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Lipid metabolism in cultured lichen photobionts with different phosphorus status

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Lipid metabolism was studied in different photobiont species from lichens by following incorporation of radiolabel from [1-^{14}C]acetate. In four algal photobionts, *Coccomyxa mucigena, C. peltigera variolosae, Trebouxia aggregata, T. erici,* polar lipids were mainly (73–90%) labelled while triacylglycerols were the most highly labelled non-polar lipid class. A rhamnose-containing lipid was found in two *Coccomyxa* species, representing about 11% of the polar lipids of *C. mucigena.* All the major algal glycosyl- and phospho-glycerides were labelled with monogalactosyldiacylglycerol and phosphatidylglycerol, respectively, being the main labelled lipids in the polar lipid classes. The photobionts were grown in media differing in their phosphate content by one hundred-fold. Low phosphate levels caused only a small decrease in the proportion of phosphoglyceride labelling—mainly in phosphatidylglycerol. However, total lipid labelling was reduced (by 83.3 and 76.6% in two *Coccomyxa* spp. and 62.1 and 27% in two *Trebouxia* spp.) for the green algae. By comparison, variations in phosphate availability had no significant effect on a *Nostoc* sp. Examination of the algal species by electron microscopy revealed phosphorus-containing granules. This reserve of phosphorus explains why the algal photobionts were able to maintain the proportion of phosphoglyceride labelling well and may be an important adaptive mechanism for lichens.

Keywords: *Coccomyxa* spp.; *Trebouxia* spp.; *Nostoc* sp.; Lichens; Photobionts; Lipid metabolism; Phosphate availability

1. Introduction

Lichens are symbiotic associations between heterotrophic fungi (mycobionts) and photosynthetic prokaryotic (cyanobacteria) or eukaryotic (algae) organisms (photobionts). They are important constituents of the vegetation of many ecosystems in the world, from the tropics to polar regions (approximately 8% of terrestrial ecosystems are lichen-dominated) and include around 17 000 species (Hale, 1983; Nash, 1996a). About 85% of lichen-forming fungi are symbiotic with green algae, approximately 10% with cyanobacteria (blue-green algae) and 3–4% are cephalodiate species which associate simultaneously with green algae and cyanobacteria (Honegger, 1996).

Lichens are well known as indicators of environmental pollution, including heavy metal deposition. They are also useful as biomonitors of atmospheric deposition for several reasons. Lichens have a wide geographic distribution and, as perennial, slow-growing and long-living organisms, maintain a fairly uniform morphology with time (Ahmadjian, 1993). They are capable of accumulating many elements to concentrations that exceed their physiological requirement due to the absence of a waxy cuticle on the surface of lichen thalli which allows the diffusion of contaminants into lichen tissues. Moreover, the capacity of lichens to lose water as a result of evaporation during dry periods may lead to a concentration of pollutants to toxic levels. In addition, photosynthesis in lichens can take place at relatively low environmental temperatures and, as a result, lichens (and their metabolism) are exposed to pollutants practically all year (Gries, 1996; Nash, 1996b). The high sensitivity of lichens is also related to their symbiotic nature that requires the metabolic balance between symbionts to be maintained. Alteration of this balance, caused by contaminants, may lead readily to a breakdown of the whole association (Gries, 1996).

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Although lichens have a low mineral nutrient demand, nutrient availability is an important factor in determining their occurrence and distribution. Phosphate (P_i) is one of the essential but least available plant nutrients in many natural ecosystems. Low phosphate availability is mainly due to its insoluble precipitation with cations or its conversion into organic complexes (Wykoff et al., 1999; Chen et al., 2000; Raghothama, 2000). Phosphorus is a key component in virtually every aspect of cell life including energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis and respiration (Raghothama, 2000). Most of these are membrane-bound processes and, therefore, are highly dependent on the integrity and function of such membranes which, in turn, are determined by their lipid composition (for review see Murata and Siegenthaler, 1998). Thus, lipid metabolism is well-known to be a key instrument in maintaining the physiological functions of membranes which allows organisms to re-adjust to environment changes and tolerate severe stresses (for reviews see Thompson, 1996; Harwood, 1998; Rama Deli and Prasad, 1999).

Phosphorus availability has been shown to cause significant changes in the lipid biosynthesis of algae and cyanobacteria (Lombardi and Wandersky, 1991; Reitan et al., 1994; El-Sheek and Rady, 1995; Güler et al., 1996; Sato et al., 2000). In general, phosphate-limitation caused a drastic reduction in membrane phospholipids and replacement of these compounds by non-phosphorus glycolipids and/or sulfolipid. In the photosynthetic bacteria, *Rhodobacter sphaeroides*, betaine lipids also increased (Benning et al., 1995). These changes represent an effective phosphate-conserving mechanism (Güler et al., 1996). An enhanced level of unsaturated fatty acids which could cause greater membrane "fluidity" has also been found in phosphorus-starved cells of the green algae *Chlorella kessleri* (El-Sheek and Rady, 1995).

In spite of widespread information about lipids and lipid metabolism in different algae, especially unicellular green species, lichen photobionts (green algae and/or cyanobacteria) have not, to our knowledge, ever been studied. Therefore, in the present work, lipid metabolism in different lichen photobionts (green algae and a cyanobacterium) has been studied as a continuation of our research on lipid metabolism in lower plants. As an example of the adaptation of such organisms to environmental stress, we have examined effects of phosphorus availability which, as described above, is a key nutrient factor for lichens.

2. Results

2.1. Lipid synthesis in algae but not in Nostoc is affected by phosphate availability

We used [1-^{14}C]acetate as a suitable precursor for lipid biosynthesis (see Roughan and Slack, 1982) and incubated all five photobionts in its presence. The two *Coccomyxa* species were both severely affected by low phosphate availability with over 75% reduction in radiolabelling (Table 1). *Trebouxia aggregata* showed a 62% reduction while *Trebouxia erici* had labelling of total lipids only reduced by 27% (Table 2). In all four algal species labelling of polar lipids accounted for 75–80% of the total under standard growth conditions. The relative percentage of polar lipid labelling was increased by low phosphate in three of the four algal species (Tables 1 and 2)—and represented about 90% of the total lipids in *Trebouxia aggregata* (Table 2).

In contrast to the green algae, the photobiont *Nostoc* sp. showed no significant effects of low phosphate either on total labelling or on the relative amounts of radioactivity in polar and non-polar lipids (Table 3).

2.2. Non-polar lipids

The non-polar lipids were separated by TLC and major lipid classes analysed for radioactivity. Triacylglycerols (TAGs) were always the main labelled class, representing 48–78% of the total non-polar lipids in the green algal species (Tables 1 and 2). Diacylglycerols (DAGs) were usually the next most abundant labelled class although *Coccomyxa peltigera variolosae* also had equivalent amounts of radiolabelled sterols and an unidentified lipid which migrated with an R_f value of 0.40 in the solvent systems mentioned above (i.e. between DAGs and free fatty acids: see Section 4.4). Wax and sterol esters were minor but significant components of the labelled non-polar lipids.

Growth in low phosphorus media caused rather small changes in the lipid classes of most algae. In general, the proportion of radioactivity in DAGs was increased and, for three species, that in TAGs decreased. For *Coccomyxa peltigera variolosae*, the percentage labelling of free sterols was reduced but in both *Coccomyxa* species that of the unknown lipid was unchanged by phosphate availability (Table 1).

By contrast, *Trebouxia erici* showed increases in the proportional labelling of sterols, DAGs and the unknown lipid which were compensated by a large decrease in that of TAGs (Table 2).

2.3. Phosphate availability causes surprisingly small changes to polar lipid labelling

Good labelling rates of most common polar lipids from [1-^{14}C]acetate were found for the two *Coccomyxa* spp. (Fig. 1) and the *Trebouxia* spp. (Fig. 2). Phosphatidylglycerol (PG) was the best labelled phosphoglyceride and phosphatidylcholine (PC) the next. Monogalactosyldiacylglycerol (MGDG) was the major glycosylglyceride labelled although this was only 9% of the total in *Trebouxia erici* (Fig. 2). Diacylglycerol tri-

Table 1
Incorporation of radioactivity from [1-^{14}C]acetate into the lipids of *Coccomyxa* spp. grown under different phosphorus regimes

	Coccomyxa mucigena		*Coccomyxa peltigera variolosae*	
Lipid	1.7 mM phosphate	0.017 mM phosphate	1.7 mM phosphate	0.017 mM phosphate
d.p.m.×10^{-4}/g fresh wt				
Total lipids	2187.4±133.8	364.6±25.4**	4093.4±209.2	957.4±62.5**
% of total labelled lipids				
Polar lipids	74.8±3.0	73.4±2.3	72.6±3.0	81.8±1.7**
Non-polar lipids	27.4±1.7	26.6±0.9	27.4±1.7	18.2±1.0*
% of total radiolabelled non-polar lipids				
Free sterols	2.3±0.4	3.8±0.5	17.4±1.3	5.9±0.8**
Diacylglycerols	18.6±2.0	26.6±2.8*	16.7±2.8	19.4±2.0
X-lipid	5.7±0.4	4.0±1.1	15.8±1.4	16.4±3.6
Triacylglycerols	73.2±1.3	65.3±3.0*	48.1±2.5	57.2±3.9**
WE+SE	0.2±0.1	0.3±0.1	2.0±0.5	1.1±0.2

Incorporation into total lipids is expressed as d.p.m.×10^{-4}/g fresh wt.

Radioactivity into non-polar or polar lipids is as% of total radioactive lipids and individual lipid classes as a% of total non-polar lipids. The algae were grown under 1.7 or 0.017 mM phosphate (see Experimental).

Abbreviations: WE+SE = wax esters + steryl esters; X-lipid = unidentified lipid (see text).

Data as means±S.D., where $n=3$ for independent samples.

*$P<0.05$, **$P<0.01$, on statistical analysis by one-way ANOVA using a Tukey test to compare results from the two phosphate media.

Table 2
Incorporation of radioactivity from [1-^{14}C]acetate into the lipids of *Trebouxia* spp. Grown under different phosphorus regimes

	Trebouxia erici		*Trebouxia aggregata*	
Lipid	1.7 mM phosphate	0.017 mM phosphate	1.7 mM phosphate	0.017 mM phosphate
d.p.m.×10^{-4}/g fresh wt				
Total lipids	1306.0±85.0	957.4±62.5***	1143.7±78.6	433.9±22.0***
% of total labelled lipids				
Polar lipids	76.0±2.0	81.5±2.2**	79.8±2.2	90.0±2.4***
Non-polar lipids	24.0±1.1	18.5±1.0**	20.3±1.6	10.0±1.3***
% of total radiolabelled non-polar lipids				
Free sterols	5.8±1.9	19.8±2.3**	4.6±0.4	9.3±1.6*
Diacylglycerols	22.4±2.9	39.1±2.4**	8.8±1.7	14.5±1.6*
X-lipid	3.7±0.5	10.5±1.3***	5.0±0.8	7.5±2.5*
Triacylglycerols	66.0±3.1	25.6±2.8***	77.8±1.6	65.2±2.1***
WE+SE	2.1±0.1	5.0±0.2	3.8±0.3	3.5±0.3

See Table 1 for details.

Statistical analysis was by one-way ANOVA using a Tukey test to compare results from the two media. *$P<0.05$, **$P<0.01$, ***$P<0.005$.

Table 3
Phosphorus availability has no significant effect on lipid labelling from [1-^{14}C]acetate in an endosymbiont *Nostoc* species

Lipid	230 μM phosphate	2.3 μM phosphate
d.p.m. 10^{-4}/g fresh wt		
Total lipids	104.8±23.5	140.8±19.5
% of total labelled lipids		
Polar lipids	89.0±1.8	88.6±2.3
Non-polar lipids	11.0±0.7	

Data as means±S.D., where $n=3$ for independent samples.

methylhomoserine (ether) lipid (DGTS) was significant in three species and contained 13% of total radiolabelling in *Coccomyxa mucigena* while both *Coccomyxa* species contained a rhamnose-glycerolipid (Fig. 1).

The *Nostoc* sp., as is well-known for cyanobacteria (Murata and Nishida, 1987; Wada and Murata, 1998), contained a simple lipid pattern with only five components. Again, MGDG was the major labelled glycosylglyceride while, in this case, PG was the only phosphoglyceride (Fig. 3).

Since low phosphate growth conditions are known to have dramatic effects on the lipids in photosynthetic bacteria (Benning et al., 1995) and higher plants (Essigmann et al., 1998; Chen et al., 2000), we were surprised to find only small effects in the algal photobionts (Figs. 1–3). Thus, in general, the proportional labelling of phosphoglycerides was remarkably preserved. The proportional labelling of PG was usually slightly decreased (except *Trebouxia aggregata*) and this was usually accompanied by an increase in that of MGDG,

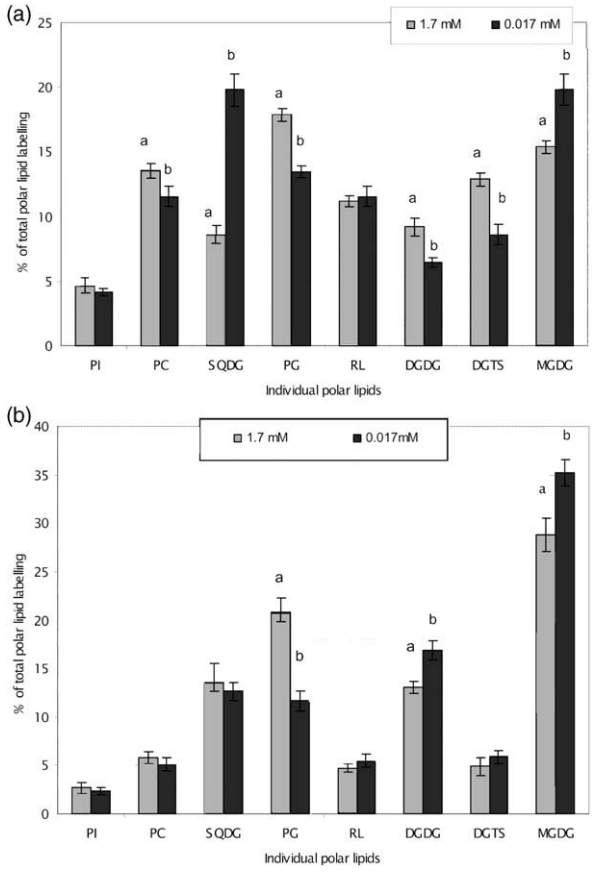

Fig. 1. Relative labelling of individual polar lipids in response to phosphorus availability for the endosymbiont green algal species *Coccomyxa mucigena* (A) and *C. peltigera variolosae* (B). Means±S.D. ($n=3$) shown. Bars with different letters are statistically different at $P<0.01$ on statistical analysis by one-way ANOVA using a Tukey test for comparison of the two phosphate levels (1.7 and 0.017 mM). Abbreviations: DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceroltrimethyl-homoserine; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; RL, rhamnose-lipid; SQDG, sulphoquinovosyldiacylglycerol.

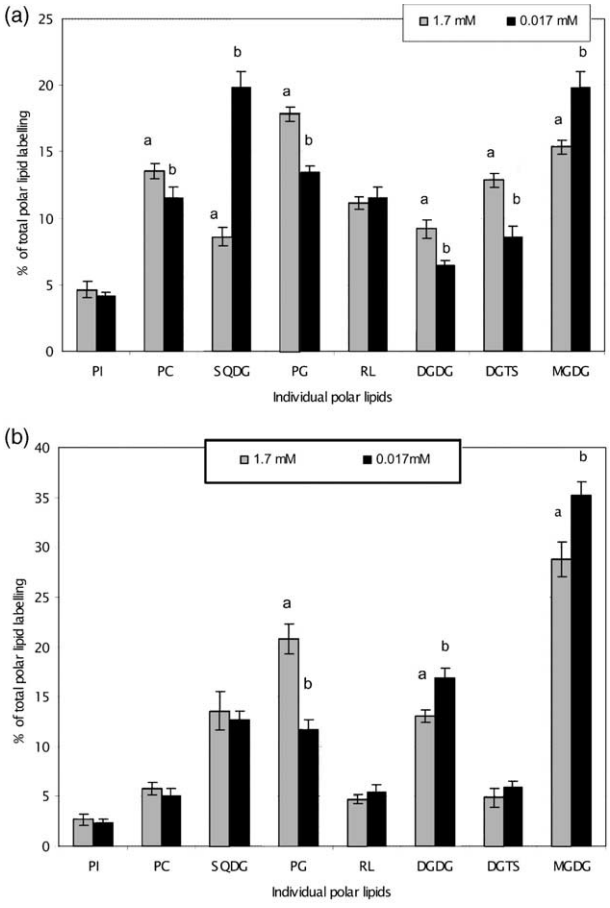

Fig. 2. Relative labelling of individual polar lipids in response to phosphorus availability for the endosymbiont green algal species *Trebouxia aggregata* (A) and *T. erici* (B). Means±S.D. ($n=3$) shown. Bars with different letters are statistically different at $P<0.01$ (a, b) or $P<0.05$ (c, d) on statistical analysis by one-way ANOVA using a Tukey test to compare the results from the two phosphate levels (1.7 and 0.017 mM). Abbreviations as for Fig. 1.

as expected. However, overall, the proportional labelling of phospholipids only changed from 33% to 29% and 30% to 20% in the two *Coccomyxa* spp. (Fig. 1) and from 60% to 58% and 78% to 68% in *Trebouxia aggregata* and *T. erici*, respectively (Fig. 2). Only in *Coccomyxa peltigera variolosae* was PG much affected and, even in this case, the other labelled phosphoglycerides were unaffected by phosphate availability (Fig. 1).

In previous work with other organisms, a decrease in the negatively charged phospholipid PG is often compensated by an increase in the anionic glycosylglyceride, sulpholipid (sulphoquinovosyldiacylglycerol; SQDG) but only in *Coccomyxa mucigena* was such a change seen (Fig. 1). No consistent changes in the labelling of DGTS or the rhamnose-lipid were caused by alteration in phosphate availability (Figs. 1 and 2).

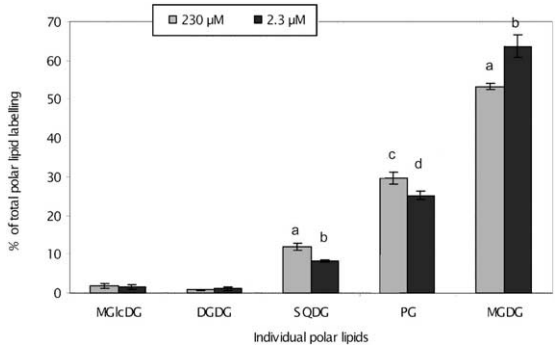

Fig. 3. Relative labelling of individual polar lipids in an endosymbiont *Nostoc* sp. grown at two phosphate levels. Means±S.D. ($n=3$) shown. Bars with different letters are statistically different at $P<0.01$ (a, b) or $P<0.05$ (c, d) on statistical analysis by one-way ANOVA using a Tukey test in comparison of results from the two phosphate levels (1.7 and 0.017 mM). Abbreviations as for Fig. 1 and MGlcDG, monoglucosyldiacylglycerol.

2.4. Fatty acids of the photobionts

The major acids found in the photobionts are shown in Table 4. All the green algae had palmitic as their main saturated fatty acid, oleate as their main monoenoic acid and linoleic and α-linolenic as the main polyunsaturated fatty acids. All these species also contained significant amounts of 16C unsaturated fatty acids, up to hexadecatetraenoate. *Nostoc* differed from the eukaryotic photobionts in containing palmitoleate as its main monoenoic component (Table 4).

When radiolabelling of the photobionts was studied, most of the endogenous fatty acids contained radioactivity. While the labelling of palmitate and palmitoleate was usually roughly in proportion to their occurrence in the algae (an exception being *C. mucigena* where palmitoleate was strongly labelled), oleate was much better labelled than the 18C polyunsaturated fatty acids. Since oleate is a precursor of the latter, such a result most probably reflects the slow sequential desaturation of 18C acids and the length of incubation used in our experiments.

The *Nostoc* sp. showed rather poor labelling of palmitoleate and no detectable labelling of polyunsaturated 18C fatty acids within the incubation period (Table 4). This indicated that desaturation was rather slow in the cyanobacterium.

When the effect of phosphate availability on endogenous fatty acid composition or their relative labelling was tested, there were very few significant effects and those changes seen were not consistent among different species (results not shown).

Table 4
Fatty acid content (wt%) and labelling from [1-^{14}C]acetate (% of total fatty acid labelling) in the endosymbiont algal species, *Coccomyxa mucigena, C. peltigera variolosae, Trebouxia erici, T. aggregata* and in a cyanobacterial, *Nostoc* sp

Fatty acid	*Coccomyxa mucigena*	*C. peltigera variolosae*
C16:0	34.0±1.3 *(32.8±1.9)*	30.0±1.9 *(38.4±1.9)*
C16:1(n-9)	6.2±1.3 *(32.8±1.9)*	4.1±0.2 *(5.8±1.4)*
C16:2(n-6)	1.4±0.3 *(n.d.)*	3.0±0.2 *(n.d.)*
C18:0	7.5±0.3 *(4.7±1.0)*	3.1±0.3 *(3.8±0.3)*
C18:1(n-9)	19.6±1.7 *(35.0±3.1)*	24.7±1.2 *(28.4±1.8)*
C16:3(n-3)	5.5±0.9 *(10.0±1.1)*	4.5±0.7 *(11.3±1.1)*
C18:2(n-6)	17.1±0.2 *(10.0±2.0)*	11.1±0.4 *(12.3±1.6)*
C18:3(n-3)	7.2±0.3 *(n.d.)*	16.1±0.6 *(n.d.)*
Others	1.5±0.5 *(n.d.)*	3.4±1.1 *(n.d.)*
	Trebouxia erici	*Trebouxia aggregata*
C16:0	32.8±2.1 *(39.4±2.3)*	20.1±1.2 *(24.2±2.3)*
C16:1(n-9)	5.1±0.5 *(2.6±0.4)*	4.7±0.3 *(4.2±0.9)*
C16:2(n-6)	2.2±0.3 *(n.d.)*	2.1±0.2 *(n.d.)*
C18:0	4.5±0.6 *(n.d.)*	4.2±0.1 *(1.3±0.4)*
C18:1(n-9)	20.0±0.6 *(50.9±3.5)*	31.0±1.3 *(55.9±2.0)*
C16:3(n-3)	6.4±0.4 *(4.1±0.8)*	5.4±0.8 *(6.7±1.1)*
C18:2(n-6)	20.0±1.7 *(3.0±0.3)*	17.7±1.0 *(7.7±1.2)*
C16:4(n-3)	3.6±0.4 *(n.d.)*	3.2±0.3 *(n.d.)*
C18:3(n-3)	4.4±0.6 *(n.d.)*	10.2±0.6 *(n.d.)*
Others	1.0±0.7 *(n.d.)*	1.4±0.9 *(n.d.)*
	Nostoc sp	
C14:0	0.7±0.1 *(n.d.)*	
C16:0	37.4±1.1 *(48.2±3.5)*	
C16:1(n-9)	16.8±1.3 *(5.6±1.4)*	
C16:2(n-6)	0.7±0.1 *(n.d.)*	
C18:0	2.1±0.3 *(14.0±1.6)*	
C18:1(n-9)	12.8±1.2 *(32.2±2.6)*	
C18:2(n-6)	14.4±0.3 *(n.d.)*	
C18:3(n-3)	13.9±0.2 *(n.d.)*	

Data as means±S.D. where $n=3$ for independent samples for algae growing at 1.7 mM phosphate and for *Nostoc* growing at 230 μM phosphate. Radiolabelling in parentheses.

Fatty acids are indicated with the number before the colon showing the number of carbon atoms, the figure afterwards denoting the number of double bonds and their positions are in brackets. n.d., none detected.

2.5. How can photobionts maintain phosphoglyceride synthesis?

Since phosphorus is such an important element for viability and because lichens survive in many harsh habitats where phosphate availability can be scarce, it makes sense if they have evolved strategies to deal with environmental phosphate deficiency. Even though growth rates for green algae were approximately halved by low phosphate (data not shown), they were able to survive for many generations although phosphoglycerides were required for cellular membranes and where overall polar lipid labelling patterns (Figs. 1 and 2) were hardly affected. Even taking into account the dramatic decrease in lipid labelling for *Coccomyxa* spp., other species (*Trebouxia erici, Nostoc*) were hardly affected.

In order to solve this puzzle, we examined the photobionts by electron microscopy (Fig. 4). The organisms all showed dense-staining granules which, were examined by X-ray probe microscopy. The granules were found to contain phosphorus, calcium and zinc at levels which were at least 20 times their cytosolic concentrations. No other metals were detected (data not shown). These structures corresponded to the polyphosphate bodies which have been reported in some other algae and cyanobacteria (Cembella et al., 1984; Tang et al., 1995; Abu-Shammala, 1999; Rangsayatorn et al., 2002). Interestingly, growth in low phosphate had little effect on the number or size of these bodies (Fig. 4). Thus, it is clear that the photosymbionts are able to utilise these bodies as a phosphorus reserve and, indeed, approximate calculation suggests that the total phosphorus available in these algae is sufficient for at least eight cell divisions whereas, during the experiments conducted, the different photobionts underwent 3–5 cell divisions (data not shown). Moreover, since the polyphosphate bodies are in the cytosol (Fig. 4) any phosphate released would be available easily for phosphoglyceride formation on the endoplasmic reticulum.

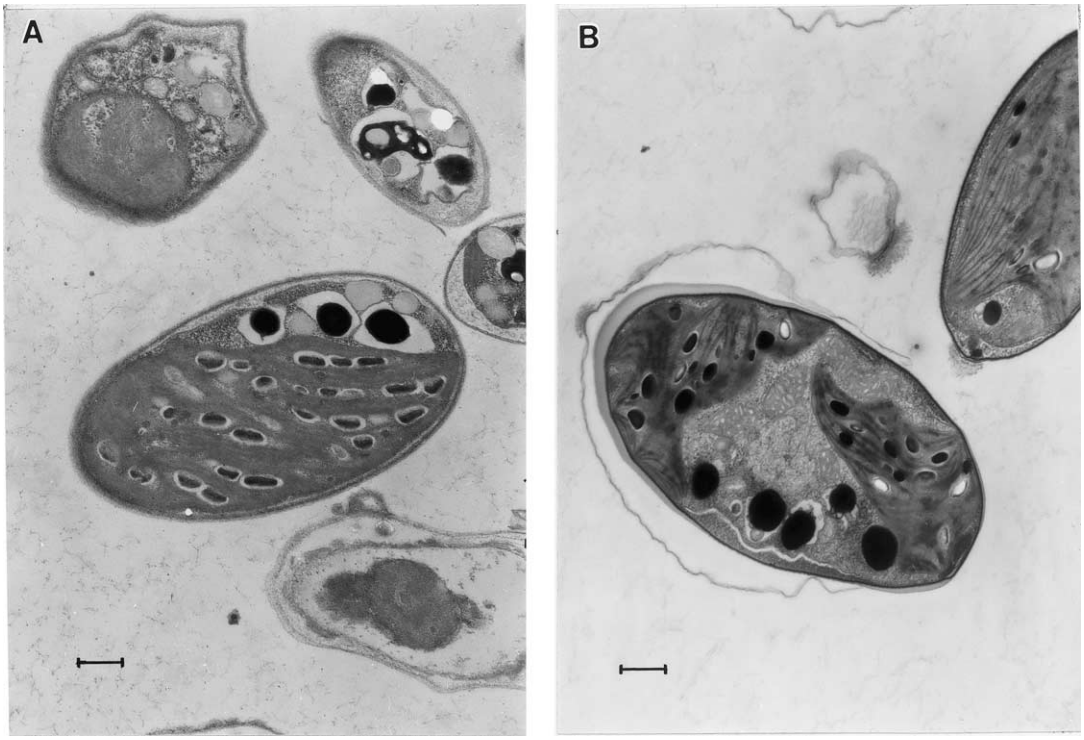

Fig. 4. Electron micrographs of *Coccomyxa peltigera variolosae* grown under normal (1.7 mM) (A) and reduced (0.017 mM) (B) phosphate concentrations (see *Experimental*). Polyphosphate deposits in the cytosol are clearly visible. The numbers and size of these deposits were not reduced significantly during growth under low phosphate for two weeks (See Experimental). Bar equals 0.5 μm.

3. Discussion

In the present study the species investigated were chosen for their different abilities to tolerate harsh environments. *Trebouxia* spp. represent an example of photobionts which are usually associated with the ecologically most successful lichen species of extreme environments (arctic, alpine, antarctic and desert ecosystems) (Honegger, 1991). Green algal photobionts of *Coccomyxa* and cyanobacterial *Nostoc* species are symbiotic partners in *Peltigera* lichens which are relatively sensitive to the environment and grow exclusively in mild moist habitats. Although *Trebouxia* and *Coccomyxa* species have a close taxonomical relationship, they form lichens with different structural and functional mycobiont-photobiont interfaces. The physiological integration between symbionts is closer in lichens with *Trebouxia* photobionts compared to lichens with *Coccomyxa*. This may be related to the fact that *Trebouxia* are rarely found free-living in natural habitats, whereas *Coccomyxa* also comprise many non-symbiotic species.

The lipid metabolism of photobionts is of considerable interest in its own right, since there have been no such studies of these important organisms before. All the algal species studied were able to accumulate the typical storage lipid, triacylglycerol, but their metabolism was much more concentrated towards the maintenance of membrane lipids (Table 1). Phospholipid synthesis was important in all organisms, with PG and then PC being labelled the best (Figs. 1–3). Total phosphoglyceride labelling was 30–35% of total polar lipids in *Coccomyxa* but 60–78% in *Trebouxia* spp. Of the remaining polar lipids, the typical chloroplast lipids MGDG, digalactosyldiacylglycerol (DGDG) and SQDG were appreciably labelled in all species. DGTS was found in all species, except *Trebouxia aggregata,* and was very minor in *T. erici* and *C. mucigena* while both *Coccomyxa* spp. contained a rhamnose-lipid. They also contained another sugar-containing, but unidentified, lipid. The presence of rhamnose- and mannose-containing lipids (which may serve to partly replace other glycosylglycerides such as MGDG) has been noted in certain red algal species (Pettitt and Harwood, 1986; Harwood and Jones, 1989; Pettitt et al., 1989).

Thus, to summarise, all the photobiont algae show common features of their lipid biochemistry but with distinct characteristics. So far as fatty acids are concerned (Table 4) the four algal species have many features in common both with regard to endogenous and to labelled acids. Interestingly, although phosphate availability has been reported to alter fatty acid composition in *Chlorella kessleri* (El-Sheek and Rady, 1995), we found only minor alterations in such compounds during the present work.

In light of previous studies on phosphate deficiency and lipid metabolism in several different organisms (Minnikin et al., 1972; Minnikin and Abdolrahimzadeh, 1974; Minnikin et al., 1974; Benning et al., 1995; Essig-

mann et al., 1998; Geiger et al., 1999; Chen et al., 2000; Sato et al., 2000), we expected growth in low phosphate media to produce large changes in phosphoglyceride synthesis and content. However, although growth and lipid labelling were impaired in the algal species, there were only minor changes in the relative rates of phosphoglyceride labelling (Table 3) and hardly any decrease in the relative labelling of PG (Fig. 3). Thus, all organisms were surprisingly robust in maintaining their phosphoglyceride synthesis in the face of low phosphate availability. We concluded that the presence of polyphosphate inclusions is important for this and may well be a significant adaptation for lichen symbionts which are often subject to mineral deficiencies (Nash, 1996b). Such polyphosphate bodies have been noted before in many microorganisms (Cembella et al., 1984; Tang et al., 1995; Torres et al., 1998; Abu-Shammala, 1999; Rangsayatorn et al., 2002). We calculated that their presence in the photobionts studied would permit membrane synthesis for many generations. However, it is also obvious that there are immediate consequences of growth under low phosphate which is also revealed in lower total lipid synthesis especially in *Coccomyxa* spp. (Table 1). Unfortunately, since there is no biochemical information available about the formation and breakdown of the polyphosphate inclusions, we cannot speculate on their metabolism and, hence, production of water-soluble compounds which can contribute to phosphoglyceride synthesis or other aspects of growth. Nevertheless, it would be interesting to examine if the growth of *Coccomyxa*-containing lichens was affected more than *Trebouxia*- or *Nostoc*-containing lichens by phosphate availability, as might seem possible from our results.

4. Experimental

4.1. Experimental material

Authentic strains of the unicellular green algae *Coccomyxa mucigena* Jaag (215-4), *C. peltigera variolosae* Jaag (216-6), *Trebouxia aggregata* (Archibald) Gärtner (219-1d) and *T. erici* Ahmadjian (32.85), were obtained from the SAG-Sammlung von Algenkulturen at the University of Göttingen. They were cultured in Bold's medium (Ahmadjian, 1967) photoorganotrophically with 1% w/v glucose at 24C under a 14 h photoperiod and 100 μE m^{-2} s^{-1} illumination. Bold's medium contained 1.7 mM phosphate and this was adjusted to 17 μM for phosphate-limitation experiments. Growth of the cultures was monitored by measuring the optical density of the cell suspension spectrophotometrically at 560 nm.

An authentic strain of *Nostoc* sp., isolated from the lichen *Peltigera horizontalis,* was kindly provided by Dr. Eckhard Loos (Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 93040 Regensburg, Germany). It was cultured in the medium described by Stanier et al. (1971) under the same temperature and illumination conditions as for the green algae. The medium normally contained 230 μM phosphate and this was adjusted to 2.3 μM for phosphate-limitation experiments.

4.2. Chemicals

Fatty acid standards were from Nu-Chek Prep. Inc. (PO Box 172, Elysian, MN 56028, USA) and silica gel G plates from Merck. Complex lipid standards were from Sigma (Poole, Dorset, UK). [1-^{14}C]Acetate, Na salt (sp. act. 1.85–2.29 GBq/mmol) was from Amersham Life Science Ltd. (Bucks HP7 9NA, UK). Other reagents were of the best available grades and were from Sigma (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK).

4.3. Incubations

To produce a biomass of algae in the exponential growth phase, 3 ml aliquots of algal cells from stock cultures were transferred to 100-ml flasks containing 50 ml fresh medium with normal or 100-fold phosphate-limited media and inoculates were incubated for 14 days under conditions as described above. Algal cells were separated by centrifugation, supernatants were decanted and 20 ml fresh glucose-free medium (with normal or limited phosphate supply) was added, followed by gently stirring, to each flask. *Nostoc* cells were separated by filtration (Whatman N4) and transferred into 20 ml fresh medium with normal or limited phosphate supply.

Cultures were used for lipid labelling by adding 135 kBq [^{14}C]acetate and incubated further for 2 h at 24 °C with 200 μE m^{-2} s^{-1} continuous illumination. Experiments were performed in triplicate with independent samples. At the end of the incubation period, cell pellets (about 300 mg of fresh weight) were harvested by centrifugation (green algae) or filtration (*Nostoc* sp.), quickly washed with distilled H_2O (2×5 ml) to remove excess radiolabel and metabolism was terminated by the addition of hot isopropanol and heating at 70 °C for 30 min. This method ensured that lipid catabolic enzymes were inactivated. Lipids were extracted twice more with hot isopropanol (three extractions) and finally with a mixture of isopropanol:chloroform (1:1, v/v) (Kates, 1986). Garbus solution (2 M KCl in 0.5 M potassium phosphate buffer, pH 7.4) was used to wash the extraction mixture (Garbus et al., 1963). Fatty acid methyl esters (FAMEs) were prepared by transmethylation of the lipid extract with 2.5% H_2SO_4 in dry methanol.

4.4. Lipid analysis

Non-polar lipids were separated by 1-D TLC on 20×20 cm silica gel G plates with double development,

first with toluene–hexane–formic acid (140:60:1, by vol.) for the whole plate height followed by hexane–diethyl ether–formic acid (60:40:1, by vol.) to half height (Hansen and Rossi, 1990).

Polar lipids were separated by 2-D TLC on 10×10 cm silica gel G plates using chloroform–methanol–water (65:25:4, by vol.) in the first dimension and then chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5, by vol.) in the second direction (Benning et al., 1995).

Plates were sprayed with 0.05% 8-anilino-4-naphthosulphonic acid in methanol and viewed under U.V. light to reveal lipids or visualized under iodine vapour. Identification was made by reference to authentic standards and confirmed using specific colour reagents (Kates, 1986).

The moieties of two unusual polar lipids stained with a positive α-naphthol reaction and were identified after hydrolysis as described by Roy and Harwood (1999). The glycerol backbone of these lipids was also confirmed by labelling from [^{14}C]glycerol and subsequent hydrolytic analysis.

Fatty acid methyl ethers (FAMEs) were analysed by radio-GLC using a Unicam GCD gas chromatograph connected via an effluent splitter to a LabLogic RAGA (LabLogic, Sheffield, UK) gas flow proportional counter. Glass columns (1.5 m×4 mm int. diam.) were packed with 10% SP-2330 on 100/120 Supelcoport (Supelco, Bellefonte, PA, USA) and run isothermally at 180 °C. Routine identification was by reference to standards and quantification was made using an internal standard of heptadecanoate and Rachel Software (LabLogic).

To differentiate some co-eluting peaks, $AgNO_3$-TLC was used to separate fatty acids on the basis of their unsaturation. The bands recovered from the TLC plates were eluted, washed (Henderson and Tocher, 1992) and re-run by radio-GLC.

To determine the double bond positions in fatty acids, their 4,4-dimethyloxazoline (DMOX) derivatives were analysed by GC–MS using a Hewlett Packard 5890 Series II Plus gas chromatograph as described previously (Guschina et al., 2002).

Radioactive counting was made using Opti-Fluor (Packard Bioscience B.V., Groningen, The Netherlands) scintillant and a Beckman 1209 Rackbeta liquid scintillation counter. Quench correction was by the external standard channels ratio method.

4.5. Ultrastructural studies

For ultrastructural studies, cells were collected by low-speed (500 rpm) centrifugation and fixed as recommended by Hayat (1986). The specimens were transferred into a mixture (1:1, by vol.) of propylene oxide and epoxy resin (Araldite CY 212) for 12 h and then embedded in pure Araldite for 48 h at 60 °C. The polymerized resin block was cut into 60 nm thick sections on an ultracut microtome using a glass knife. The sections were counterstained in lead citrate and uranyl acetate prior to an ultrastructural examination under a Philips EM 208 transmission electron microscope.

X-ray microanalysis was carried out using a Jeol 1210 transmission electron microscope fitted with a Link ISIS X-ray analyzer.

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