [22]. They were prepared, and the undefined lipid components were extracted with organic solvents, as described [21,23,24]. The undefined media components were from Difco Laboratories and fatty acid poor bovine serum albumin (BSA), fraction V, was from Boehringer Mannheim. The radioactively labelled compounds (Table 1) were purchased from Amersham International. Labelled and nonlabelled fatty acids and cholesterol from sterile ethanol stocks were mixed prior to supplementation. The cells were adapted to all the different growth media and temperatures by five daily consecutive transfers (2% v/v inoculum). Cells were harvested after 24 h of growth [23]. The alkane n-dodecane and/or the $C_{16}EO_8$ detergent (see Tables 2 and 3) was added 6 h before the harvest [25].

Table 1
Composition of the growth media

Basal components
Tryptose medium (T):
Tryptose [a] 20 g/l; fatty acid poor BSA 4 g/l; glucose 7 g/l; Tris 5 g/l; NaCl 5 g/l; penicillin G 60 mg/l
Heart infusion broth medium [b] (HIB):
Heart infusion broth [a] 12 g/l; peptone [a] 5 g/l; yeast extract [a] 5 g/l; fatty acid-poor BSA 4 g/l; glucose 2.5 g/l; Tris 3.7 g/l; penicillin G 60 mg/l
Specific supplements
[^{3}H]16:0 120 μM (30 μCi/l) plus [^{14}C]18:1*c* 30 μM (10 μCi/l)
[^{14}C]18:1*c* 150 μM (10 μCi/l)
[^{3}H]cholesterol 15 μM (30 μCi/l)
$MgCl_2$ 15 μM
n-Dodecane 1.5 mM
Poly(oxyethylene) alkyl ether detergent ($C_{16}EO_8$) 40 μM

[a] Lipid-extracted components, see Materials and methods.
[b] pH adjustments result in the addition of NaCl to the HIB medium [21].

Analyses. Cell growth was monitored by phase contrast light microscopy and by the turbidity at 540 nm, the decrease of the pH value, and the protein content of cells and isolated membranes. Membranes were prepared according to [23] and the polypeptide composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described [26]. Several individual membrane proteins were identified by monospecific antibodies on immunoblots and all proteins were quantified by scanning of stained and dried gels [27].

Membrane lipids were extracted by chloroform/methanol (2:1, v/v) and separated by thin-layer chromatography (TLC) on Merck silica gel H pre-coated plates in the solvent system chloroform/methanol/water (80:25:4, v/v). This system enables the separation of cholesterol from the nonpolar and polar lipids of *A. laidlawii*. The individual lipids were quantified by liquid scintillation double-channel counting [26].

The acyl chain composition of the membrane lipids was determined by gas-liquid chromatography (GLC) as in [28], with a Carlo Erba Instruments Model HRGC 5300-HT apparatus equipped with a capillary fused silica column coated with Supelcowax™ 10.

Experimental design. For the experimental settings of the manipulated variables (shown in Table 2) a fractional factorial design (FFD) was used. This enables the effect of many factors (independent variables) to be investigated simultaneously and with few experiments. In the present design, eight variables were investigated

Table 2
The experimental design variables

Design variables	Low level (−1)	High level (+1)
x_1 Growth temperature (°C)	28	37
x_2 16:0/18:1*c* (μM/μM)	0/150	120/30
x_3 $MgCl_2$ (mM)	0	15
x_4 n-Dodecane (mM)	0	1.5
x_5 $C_{16}EO_8$ detergent (μM)	0	40
x_6 Cholesterol (μM)	0	15
x_7 *A. laidlawii* strain	A-EF22	B-PG9
x_8 Growth medium [a]	HIB	T

[a] See Table 1 for composition.

at two different levels (Table 2); the variables and their levels are given in Table 3.

Multiple regression. In the analysis of the FFD, the relationship between a measured biological response variable y (dependent variable), and the m ($m = 1 \ldots 8$)

Table 3
Coded variable settings for the experiments

Culture number	Design variables (from Table 2)							
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8
1	−1	−1	−1	−1	−1	−1	−1	+1
2	+1	−1	−1	−1	−1	+1	+1	+1
3	−1	+1	−1	−1	−1	+1	+1	−1
4	+1	+1	−1	−1	−1	−1	−1	−1
5	−1	−1	+1	−1	−1	+1	−1	−1
6	+1	−1	+1	−1	−1	−1	+1	−1
7	−1	+1	+1	−1	−1	−1	+1	+1
8	+1	+1	+1	−1	−1	+1	−1	+1
9	−1	−1	−1	+1	−1	−1	+1	−1
10	+1	−1	−1	+1	−1	+1	−1	−1
11	−1	+1	−1	+1	−1	+1	−1	+1
12	+1	+1	−1	+1	−1	−1	+1	+1
13	−1	−1	+1	+1	−1	+1	+1	+1
14	+1	−1	+1	+1	−1	−1	−1	+1
15	−1	+1	+1	+1	−1	−1	−1	−1
16	+1	+1	+1	+1	−1	+1	+1	−1
17	−1	−1	−1	−1	+1	−1	−1	−1
18	+1	−1	−1	−1	+1	+1	+1	−1
19	−1	+1	−1	−1	+1	+1	+1	+1
20	+1	+1	−1	−1	+1	−1	−1	+1
21	−1	−1	+1	−1	+1	+1	−1	+1
22	+1	−1	+1	−1	+1	−1	+1	+1
23	−1	+1	+1	−1	+1	−1	+1	−1
24	+1	+1	+1	−1	+1	+1	−1	−1
25	−1	−1	−1	+1	+1	−1	+1	+1
26	+1	−1	−1	+1	+1	+1	−1	+1
27	−1	+1	−1	+1	+1	+1	−1	−1
28	+1	+1	−1	+1	+1	−1	+1	−1
29	−1	−1	+1	+1	+1	+1	+1	−1
30	+1	−1	+1	+1	+1	−1	−1	−1
31	−1	+1	+1	+1	+1	−1	−1	+1
32	+1	+1	+1	+1	+1	+1	+1	+1

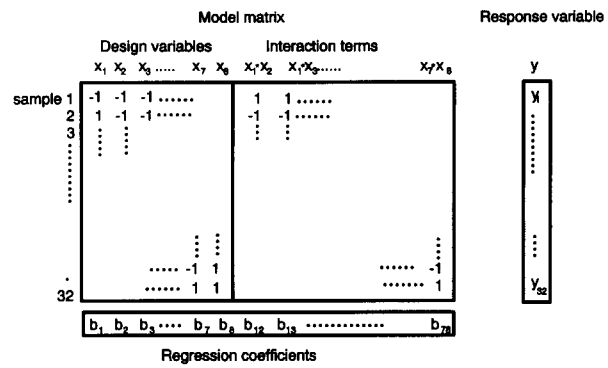

Fig. 1. The regression coefficients b_m and b_{mn} for the eight design variables x_m in their coded form (from Table 3) and their cross-terms ($x_m x_n$ $m \neq n$) are determined by linear multiple regression for a lipid variable y (mol%), see Eqs. (1) and (2). The regression coefficients for these models are given in Table 4.

independent design variables x_m (experimental settings) in their coded form (see below and Fig. 1) and their interaction terms $x_m x_n$ ($n = 1 \ldots 8$; $m \neq n$), is approximated with the model:

$$y = b_0 + \Sigma b_m x_m + \Sigma b_{mn} x_m x_n + e \quad (1)$$

The regression coefficients are determined by linear multiple regression, where the independent variables x are expressed in their coded form, i.e.,

$$x_m = (l_m - l_i)/0.5 \mathrm{d}l_m \quad (2)$$

Here l_m is the original variable setting, l_i the mean value of the high and low variable setting, and $\mathrm{d}l_m$ the difference between the high and low settings.

In Eq. (1), b_0 is the intercept, b_m are the regression coefficients for the linear terms (x_m), and b_{mn} are the coefficients for the two factor interactions or cross terms ($x_m x_n$), ($m \neq n$). In a FFD the regression coefficients usually are not expressing pure effects from an independent variable. However, the confounded pattern among the independent variables and their interaction effects can easily be determined from the used FFD, see Box et al. [29] for details.

In the present model, where linear terms and two factor interactions are estimated, the residuals (e) can be used for statistical tests, e.g., giving the significance of the regression coefficients. For more details of the analysis of the factorial design, see [29]. The data analysis was performed with the statistical package MODDE (Umetri AB, Umeå, Sweden).

PLS discriminant analysis. The differences between the A-EF22 and B-PG9 strains with respect to all measured variables were monitored by a partial least-squares (PLS) discriminant analysis [15,16]. This was preferred to multiple regression-based discriminant analysis since in the present discriminant analysis problems with many correlated variables may occur. PLS is a projection method similar to principal component

Table 4
Regression coefficients from the analysis of the 2^{8-3}_{IV} fractional factorial design

	Column A MGlcDAG	B DGlcDAG	C MGlcDAG/ DGlcDAG	D MAMGlcDAG + MAPGlc	E PG	F GP-der. [b] + O-PG	G Phospho-lipids	H Neutral fraction	Row
Model parameters [a]									
Constant	25.56	22.14	1.74	5.57	25.76	16.15	41.90	4.81	1
Temperature (Tem)	–	2.17	–	−1.34	–	–	–	–	2
16:0/18:1*c* (FA)	–	−3.17	0.17	4.60	−3.09	−1.56	−4.66	3.25	3
$MgCl_2$ (Mg)	–	–	0.26	–	−1.23	1.96	–	–	4
n-Dodecane (Dod)	–	1.84	–	−1.81	–	–	–	−0.82	5
$C_{16}EO_8$ (Det)	2.91	−2.41	0.65	−1.03	1.80	–	–	−0.84	6
Cholesterol (Cho)	−2.22	–	–	–	–	−1.60	−1.76	2.80	7
Strain (Str)	–	−9.11	0.88	2.74	–	4.58	5.74	–	8
Medium (Med)	–	–	–	–	2.00	1.86	3.85	−2.33	9
Tem*FA; Dod*Str; Mg*Cho	2.38	–	–	−1.38	−1.42	–	–	0.88	10
Tem*Mg; FA*Cho	–	–	0.21	–	−1.29	−1.00	−2.30	2.59	11
Tem*Dod; FA*Str	−5.07	–	–	3.23	1.32	–	1.72	0.67	12
Tem*Det;	–	–	0.24	–	–	–	–	–	13
Tem*Cho; FA*Mg	–	–	–	–	1.60	–	–	–	14
Tem*Str; FA*Dod	4.19	−1.66	0.37	−1.89	–	–	–	–	15
Tem*Med	–	–	0.23	–	–	–	–	–	16
FA*Det	–	–	–	−1.05	–	1.19	–	−0.70	17
FA*Med	–	–	–	–	–	–	1.51	−1.41	18
Mg*Dod; Cho*Str	–	–	–	–	−1.23	–	−1.66	0.78	19
Mg*Det	–	–	0.14	–	–	–	–	–	20
Mg*Str; Dod*Cho	–	–	0.26	–	–	–	–	–	21
Mg*Med	–	–	0.28	–	–	–	–	–	22
Det*Cho	–	–	–	–	1.68	–	2.18	−1.41	23
Det*Str	2.49	−1.49	0.59	−1.01	1.82	–	1.74	−1.75	24
Det*Med	–	–	0.18	–	–	–	–	1.06	25
Cho*Med	–	–	–	−0.91	–	–	–	−1.90	26
Str*Med	–	–	–	–	–	–	–	−0.64	27

[a] The regression coefficients in Eq. (1); the larger coefficient, the stronger impact of a specific condition (cf. Table 2). The model intercept b_0 (row 1), the coefficients for the linear terms b_m (rows 2–9) and the interaction terms b_{mn} (rows 10–27). Two interaction terms (Dod*Det and Dod*Med) gave no significant regression coefficients. Only main effects and two-factor interactions are shown.

[b] The GP-derivatives are GPMGlcDAG and GPDGlcDAG.

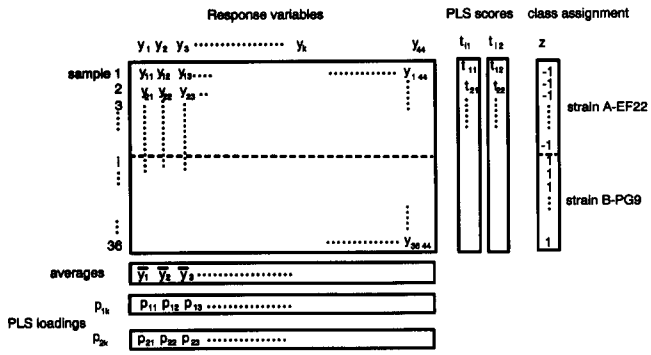

Fig. 2. The variable measurements for the fourty-four responses (y) are projected on a vector z which assign the samples to strain A-EF22 ($z = -1$) and B-PG9 ($z = +1$). The projection is expressed in two latent PLS score vectors. The loading vectors p give the variable influence on the score vectors. The y-variables were mean-centered (y) and scaled to unit variance. The sample-oriented (t_2 vs. t_1) projection is given in Fig. 5a and the corresponding variable-oriented projection in Fig. 5b.

analysis (PCA) which creates sample-oriented (here denoted t_{i2}/t_{i1}; $i = 1$–36) as well as variable-oriented (here denoted p_{2k}/p_{1k}; $k = 1$–44) low-dimensional projections of a multivariate data set, see Fig. 2. The major difference from PCA is that the known class-belongings of the samples are utilized in the projection of the multivariate data into the new low dimensional space. The sample-oriented projection can be inspected for groupings among the samples and the variable-oriented projection gives information of which variables that contribute with class separating information. The number of statistically significant dimensions are determined here using cross-validation [30]. In the present PLS discriminant analysis, a z-vector was formed by giving samples containing strain A-EF22 a z-value $= -1$ and the strain B-PG9 samples a z-value $= +1$. In the PLS analysis the 44 y-variables characterizing the membrane were used as predictor variables (see Fig. 2).

3. Results

Eight design parameters were chosen in the present study (Table 2). The tryptose (T) and heart infusion broth (HIB) media have been used in a number of investigations of the A-EF22, B-JU and B-PG9 strains, respectively (reviewed in [6]). Defined media exist but are rarely used since they contain many components and yield poor growth. Growth temperature, the ratio between saturated and unsaturated acyl chains, and the addition of foreign molecules like n-dodecane, $C_{16}EO_8$ poly(oxyethylene) alkyl ether detergent, and cholesterol, are all known to affect the membrane lipid composition in strain A-EF22 in a predictable manner [7,31,32]. For strain B-PG9 growth temperature, fatty acid supplementation, and cholesterol content have been studied [9,33]. The influence of Mg^{2+} in A-EF22 has also been investigated [8].

For the experimental settings a 2^{8-3}_{IV} FFD was used. No special selection among the possible 2^{8-3} designs has been done, since no a priori information was available for the existence of specific interactions. The FFD is a so called resolution IV design [29], which means that the main effects are confounded with three-factor interactions, but not with two-factor interactions. However, the latter are confounded with other two-factor interactions (Table 4). For each of the 32 experiments in the design totally 44 response variables, monitoring the membrane composition, were measured. Appropriate concentrations for design variables number 3 to 6 (Table 2) were selected in separate multivariate experiments. This was necessary since several of these additives each elicits strong responses in the lipid composition and also may impair the cell growth when combined at high concentrations. Hence, the concentrations of these additives had to be decreased when present in the same culture. The eight chosen variables were investigated at two different levels (-1 and $+1$ in Table 2) in the present FFD, and the combination of these variables are shown in Table 3. Strain B-PG9 grew well in both T and HIB media, whereas the growth of strain A-EF22 was impaired in HIB medium (see below); the latter effect was evident also by titrating HIB medium into T medium (data not shown).

3.1. Polar headgroup and acyl chain composition

The following major membrane lipids have been shown to occur in the two *A. laidlawii* strains: glycerophosphoryldiglucosyldiacylglycerol (GPDGlcDAG), phosphatidylglycerol (PG), diglucosyldiacylglycerol (DGlcDAG), and monoglucosyldiacylglycerol (MGlcDAG) [20,34,35], see Fig. 3. Both strains also synthesize a minor fraction of neutral lipids, which is dominated by diacylglycerols (DAGs) and free fatty acids for strain A-EF22 grown in lipid-extracted T medium (Wieslander, Å. and Rilfors, L., unpublished results). Strain A-EF22 synthesizes one unique membrane lipid, the MGlcDAG derivative 3-*O*-acylmonoglucosyldiacylglycerol (MAMGlcDAG) [20,35], see Fig. 3. Likewise, the strain B-PG9 synthesizes two unique lipids, *O*-aminoacylphosphatidylglycerol (*O*-PG) and 2-*O*-acyl-1-*O*-polyprenyl-α-D-glucopyranoside (MAPGlc) [34,36], the former only in small amounts. *O*-PG usually contains an ester-linked alanine and is thus zwitterionic. Finally, glycerophosphorylmonoglucosyldiacylglycerol (GPMGlcDAG) was observed in both strains.

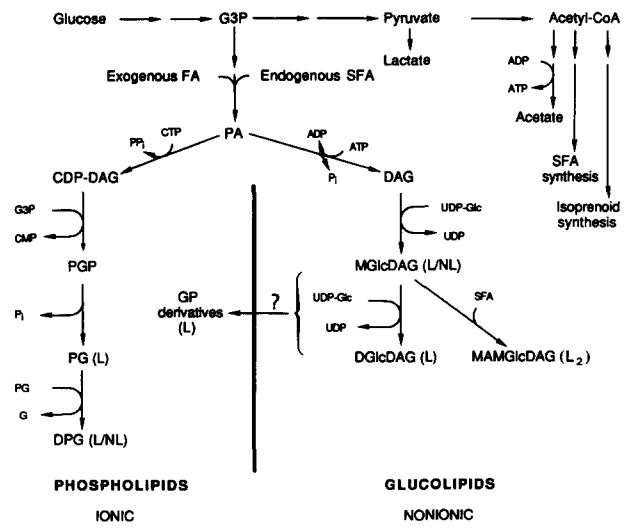

Fig. 3. Tentative pathways for the biosynthesis of membrane lipids in *Acholeplasma laidlawii* A-EF22. Abbreviations used: P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; G, glycerol; GP, glycerophosphoryl; G3P, *sn*-glycero-3-phosphate; PGP, phosphatidylglycerophosphate; FA, fatty acids; SFA, saturated fatty acids; L, lamellar phase; NL, nonlamellar phase; L_2, reversed micellar phase; and ?, suggested pathway. Endogenous SFA and exogenous FA are incorporated from their acyl carrier protein and CoA derivatives, respectively. Modified from [7].

Multiple regression

The relationships between the independent design variables and each of eight selected lipid variables (mol% of major lipids) were modelled with multiple regression according to the theory in Materials and methods and Fig. 1. The statistically significant regression coefficients for the design variables from the analysis of the FFD, together with their confounding pattern, are given in Table 4; the larger regression coefficient, the stronger impact of a specific condition. Empty spaces indicate that the regression coefficients are not significant. The main aim of a FFD is to make a broad screening of the dominating variables with a minimum number of experiments. However, due to the confounding pattern among the variables, this is to some extent done at the expense of interpretability. Thus, we will only discuss the dominating effects in this paper. From the sizes and signs of the regression coefficients a number of conclusions can be drawn.

MGlcDAG and DGlcDAG

It has been shown that MGlcDAG has pronounced abilities to form nonlamellar phase structures such as reversed cubic (I_{II}) and reversed hexagonal (H_{II}) phases, whereas DGlcDAG only forms lamellar phases under similar conditions [24,31,37,38]. The molar fraction of MGlcDAG, and the type of acyl chains in all the membrane lipids, are important for the phase equilibria [6,31]; a larger fraction of MGlcDAG, and unsaturated acyl chains, or higher temperature all shift the equilibria towards nonlamellar structures. The glucolipid composition is regulated in vivo as a response to several factors, such as growth temperature, acyl chain composition, and the presence of different foreign molecules ([6] and references therein), known to affect the phase equilibria of synthetic membrane lipids [14,39,40]. Thereby, similar phase equilibria are maintained for the total lipid mixtures [31]. The regulation is probably achieved at one or a few specific steps in the lipid synthesis, since MGlcDAG is the immediate precursor to DGlcDAG [41,42], see Fig. 3.

As seen in Table 4, the molar fraction of DGlcDAG (column B) was, according to the order of the size of the regression coefficients, above all dependent upon the strain (minus = A-EF22, cf. Table 2), the acyl chain composition (minus = 18:1*c*), the presence of detergent (minus = 0 mM) and n-dodecane (plus = 1.5 mM), and the growth temperature (plus = 37°C). Hence, the fraction of this lipid was higher in strain A-EF22, and was higher in both strains with unsaturated acyl chains, in the absence of detergent, and at high temperatures. In contrast to the coefficients for DGlcDAG, two interaction terms are the most influential ones for the molar fraction of MGlcDAG (column A), but the alternative interpretations cannot be resolved by the present design. However, the regression coefficients at rows 6 and 7 in column A (Table 4) clearly show that the fraction of MGlcDAG is increased by the presence of detergent, and decreased by cholesterol, in both strains. The positive interaction term (row 24 in Table 4) indicates that the response to the detergent is strongest in the B strain. The molar ratio MGlcDAG/DGlcDAG was substantially higher in the B strain (row 8 in column C of Table 4), and the presence of detergent raised this lipid ratio in both strains but more pronounced in the B strain. A weaker, but significant, increase in the glucolipid ratio was also obtained by the presence of Mg^{2+} or saturated fatty acids (SFAs). It can be noted that the variations in the relative amounts of MGlcDAG and DGlcDAG more resemble the variations in the fraction of DGlcDAG than the fraction of MGlcDAG, and that the growth medium (T or HIB) had no significant influence on these three variables (Table 4).

MAMGlcDAG and MAPGlc

These two unique glucolipids both have high melting temperatures and strong abilities to form reversed aggregate structures [38,43]. They also migrate to similar positions in the thin-layer chromatography system used here. Therefore, these two lipids were treated as one response variable in the multivariate analysis. Most probably, MGlcDAG is the immediate precursor to MAMGlcDAG [35], Fig. 3. Table 4 (column D) convincingly shows that the syntheses of MAMGlcDAG or MAPGlc were stimulated when *A. laidlawii* is grown

on SFAs, and that the molar fraction of MAPGlc was larger in strain B-PG9 than the molar fraction of MAMGlcDAG in strain A-EF22 under similar growth conditions (row 8). The negative regression coefficient for n-dodecane (row 5) implies that the presence of this alkane decreased the synthesis of these two glucolipids, which was opposite to its effect on the synthesis of DGlcDAG. Likewise, at an increase of the growth temperature (row 2) the syntheses of MAMGlcDAG or MAPGlc were decreased, whereas the synthesis of DGlcDAG was increased, respectively.

Phospholipids

The two *A. laidlawii* strains synthesize three different anionic lipids, namely PG, GPDGlcDAG, and GPMGlcDAG, plus the minor (and zwitterionic) *O*-PG in strain B. Since the phospholipid PG in most cases is the dominating anionic lipid it was quantified separately, whereas the other three lipids were quantified as a pool. The phospholipids (column G in Table 4) constituted a larger fraction in strain B-PG9 than in strain A-EF22 (cf. row 8); this difference did not hold for PG but was valid for the other phospholipids (columns E and F in Table 4). The synthesis of anionic lipids was stimulated with unsaturated acyl chains in both strains (row 3), and was most pronounced for PG (column E). In constrast to the glucolipids, the molar fraction of the phospholipids was clearly affected by the type of growth medium (row 9), being largest for cells grown in the T medium. It can also be seen in Table 3 that the presence of Mg^{2+} had opposite effects on the molar fraction of PG and the other three phospholipids (row 4). Finally, the presence of the detergent stimulated the synthesis of PG, especially in the B strain (rows 6 and 24).

Neutral lipids

This lipid fraction is usually minor, but occasionally reached 5–10 mol% in the present experiments. According to column H in Table 4, the amount of this fraction was increased by the presence of saturated acyl chains (row 3) and cholesterol (row 7). The synthesis of the neutral lipids was also stimulated in the HIB medium (row 9).

Acyl chain composition

It has been shown that *A. laidlawii* can synthesize SFAs but not unsaturated fatty acids (UFAs) [44]. This synthesis is partially inhibited by exogenous SFAs in strain A-EF22 [23]. Several strains demand the presence of UFAs, branched-chain, or cyclopropane fatty acids in order to grow properly ([10,45] and references therein). The ratio labelled palmitoyl (16:0) to oleoyl (18:1*c*) chains could only be determined for half of the cultures due to the experimental design (Tables 2 and 3). The regression coefficients of the FFD show that the ratio 16:0/18:1*c* is higher in the B strain than in the A strain. GLC analysis (data not shown) of the acyl chain composition in cells from cultures supplemented with 18:1*c* only, revealed a larger fraction of 16:0 in the B strain than in the A strain, and in the HIB medium compared to the T medium. This 16:0 must be derived from the endogenous synthesis, since this fatty acid was not supplemented to these cultures. One method to evaluate this endogenous synthesis is by biosynthetic incorporation of radioactive acetate [46], see Fig. 3. Such experiments showed above all that the incorporation of acetate into the lipid acyl chains is higher for cells grown in the HIB medium (data not shown). Generally, the fraction of acyl chains shorter than 16:0 was larger in cells grown in HIB media and in the B strain. Hence, the degree of acyl chain saturation is higher in the membrane lipids of the B strain, and the average acyl chain length is shorter.

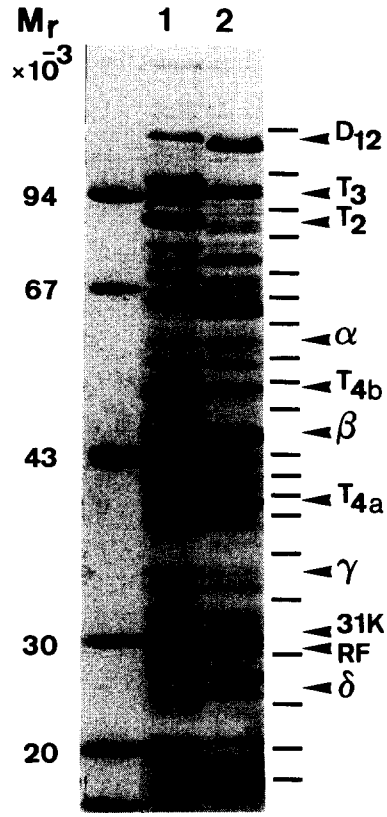

Fig. 4. Membrane proteins from *Acholeplasma laidlawii* strain A-EF22 (lane 1) grown in T-medium (culture 20), and strain B-PG9 (lane 2) grown in HIB-medium (culture 28), and separated by SDS-PAGE. Proteins in the Coomassie-stained and dried gels were quantified by gel scanning. Different zones, defined by horizontal bars, were named after the dominating proteins in strain A-EF22 and labelled by letters. Several of the proteins were identified here by monospecific (polyclonal) antibodies after immunoblotting, see [51].

3.2. Differences between strains A-EF22 and B-PG9

Table 5 summarizes the results obtained with the cultures 33–36. The two *A. laidlawii* strains were grown under identical conditions in HIB and T media with intermediate levels of all supplements. The cellular yields were significantly higher for strain B-PG9 in both media and the amounts of cholesterol, the fraction of GPMGlcDAG, GPDGlcDAG and *O*-PG, as well as the fraction of MGlcDAG, were larger for the B strain, whereas the fraction of DGlcDAG was substantially smaller (Table 5). These data lead to a larger fraction of phospholipids, and a considerably higher fraction of MGlcDAG versus DGlcDAG for the B strain in both media. Furthermore, the fractions of short acyl chains and endogenously synthesized 16:0 were larger in the B strain. The short chains are also most likely of endogenous origin; this is corroborated by the increased incorporation of radioactive acetate into the membrane lipids (above). The A strain grew less efficiently in the HIB medium, and the fraction of anionic lipids was larger in the T medium. The glucolipid composition was also affected by the media, since the fraction of MGlcDAG was higher, and the fraction of DGlcDAG smaller, in the HIB medium for both strains (Table 5). The influences of the growth media were verified for the A strain in separate experiments by mixing the two media in different proportions, and by adding 16:0 and 18:1*c* in different ratios (data not shown). These experiments also revealed that the fraction of 18:1*c* acyl chains incorporated into the membrane lipids increased with the fraction of T medium. Moreover, the average acyl chain length varied. For example, the acyl chains were approximately one carbon atom longer in cells grown in pure T medium with 60 μM 16:0 and 90 μM 18:1*c*, than in the corresponding HIB medium.

In order to evaluate the extent of differences between the *A. laidlawii* strains A-EF22 and B-PG9, a PLS discriminant analysis for the 36 cultures of Tables 3 and 5 was performed on 44 cell variables; cell growth (4 variables); polar headgroup (8 variables) and acyl chain (22 variables) composition; and membrane protein composition (10 variables). In PLS [47] the known class-belongings of the samples are utilized in the projection of the multivariate data into the new low-dimensional space (see Materials and methods and Fig. 2). Ten regions in the SDS gels, each dominated by identified major membrane proteins [27,48–50], were selected for the multivariate data calculations (Fig. 4). Generally, the B strain exhibited larger variations in the protein composition than the A strain; the amounts

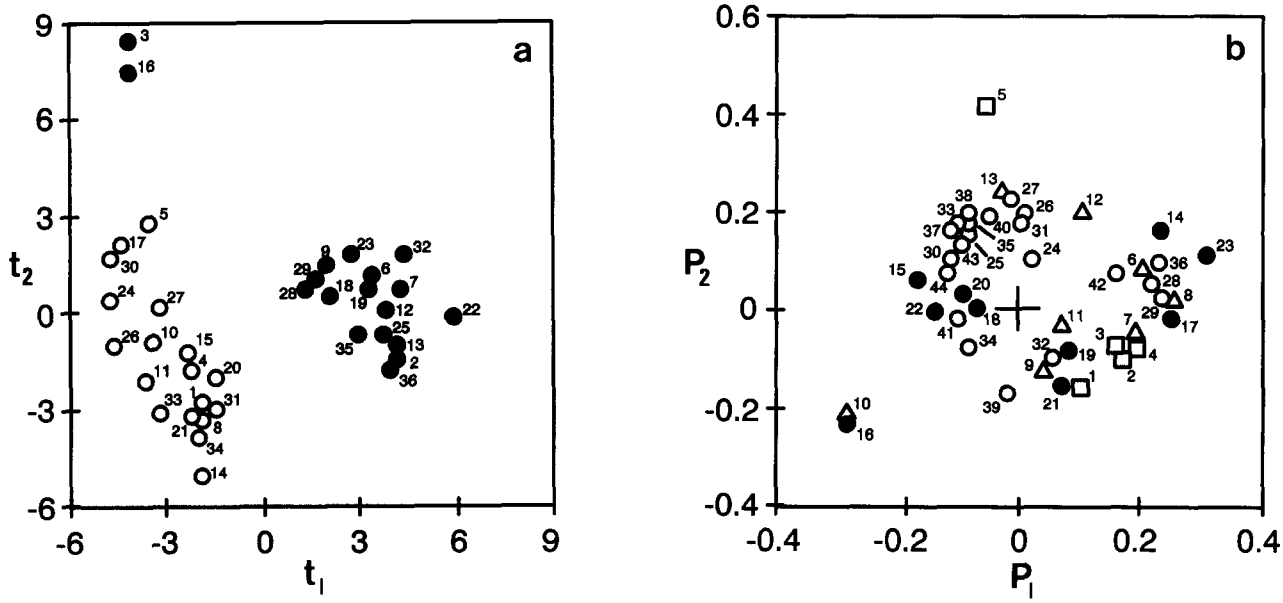

Fig. 5. PLS discriminant analysis of the differences between *A. laidlawii* strains A-EF22 and B-PG9. (a) Sample-oriented score plot (t_2/t_1) for the 36 cultures described by Tables 3 and 5; ○, A-EF22; and ●, B-PG9. (b) Variable-oriented loading plot (p_2/p_1) for the 44 analyzed variables (from each culture) contributing to the separation of the strains. Here the most discriminant variables are those with high absolute values of p_1, because the t_1-vector discriminates the A and B strains (cf. 5a). □, cell growth variables; △, lipid composition; ●, membrane protein composition; and ○, acyl chain composition. Keys: 1, turbidity of culture (540 nm); 2, 3 and 4, amounts of cell protein, membrane protein and polar lipids, respectively; 5, incorporation of [^{3}H]acetate; 6, ratio of MGlcDAG/DGlcDAG; 7, fraction of ionic lipids; 8, amounts of GP-derivatives plus *O*-PG; 9–13, fractions of PG, DGlcDAG, MGlcDAG, MAMGlcDAG and neutral lipids, respectively; 14–23, protein peaks (from gel scanning, cf. Fig. 4) D_{12}, T_3, T_2, ATPase subunit α, T_{4b}, ATPase subunit β, T_{4a}, ATPase subunit γ, 31K plus flavoprotein (RF), ATPase subunit δ, respectively, cf. [51]; 24, ratio of 16:0/18:1*c* (from isotopes); 26, 28, 29, 32, 36, 38, 39 and 40, fractions of acyl chains 12:0, 14:0, 15:0, 16:0, 17:0, 18:0 18:1*c* and 18:2*c*, respectively (from gas-liquid chromatography); other numbers (between 25 and 44), unidentified acyl chains.

Table 5
Membrane lipid composition in strains A-EF22 and B-PG9 from T and HIB growth media

	Culture number			
	33	34	35	36
Strain	A-EF22	A-EF22	B-PG9	B-PG9
Growth medium [a]	HIB	T	HIB	T
Turbidity (540 nm)	0.38	0.55	0.51	0.56
Cell protein (μg/ml)	95	206	333	348
Membrane protein (μg/ml)	41	56	96	226
Polar lipids [b] (nmol/ml)	15.7	22.3	40.8	56.3
Cholesterol (nmol/ml)	0.57	2.00	2.41	2.42
Glucophospholipids [c] (mol%)	4.7	7.1	20.1	22.5
PG (mol%)	24.2	30.6	23.0	21.5
DGlcDAG (mol%)	43.4	42.2	17.0	22.0
MGlcDAG (mol%)	18.1	14.8	38.0	31.9
MAMGlcDAG (mol%)	7.5	2.9	–	–
MAPGlc (mol%)	–	–	0.6	1.2
Neutral lipids [d] (mol%)	2.1	2.4	1.2	0.9
Phospholipids (mol%)	28.9	37.7	43.1	44.0
Ratio MGlcDAG/DGlcDAG	0.42	0.35	2.23	1.45
Short acyl chains [e] (mol%)	9	7	12	14
Endogenous [f] 16:0 (mol%)	5	9	22	21

[a] Media 33–36 were supplemented with intermediate levels of all additives, i.e., 90 μM 16:0 plus 60 μM 18:1*c*, 3.75 mM $MgCl_2$, 0.375 mM n-dodecane, 10 μM $C_{16}EO_8$ detergent, and 3.75 μM cholesterol (see Table 1 for the other basal media components), and the cells were grown at 32.5°C.
[b] The polar lipid fraction consists of all lipids except the neutral fraction and cholesterol.
[c] The glucophospholipids are GPMGlcDAG and GPDGlcDAG. *O*-PG was included in this fraction in the calculations.
[d] The neutral lipid fraction consists mainly of diacylglycerols and free fatty acids.
[e] Acyl chains with fewer than 16 carbon atoms as determined by GLC.
[f] 16:0 chains endogenously synthesized; calculated by a combination of isotope and GLC data.

of two major proteins (T_2 and T_{4a}) varied ten-fold in the B strain. At least four major proteins also have apparent molecular masses which are 2 kDa smaller than for the corresponding proteins in the A strain (proteins D_{12}, T_2, E2p, and the β subunit of the $(Na^+ + Mg^{2+})$-ATPase), see Fig. 4. The major acyl protein T_3 has an apparent molecular mass in the B strain which is only half of that observed in the A strain.

The results of the PLS analysis are presented as sample-oriented and variable-oriented plots. Fig. 5a clearly shows that the two *A. laidlawii* strains separate from each other. According to cross-validation the class separation is highly significant since 56% of the variance in the *z*-vector is explained by the first and most class-separating PLS dimension. A cross-validated explained variance of about 10% corresponds to a significance of $P = 0.05$ [52]. The B strain is an inhomogenous class since the cultures 3 and 16 separate from the other B strain cultures; the cell growth in the cultures 3 and 16 was very poor. If the strongly deviating cultures 3 and 16 in Fig. 5a are not considered, the class-separating information between the strains is along the t_1-vector. The variables that contribute most information to the t_1-vector are those with high absolute p_1 values. Thus, most class-separating information (Fig. 5b) is found in variables 10 (molar fraction of DGlcDAG) and 16 (acyl protein T_2), which both have higher values in strain A-EF22; and in variables 12 (molar fraction of MGlcDAG), 14 (acyl protein D_{12}), 23 ($(Na^+ + Mg^{2+})$-ATPase subunit δ), 42 (molar fraction of unidentified long FA), 6 (molar ratio MGlcDAG/DGlcDAG), 36, 28 and 29 (molar fraction of 17:0, 14:0 and 15:0, respectively), and 8 (molar fraction of GP-derivatives and *O*-PG), which all have high values in strain B-PG9. These results clearly demonstrate distinct differences between the two strains in the chemical composition of the membrane.

4. Discussion

Regulation of lipid composition

The regulation of the membrane lipid composition in the strains A-EF22 and B-JU have been interpreted in relation to a theory describing the self-assembly and phase equilibria of amphiphilic molecules [7,39]. The following conclusion has been drawn: When the internal or environmental conditions are altered in such a way that the phase equilibria of the membrane lipids are shifted towards either nonlamellar or lamellar phases, the cells respond by changing their lipid composition so that the phase equilibria are shifted back towards the initial state [6,7,31,38,39]. Moreover, the fraction of anionic lipids seems to be regulated in these two strains in order to maintain a constant, negative lipid surface potential [8]. These packing properties are acting in the membrane lipid bilayer and are metabolically set to yield a certain packing frustration of the molecules and causing a negative curvature of the lipid mixtures [6]. The above mentioned regulation mechanisms have not been consistently observed in *A. laidlawii* strain B-PG9; this is most obvious for the anionic lipid fraction [9,33].

Strains A-EF22 and B-PG9

The regression coefficients shown in Table 4 are based upon the combined results obtained from the two strains (see theory in Materials and Methods). The regulation of the lipid composition observed here in these strains is in most cases in good agreement with the results obtained previously and the conclusions stated above for strains A-EF22 and B-JU. Supplementations of the growth media with Mg^{2+}, n-dodecane, detergent, and cholesterol, and the difference in growth temperature, were all smaller than in the previous experiments. Hence, some metabolic responses are

likely to be weaker in this multivariate study and may not yield significant regression coefficients.

Despite a generally higher ratio of MGlcDAG to DGlcDAG in strain B-PG9 (Tables 4 and 5), the ratio was increased in both strains with detergent, Mg^{2+}, and SFA due to a decrease in especially DGlcDAG amounts (Table 4). The effects of these three additives have been observed previously in strain A-EF22 [7,8,25], and for SFA also for strain B-PG9 [9]. Presence of the $C_{16}EO_8$ detergent in the membranes, or of more saturated acyl chains in the lipids, both promote lamellar phases. The relative decrease of DGlcDAG versus MGlcDAG is thus expected.

The synthesis and regulation of MAMGlcDAG in strain A-EF22 also seems to be governed by the phase equilibria of the membrane lipids. This unusual glucolipid is only synthesized in substantial amounts in membranes enriched in SFA chains of medium length (Table 4) and especially at the expense of MGlcDAG. The glucolipid has a pronounced ability to form reversed aggregate structures [38], a property probably used by the cells since the phase equilibria of MGlcDAG are shifted towards lamellar phases under these conditions [24,38,53]. The observed reduction of the amounts of MAMGlcDAG in the presence of n-dodecane (Table 4) supports this conclusion since the alkane induces the formation of nonlamellar phases in synthetic as well as biological lipid-water systems [25,54]. Likewise, the synthesis of MAPGlc was strongly stimulated in strain B-PG9 by the presence of SFA in the growth media (Table 4). MAPGlc is more prone to form nonlamellar phases than MGlcDAG [43] which becomes less prone to do this with SFA chains [38]. The present results consequently indicate that the relative amounts of the three glucolipids in each of the strains, although metabolically set at different levels, are regulated in relation to the equilibria between lamellar and nonlamellar phases.

The fraction of phospholipids increased in both strains when the cells were fed with an UFA only (Table 4). Inspection of the data from the individual cultures revealed that this response was stronger for the A-EF22 strain. These observations are in agreement with previous data for the A strain [7,8]. However, no results for the total ionic lipid fraction, but only for PG, have been reported previously from strain B-PG9 [9,33]. For one stimuli here, a significant regression coefficient was obtained only for one of the anionic lipids (column E, row 6 in Table 4) and for another stimuli even opposite effects were obtained for different anionic lipids (cf. columns E and F, row 4 in Table 4).

B-JU and other strains

When strain B-JU is grown in the T medium the fractions of MGlcDAG and DGlcDAG and the fraction of anionic lipids are regulated similarily as in strain A-EF22 [55,56]. The extent of incorporation of cholesterol into the membranes of 20 other, different *A. laidlawii* strains is inversely proportional to the relative amounts of MGlcDAG and MAMGlcDAG [57]. These results seem to be in agreement with a regulatory mechanism based on lipid phase equilibria since cholesterol can induce the formation of nonlamellar phases in various lipid-water mixtures [39], cf. above.

Lipid regulatory mechanisms

Three basal variables, the fractions of MGlcDAG and DGlcDAG and the fraction of anionic lipids, are set at different levels in the two strains. The significant regression coefficients of Table 4 clearly show that for both strains the fraction made of DGlcDAG vary more than the fraction of MGlcDAG. This indicates that the single metabolic step between MGlcDAG and DGlcDAG (Fig. 3) has an important regulatory function. The fraction of MGlcDAG is higher in strain B-PG9 than in A-EF22 (and in B-JU). A probable explanation to this is the fact (Table 5) that B-PG9 has a larger fraction of endogenous short and saturated acyl chains, and a lower fraction of incorporated exogenous 18:1*c* chains, in the lipids. Such differences will all shift the phase equilibria of synthetic as well as native membrane lipids towards lamellar phases. This has been shown for MGlcDAG [24,31,37,38,53,58,59], phosphatidylethanolamines [60,61], and monoacylglycerols [62]. It is therefore reasonable that strain B-PG9 has a larger fraction of MGlcDAG since MGlcDAG has a more pronounced ability than DGlcDAG to form nonlamellar phases due to its smaller polar headgroup structure and lateral surface area per molecule [24,31,37]. Support for this conclusion comes from a series of A-EF22 membrane polar lipid samples, with varying acyl chain length and unsaturation (including 16:0/18:1*c* supplements −1 and +1 in Table 1) but with physiologically compensated MGlcDAG and DGlcDAG fractions, having similar lamellar to nonlamellar phase transition temperatures [31]. Nonlamellar phases can also be observed in B-PG9 polar lipid mixtures at similar conditions [63]. In addition to the strain properties, the growth medium also influences the membrane lipid composition, yielding more saturated and shorter acyl chains in the HIB medium (see Results). Such acyl chains shift the equilibria of membrane lipids towards lamellar phases, and this is a probable reason for the larger fraction of MGlcDAG obtained in the HIB media.

The larger fraction of anionic lipids in cells grown in the T medium is most likely dependent upon the higher concentration of Na^+ in this medium. It has been shown previously that an increased Na^+ concentration will decrease the surface potential of *A. laid-*

lawii A-EF22 lipids due to a screening of the anionic lipid headgroups [8]. In this investigation the B strain exhibited a weaker response in the anionic lipid fraction as a function of the degree of acyl chain unsaturation in the lipids (see Discussion above). Strain B-PG9 synthesizes a larger fraction of anionic lipids in both growth media. Since the increase in the surface potential at physiological ionic strengths levels off when the fraction of an anionic lipid reaches 40–50 mol% [8], an active regulation of the fraction of anionic lipids may not be as motivated in strain B-PG9 as it is in strain A-EF22.

From this investigation we can conclude that: (1) There are differences between *A. laidlawii* strains A-EF22 and B-PG9 in the amounts of the major membrane lipids and proteins, and in the molecular masses of certain membrane proteins. Some of these differences can be enhanced by the type of basal growth medium used. (2) Under most conditions analyzed, the lipid composition in both strains is regulated in accordance with a proposed mechanism for strain A-EF22. (3) The large differences in molar fractions of MGlcDAG (nonlamellar) and DGlcDAG (lamellar) between the strains are most likely explained by the inherent differences in acyl chain length and unsaturation in A-EF22 and B-PG9. The chemical structure of the acyl chains affects the bilayer to nonbilayer balance of the membrane lipid mixtures, which in turn is being compensated for by the active metabolic settings of the molar fractions of MGlcDAG and DGlcDAG at different levels in the two strains. (4) The differences among the membrane proteins in the two strains observed here, may also affect the packing of the lipids and thus the regulation. Similar interpretations as those made for the principles of lipid regulation in *A. laidlawii* A-EF22, can be made from much less extensive studies of several other prokaryotes; *Pseudomonas fluorescens* [64], *Bacillus megaterium* [65], *Clostridium butyricum* [66] and *Clostridium acetobutylicum* [67]. Despite the wealth of information about the *E. coli* lipid enzyme genes and corresponding mutants as compared to other prokaryotes [68,69], the mechanisms and principles for the regulation of the membrane polar lipid composition in *E. coli* have hitherto remained a black box [70]. However, a recent analysis of certain *E. coli* mutants strongly indicates a regulation according to similar principles [71,72] as the ones in *A. laidlawii* discussed here.

Acknowledgements

This work was supported by the Swedish Natural Science Research Council. We thank Siv Andersson and Karin Stjernholm for technical assistance.

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