Diacylglyceryl-N,N,N-trimethylhomoserine-dependent Lipid Remodeling in a Green Alga, Chlorella Kessleri

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Article

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Diacylglyceryl-\(N,N,N\)-trimethylhomoserine-dependent lipid remodeling in a green alga, 
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Abstract: Membrane lipid remodeling contributes to environmental acclimation of plants. In a green lineage, a betaine lipid, diacylglyceryl-\(N,N,N\)-trimethylhomoserine (DGTS), is included exclusively among green algae and non-flowering plants. Here we show that, a green alga, \textit{Chlorella kessleri}, reported to exceptionally possess no DGTS, synthesizes it specifically under phosphorus-deficiency conditions through the eukaryotic pathway via the ER. Simultaneously, phosphatidylcholine and phosphatidylethanolamine, which are similar to DGTS in its zwitterionic property, are almost completely degraded to release 18.1\% cellular phosphorus, and to provide its diacylglycerol moieties for a part of DGTS synthesis. Above lipid remodeling system that substitutes DGTS for extrachloroplast phospholipids to lower the P-quota operates through expression induction of the gene for \textit{BTA1} that is functionally identified as responsible for DGTS synthesis, and those for phospholipid breakdown. Investigation of this lipid remodeling is necessary in a wide range of lower green plants for a comprehensive understanding of their phosphorus-deficiency acclimation strategies.

Key words: betaine lipid, \textit{BTA1}, \textit{Chlorella kessleri}, diacylglyceryl-\(N,N,N\)-trimethylhomoserine, extraplastid lipids, lipid remodeling, phosphatidylcholine, phosphatidylethanolamine, Pi-recycling, phospholipase C.
Introduction

Polar lipids that form lipid bilayers are the foundation for the construction of membranes, where membrane proteins are embedded to contribute to the functionality of the membranes. Information has accumulated over two decades on the remodeling of the membrane lipids crucial for the acclimation to some environmental stresses in photosynthetic organisms or for the compensation of their mutational loss of some polar lipid\(^1,2\). This lipid remodeling includes phosphorus (P)-limitation stress induced replacement of one anionic lipid, phosphatidylglycerol (PG), with another anionic and non-P lipid, sulfoquinovosyl diacylglycerol (SQDG), in the membranes of plant chloroplasts and in those of some bacteria including the postulated ancestor of chloroplasts, cyanobacteria. The replacement of PG by SQDG is regarded to maintain the charge balance of photosynthetic membranes at a certain level for the proper performance of photosynthesis\(^3\). Meanwhile, in seed plants, in particular, in their roots and shoots, P-limitation stress also induces a distinct lipid remodeling that substitutes another non-P lipid, digalactosyl diacylglycerol (DGDG), for phosphatidylcholine (PC) in extraplastid membranes\(^1\).
Diacylglyceryl-$N,N,N$-trimethylhomoserine (DGTS), which is one of membrane lipids, possesses both a positively charged trimethylammonium group and a negatively charged carboxyl one. This non-P betaine lipid is therefore categorized as a zwitterionic lipid. DGTS is prevalently distributed in evolutionarily lower photosynthetic organisms in a green lineage, including green algae and non-flowering plants such as mosses and ferns. Since no DGTS-containing seed plants have been found, it seems that DGTS plays some role specific in lower green plants. DGTS is similar to another zwitterionic lipid, phosphatidylcholine (PC), with respect to chemical and biophysical properties. It is generally accepted that, in green plants including lower ones, PC is localized mainly at extraplastid membranes, as observed in seed plants. Meanwhile, information on the subcellular localization of DGTS is restricted to some green algal species, including, e.g., its occurrence in plasma membranes in a green alga, *Dunaliella salina*. In this context, it is of note that the DGTS content tended to be high in species that possess a low content of PC (Fig. 1a, b), which would be interpreted as reflecting functional substitution of DGTS for PC in extrachloroplast membranes.

Concerning green algae, *Dunaliella* and *Chlamydomonas* species that belong to the Chlorophyceae possess DGTS as one of the major lipids that represents as high as 8.4 to 24.3 mol% of total polar lipids, the PC content being low (Fig. 1). In particular,
Chlamydomonas reinhardtii and Chlamydomonas moewusii are extreme in exclusively possessing DGTS with no PC. In contrast, DGTS was lower in quantity than PC in the green algal species of the Treboxiophyceae, as in Parietochloris incisa (Fig. 1). This tendency was more obvious in Chlorella species in particular: DGTS amounted to only 1.3% in Chlorella fusca, and, above all, it was absent in Chlorella kessleri (Parachlorella kessleri previously known as C. vulgaris) and Chlorella pyrenoidosa with high contents of PC (Fig. 1).

DGTS was observed in other species than lower green plants, although within taxonomically narrow ranges. Several species including the secondary endosymbiotic algae of a red lineage, Nannochloropsis oceanica, an anoxygenic photosynthetic bacterium, Rhodobacter sphaeroides, and a fungus, Flammulina velutipes, were shown to remodel lipids by substituting DGTS for PC upon P-limitation stress. Moreover, a DGTS-loss mutation caused cells to be repressed as to acclimating growth under P-limited conditions in N. oceanica. To our knowledge, however, information on lipid remodeling is scarce in lower green plants under P-limited conditions, despite its potential involvement in their mechanism of acclimation to the P-stress.

C. kessleri is industrially attractive because of its ability to synthesize triacylglycerol (TG) at high levels. We previously reported that the membrane lipid metabolism in
C. kessleri is similar to that in a seed plant, Arabidopsis thaliana, rather than to in another green alga, C. reinhardtii. First, in C. kessleri as well as in A. thaliana, PC is present with DGTS completely absent whereas, in C. reinhardtii, DGTS contrarily exists in the absence of PC. Second, the lipid synthesis in chloroplast membranes depends on the cooperation of two lipid biosynthetic pathways, i.e., the prokaryotic pathway within chloroplasts and the eukaryotic pathway via the ER in C. kessleri like in A. thaliana, whereas it proceeds predominantly through the prokaryotic pathway in C. reinhardtii. Intriguingly, during our study on polar lipids of C. kessleri under stress conditions for the induction of TG accumulation, an unidentified lipid with a similar Rf value to that of DGTS was found to appear (Supplementary Fig. 1), which indicated the necessity to reevaluate the membrane lipid metabolism in C. kessleri.

This study investigated lipid remodeling for acclimation to P-starved conditions in C. kessleri, in view of well-known quantitative increases of DGTS under these P-stress conditions, and regulatory expression patterns of the genes involved in this remodeling. The results were interpreted as showing that C. kessleri is endowed with a sophisticated regulatory mechanism to almost completely replace extraplastid phospholipids including PC with DGTS upon necessity, and that this lipid remodeling is responsible for Pi recycling.
Results

P-starvation induced cell growth defect in *C. kessleri*. This study investigated the polar lipid composition under conditions of P-starvation (-P) in *C. kessleri* to reevaluate its lipid metabolim. We first examined the effects of -P on cell growth in *C. kessleri*. As compared with P-repletion (+P) conditions, -P ones had little deleterious effect on cell growth for the first 24 h, however, causing retardation of cell growth for the next 48 h (Fig. 2a). In line, the chlorophyll (Chl) content of the culture increased more slowly under -P conditions than under +P ones (Fig. 2b). The cellular Chl content on an OD\(_{730}\)·ml basis therefore remained lower under -P conditions than under +P ones throughout the culturing (Fig. 2c). Meanwhile, the total cellular P content, which had initially been 157.1 nmole/(OD\(_{730}\)·ml), drastically decreased to 24.9 nmole/(OD\(_{730}\)·ml) in 48 h under -P conditions, relative to a mild reduction to 112.6 nmole/(OD\(_{730}\)·ml) under +P conditions (Fig. 2d). Despite the severe shortage of P, the survival ratio was little affected in -P cells (95.1%, c.f., 96.4% in +P cells), which demonstrated proper -P-acclimation of *C. kessleri* cells (Fig. 2e).
**P-starvation induced lipid remodeling in** *C. kessleri*. We then investigated whether or not the mechanism of *C. kessleri* cells for acclimating to -P stress involves lipid-remodeling. TLC analysis exhibited that the shift of *C. kessleri* cells from +P to -P conditions brought about the appearance of a novel lipid at a substantial level at 24 h, followed by persistent maintenance of the level for the next 48 h (Lipid X, Fig. 3a, b). The novel lipid was subjected to ESI-MS² analysis. Its ESI mass spectra showed two protonated positive ions, m/z 737 and 761, and their respective sodium adduct ions, m/z 759 and 783 (Fig. 4a). Meanwhile, DGTS prepared from *C. reinhardtii* gave protonated m/z 735 and 737 signals and their sodium adducts, m/z 757 and 759 (Fig. 4b). It was of note that the product ion spectrum at m/z 737 for *C. kessleri* exhibited product ions of m/z 144, 162 and 236, which were common to those of m/z 735 in *C. reinhardtii* (Fig. 4c, d). These results allowed us to identify the novel lipid as DGTS, compatible with the previous report. Accordingly, it was interpreted that the product ions, m/z 456 and 474, and m/z 480 and 498, resulted from the loss of 18:2 and that of 16:0, respectively, in *C. kessleri* (Fig. 4c). Similarly, the product ion spectrum of m/z 761 exhibited three signals (m/z 144, 162 and 236) leading to its identification as DGTS, and two signals for the loss of 18:2 (m/z 480 and 498) (Fig. 4e). These results, together with fatty acid analysis at the
$sn$-2 position (see below), clarified that the DGTS molecular species in P-starved $C. kessleri$ consisted predominantly of $sn$-1 16:0/$sn$-2 18:2 and $sn$-1 18:2/$sn$-2 18:2 species.

Subsequent quantitative GC analysis of the constituent fatty acids in individual polar lipids showed drastically different lipid compositions between +P and -P cells: PC and phosphatidylethanolamine (PE) amounted to 11.3 and 5.6 mol%, respectively, relative to total polar lipids in +P cells with no DGTS, whereas DGTS accumulated to 21.0 mol% in -P cells with disappearance of PC and PE (Fig. 3c). Another phospholipid, PG, amounted to 12.6 mol% in +P cells, however, it decreased to only 2.3 mol% in -P cells. Besides, -P cells showed a lower content of monogalactosyl diacylglycerol (MGDG) (36.2 mol%, c.f., 43.6 mol% in +P cells) with higher ones of DGDG (24.0 mol%, c.f., 14.9 mol% in +P cells) and SQDG (16.5 mol%, c.f., 12.0 mol% in +P cells). It thus turned out that $C. kessleri$, as well as other lower green plants, can synthesize DGTS, and that this ability is displayed under -P conditions, but not under +P conditions. Besides, typical lipid remodeling at chloroplast membranes occurred in $C. kessleri$, as reported in other photosynthetic organisms, such that PG decreased with a concomitant increase of SQDG, probably to keep the charge of the membranes at a certain level (Fig. 3a-c; Sato, 2004). Based on the Pi content of total cellular fraction and those of individual phospholipids in +P cells, the quantitative proportions of phosphate contents in individual phospholipids
to the total cellular fraction one was estimated to reach 12.1, 6.0, and 13.5% Pi in PC, PE, and PG, respectively (Fig. 3d). Therefore, PC and PE totally accounted for 18.1% and 17.6% of total Pi in +P cells and -P cells, respectively (Fig. 3d).

**-P-induced DGTS synthesis via prokaryotic pathway.** Individual lipids in +P cells showed their characteristic fatty acid compositions, as previously reported (Fig. 5a-f). Concerning chloroplast glycolipids, MGDG, in particular, and DGDG contained substantial amounts of unsaturated C_{16} and/or C_{18} acids such as 16:3 and 18:3 (Fig. 5a, b). SQDG had 16:0 and 18:2 as major fatty acids while PG mainly included a sole trans-unsaturated fatty acid, 16:1(3\,t), besides 16:0 and 18:2 (Fig. 5c, d). As to extrachloroplast lipids, PC and PE, mainly contained 16:0 and 18:2 (Fig. 5e, f). Meanwhile, MGDG in -P cells, as compared with that in +P ones, contained 18:3 and 16:3 more abundantly at the expense of 18:2 and 16:2, with DGDG and SQDG respectively displaying only small effects of -P, if any, on their fatty acid compositions. PG was characteristic to demonstrate a higher content of 16:1(3\,t) in -P cells than in +P ones, at the expense of 16:0. Intriguingly, the fatty acid composition in DGTS in -P cells was almost the same as those in PC and PE in +P cells, such that 16:0 and 18:2 amounted to 20-30 and 55 mol%, respectively (Fig. 5e-g).
Membrane lipids are synthesized through the prokaryotic and eukaryotic pathways in *C. kessleri* as well as in *A. thaliana*\(^{17,26}\). The prokaryotic and eukaryotic pathways are responsible for the synthesis of polar glycerolipids with C\(_{16}\) and C\(_{18}\) acids esterified, respectively, at the *sn*-2 position of the glycerol backbone. DGTS was then analyzed as to the fatty acid composition at the *sn*-2 position to estimate the proportions of prokaryotic and eukaryotic lipids (Fig. 5h). The results indicated that the *sn*-2 position was occupied almost exclusively by C\(_{18}\) acids, and strongly implied that DGTS, similar to PC and PE, was synthesized almost exclusively through the eukaryotic pathway.

**The metabolic mechanism of -P-induced DGTS accumulation in *C. kessleri***. The structural similarity of DG moieties of DGTS, and PC and PE raised the possibility that PC and PE release their fatty acid and/or DG moieties upon their degradation for DGTS synthesis. Quantitative changes in these three zwitterionic lipids were then chased for a shorter term within 24 h after the shift of +P cells to -P conditions (Fig. 6a, b). DGTS accumulation steeply increased from an initial zero level in 24 h of -P stress, both PC and PE concomitantly steadily decreasing to less than 10% the initial levels at 24 h in the cells when estimated per on the basis of OD\(_{730}\)·ml (Fig. 6a). It was of note that the accumulated content of DGTS was close to the total content of PC and PE decreases, which was
compatible with the results in Fig. 3c (48 h). However, the increased level of DGTS at 24 h was more than ca. 5-fold higher than the sum of the initial levels of PC and PE when estimated per ml culture (Fig. 6b). These results raised the possibility that fatty acids or DG moieties for DGTS synthesis were supplied partially by preexisting PC and PE through their degradation, and more abundantly by other metabolic sources like de novo fatty acid synthesis.

The effects of metabolic inhibitors on -P-induced DGTS accumulation were investigated to gain an insight into the metabolic mechanism of lipid remodeling (Fig. 6c). Cycloheximide, an inhibitor of 80S ribosomes, almost completely repressed DGTS accumulation under -P conditions, whereas chloramphenicol, an inhibitor of 70S ribosomes in chloroplasts or mitochondria, caused only low repression of DGTS accumulation by 24.1%. These results implied that -P-induced DGTS accumulation depended almost completely on the synthesis of nuclear-genome encoded proteins, and not so greatly on that of chloroplast- or mitochondria-genome encoded proteins. Intriguingly, cerulenin, an inhibitor of β-ketoacyl-ACP synthase of fatty acid synthase, inhibited DGTS accumulation by 27.3%, which inferred involvement not only of de novo synthesized fatty acids, but also of fatty acids originating from pre-existing lipids, in the DGTS accumulation, as was above suggested. Meanwhile, DGTS accumulation was
repressed by 89.0% on application of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthesis, or through a shift of the cells to dark conditions, which suggested the dependence of DGTS accumulation almost completely on photosynthesis.

Identification of the gene for DGTS synthesis in C. kessleri. We then searched for the gene for DGTS synthesis in the genomic DNA sequence of C. kessleri with the use of the protein product of the C. reinhartii BTA1 gene (CrBTA1) as a query. We found a DNA region that could encode a protein highly homologous to CrBTA1. The cDNA covering this region was synthesized through 5’- and 3’-RACE PCR with the use of total RNA isolated from C. kessleri cells starved of P, as the template. The primers for 5’- and 3’-RACE PCR were set such that a partially overlapping DNA regions were generated, thereby 1.5-kbp and 2.0-kbp DNA fragments being generated. Subsequent determination of the obtained PCR products revealed that the full-length DNA was 2073 bp long, which was postulated to encode a 78.8 kDa protein that comprised 631 amino acid residues. This protein was composed of two domains that were homologous to the bacterial BtaB (S-adenosylmethionine-diacylglycerolhomoserine-N-methyltransferase) and BtaA (S-adenosylmethionine-diacylglycerol 3-amino-3-carboxypropyl transferase) proteins at the N- and C-terminal halves, respectively (Fig. 7a). This homolog of C. kessleri was thus
characterized as type B like *C. reinhardtii* BTA1 (CrBTA1), distinct from type A composed of N-terminal BtaA- and C-terminal BtaB-like domains, and showed 62.1% identity in amino acid sequence with CrBTA1. Furthermore, the consensus sequence, VD, for the binding of S-adenosyl methionine as the substrate was conserved in both the BtaA- and BtaB-like domains (Fig. 7a). The coding region of the full-length cDNA was divided into 18 exons by 19 introns in the genome (Fig. 7b). Owing to these sequence characteristics of this protein product, it was strongly suggested that this homologous gene encodes BTA1 for DGTS synthesis. We then functionally characterized this gene through overexpression of its cDNA in *E. coli* cells that intrinsically lack DGTS. Upon induction of the corresponding protein, a novel lipid was found to appear in *E. coli* cells (Fig. 7c), which was thereafter identified as DGTS (Fig. 7d, e). These results proved that the homologous gene of *C. kessleri* encodes the BTA1 protein, and thus the gene was designated as *CkBTA1*. 

Molecular phylogenetic analysis indicated that the BtaA domain of CkBAT1 is closely related to the counterparts of green algae that belong to the Trebouxiophyceae and Chlorophyceae, and also to those of non-flowering plants, ferns and mosses (Fig. 8a). However, the clade of green algae including *C. kessleri* was far away from the clade of another green algal group, the Prasinophyceae, and secondary endosymbiotic algae, but
rather was close to the clade of fungi. A similar trend was observed for the BtaB domain (Fig. 8b). Meanwhile, bacterial BtaA and BtaB showed single clades, respectively.

Transcriptional upregulation of the genes for -P-induced lipid remodeling. Semi-quantitative analysis of mRNA levels as to individual genes was then performed (Fig. 9). CkBTA1 mRNA level was very low under +P conditions, consistent with the complete absence of DGTS, and increased under -P conditions with time, in line with DGTS accumulation. Meanwhile, the degradation of PC and PE might be catalyzed by non-specific phospholipase C (NPC) and phospholipase D (PLD)\(^1\). A search for corresponding genes in the \textit{C. kessleri} genome, with \textit{A. thaliana} orthologs of NPC and PLD genes as queries, led us to find that \textit{C. kessleri} has no homologous genes. Alternatively, \textit{C. kessleri} contained two genes coding for proteins homologous to glycerophosphodiester phosphodiesterase with possible PLC activity in \textit{C. reinhardtii} \(^30\). The expression of one of these candidate PLC genes (Cre03.g203600.t1.2, \textit{PLCc1}), similar to that of \textit{CkBTA1}, was strictly repressed at the transcript level under +P conditions, and was up-regulated with time after the shift to -P conditions, consistent with the progress of the degradation of PC and PE. Meanwhile, the other candidate gene (Cre16.g68350.t1.3, \textit{PLCc2}) was little affected in its mRNA level, irrespective of the conditions of P. There was the
possibility that phosphocholine and phosphoethanolamine, which might be the
degradation products of PC and PE, respectively, by the PLC activity of PLCc1, were
subjected to the action of phosphatase for Pi release. In accordance with this idea, the
mRNA level of the candidate of phosphatase gene PLP$^{31,32}$ was markedly up-regulated
from an almost undetectable level upon -P stress (Fig. 9). These observations implied that
the genes responsible for the lipid remodeling were subjected to up-regulation at least at
their transcript levels, which seemed compatible with the dependence of DGTS
accumulation on the synthesis of proteins encoded by nuclear genes (Fig. 6c).

Discussion

Taxonomical distribution of DGTS in primary endosymbiotic algae. The presence
of DGTS has been reported in many lower green plants, with the rare exceptional
observation of no detection of it in C. kessleri and C. pyrenoidosa. However, this study
demonstrated that C. kessleri cells are able to synthesize DGTS, and that this ability is
displayed under -P conditions, but not under +P ones (Figs. 3, 4 & 6). This -P-stress
specific DGTS appearance is the reason why DGTS has never thus far been detected in
It will be necessary to reevaluate the previously reported absence of DGTS in *C. pyrenoidosa*, which might lead to the novel notion of a general ability of DGTS synthesis in green algae.

In contrast to the prevalent distribution of DGTS in green algae, the presence of DGTS has been reported within only a taxonomically limited range in red algae. In a red microalga, *Galdieria sulphuraria*, DGTS was one of the major polar lipids, which was intriguing with respect to its synthetic pathway since this alga possesses no *BTA1* homolog in its genome (https://phycocosm.jgi.doe.gov/Galsul1/Galsul1.home.html). However, the presence of DGTS have been ruled out in other red microalgae.

Concerning red macroalgae, the presence of DGTS might have been arguable, because of its detection only in limited species, and, if any, at very low levels. The question of red macroalgal DGTS would be settled with the use of the lipidomic technique. Overall, the taxonomical distribution of DGTS should be carefully examined from both biochemical and genetical aspects, as shown by this study. The use of -P conditions would be necessary for biochemical detection of DGTS and its identification in case of strict regulation of the responsible gene, as observed in *C. kessleri*.

Physiological significance of lipid remodeling in *C. kessleri* cells in response to
**-P stress.** To our knowledge, information on the effects of -P on the DGTS contents in lower green plants is limited to that in *C. reinhardtii*. Distinct from non-green photosynthetic and non-photosynthetic organisms known to show -P-induced DGTS increases, *C. reinhardtii* showed little effect of -P on the DGTS content, which might imply that this green alga has adapted to -P-stress in freshwater habitats by losing PC. Our study therefore demonstrated -P-specific induction of DGTS synthesis in *C. kessleri* among lower green plants, as a unique lipid remodeling pattern that accompanies almost complete degradation of PC and PE at extrachloroplast membranes. In vitro assay demonstrated that DGTS was synthesized not in isolated chloroplasts, but in microsomal fractions in *C. reinhardtii* whereas the counterpart of BTA1 is localized at the ER in a fungus, *F. graminearum*. It is therefore highly probable that CkBTA1 contributes to DGTS synthesis at extrachloroplast membranes such as ER ones through the eukaryotic pathway (Fig. 5h). Accordingly, DGTS as well as PC and PE would be present mainly at extrachloroplast membranes in *C. kessleri*, as previously reported in other algal species. In this context, it was of note that the relative contents of these two phospholipids, PC and PE, in +P cells were very close to that of DGTS in -P cells (Figs. 3c & 6a). Since DGTS is similar to PC in zwitterionic and biophysical properties, and also to PE in the
zwitterionic one to PE\(^7\), we propose that DGTS functionally substitutes for PC and PE in

\(C.\ kessleri\) at extrachloroplast membranes under -P conditions.

Seed plants adopted another lipid remodeling system at extraplastid membranes in roots and shoots to replace phospholipids such as PC with DGDG\(^1\). The DGDG content increased also in \(C.\ kessleri\), however, by only 1.6-fold in -P cells relative to in +P ones (Fig. 3c; c.f., >8- fold increase of DGDG in roots of \(A.\ thaliana\)^\(^{41}\)). This small increase of DGDG might reflect the lipid remodeling at chloroplast membranes, in view of a similarly small increase of the DGDG content by 1.2-fold in -P cells of a cyanobacterium, \(Synechocystis\) sp. PCC 6803\(^{42}\). These results thus prompted us to conclude that \(C.\ kessleri\), quite different from seed plants, utilizes DGTS as a main player in the -P-responsive lipid remodeling at extraplastid membranes.

The expression of \(PLC_{c1}\) as the PLC candidate was induced under -P conditions, concomitantly with \(PLP\) mRNA up-regulated. Induced \(PLC_{c1}\) and PLP might cooperatively degrade PC and PE to release DG and Pi (Figs. 3 & 9). In line with the possible DG release, the chemical structure of DG moieties as to fatty acids of PC and PE was similar to that of DGTS. Therefore, these observations, together with the partial induction of DGTS synthesis with de novo synthesis of fatty acids being inhibited (Fig. 6c), prompted us to propose the involvement of \(PLC_{c1}\) in a part of DGTS synthesis.
through release of DG (Fig. 6b). The up-regulation of \( PLC_{C1} \) expression was reminiscent of the role of PLC in DG supply through PC degradation in the synthesis of DGTS and DGDG in a bacterium, \( Sinorhizobium \) meliloti, and \( A. \) thaliana, respectively, under -P conditions\(^{43,44} \). Identification of the actual enzymatic activity of \( PLC_{C1} \) awaits a future study.

In view of the Pi content included in PC and PE (18.1% of total cellular Pi), the involvement of PLP in their degradation would enable \( C. \) kessleri cells to enlarge a Pi-pool under -P conditions, thereby giving a great advantage for -P acclimation. Major phospholipids, i.e., PC, PE, and PG, accounted for 31.6% of total cellular Pi in \( C. \) kessleri, which was similar to the counterparts occupying 23% of total Pi in the photosynthetic tissues in seed plants (Fig. 3d)\(^{45} \). Besides phospholipids, nucleic acids occupy 35% total Pi in photosynthetic tissues of seed plants, with more than 85% of it being attributed to rRNA. Accordingly, major RNases, RNS1 and RNS2, played important roles in rRNA degradation for remobilization of Pi\(^{46} \). It will be necessary in the future to investigate other Pi-scavenging systems than DGTS-lipid remodeling in \( C. \) kessleri as a model green alga, which would lead to a comprehensive understanding of Pi-sequestering systems that is necessary for obtaining the whole picture of the -P-acclimation mechanism in lower green plants.
Evolutionary insight into lipid remodeling in a green lineage. The convincing proof of DGTS occurrence in lower green plants, together with increasing information on their possession of \textit{BTA1} homologs, prompted us to propose that the DGTS synthesis ability appeared in green algae during the evolution of a green lineage. However, the origin of \textit{BTA1} seems different between the Chlorophyceae and Treboxiophyceae, and the Prasinophyceae, as judged from the respective molecular phylogenetic characteristics of the BtaA and BtaB domains, and the fusion order of these two domains in BTA1 proteins (Fig. 8). From the phylogenetic trees of the BtaA and BtaB domains in BTA1, it might be interpreted that, through the evolution of a green lineage, type B BTA1 was first acquired by some ancestral green alga, and thereafter inherited by the Chlorophyceae and Treboxiophyceae, and finally ferns and mosses, however, without its further inheritance to seed plants.

As to the composition of zwitterionic lipids, DGTS and PC, lower green plants possessing type B BTA1 can be grouped into three groups, i.e., those containing DGTS only, both DGTS and PC, and PC only, respectively, under +P conditions. One idea is that the first species originally containing PC acquired the \textit{BTA1} gene, and then evolved to persistently possess both DGTS and PC, as in, e.g., \textit{D. salina}. Later, it seems that green
algae selected one of these two zwitterionic lipids for their membrane construction under +P conditions: some species like *C. reinhardtii* have evolved to contain DGTS only by abandoning PC synthesis with the loss of the PC synthesis genes\(^{39}\), while other species like *C. kessleri* have chosen PC by repressing the expression of *BTA1*. It is possible that some ancestral lower green plant like *C. kessleri* lost the *BTA1* gene to evolve into extant seed plants, with the acquisition of the DGDG-utilizing lipid remodeling system. The acquisition of this new remodeling system would have had a beneficial effect on nitrogen (N)-economization, and thus on acclimation to N-limitation stress generally encompassing plant habitats, since DGDG, which unlike DGTS includes no N atom. Simultaneously, complete replacement of DGTS with PC, along with the selection of DGDG-utilizing lipid remodeling, might have merited the evolutionary appearance of extant seed plants, since a variety of their biological processes, such as stress acclimation and plant development, are mediated through PC-related signaling pathways with the actions of phospholipases\(^{47}\).

**Methods**
Strain and growth conditions. C. kessleri 11 h was photoautotrophically grown with 4-fold diluted Gamborg's B5 (GB5, +P) medium or the -P medium made through replacement of NaH$_2$PO$_4$H$_2$O by equimolar KCl$^{23,24}$. The OD$_{730}$ value and Chl content were measured to monitor cell growth$^{24}$. The cells were precultured in +P medium to an OD$_{730}$ value of ca. 0.5, and then adjusted to an OD$_{730}$ value of 0.2 with +P or -P medium for further growth. The survival ratio of the cells in the culture was examined through 5 µM SYTOX-staining (Invitrogen Molecular Probes$^{48}$). When indicated, the culture was supplemented with cycloheximide (8 µg·ml$^{-1}$), chloramphenicol (100 µg·ml$^{-1}$), cerulenin (10 µM), and DCMU (50 µM) as metabolic inhibitors, or shifted to dark conditions, simultaneously with the commencement of -P culturing.

Quantitation of Pi in C. kessleri cells. Cells were harvested from 20 ml culture by centrifugation after washing three times with -P medium, and thereafter disrupted with a Beads Crusher µT-12 (Taitec, Saitama, Japan) in 2 ml of extraction buffer comprising 5 mM HEPES-NaOH, pH 7.5, and 10 mM NaCl$^{24}$. To 100 µL of each whole cell extract, 2 mL of 15 mM potassium peroxodisulfate and 5 mL of distilled H$_2$O were added, the resultant solution being subjected to autoclave treatment (121ºC, 30 min) for the release of Pi from P-containing compounds. To 100 µl of the autoclave-treated solution, 300 µL
of a 1.22% malachite green G (Wako, Tokyo) solution and 600 µl of distilled H₂O were
added, and then the resultant solution was left to stand for 30 min. The Pi content was
determined in the marachite-green stained solution through spectroscopic measurement
of the absorbance at 639 nm⁴⁹.

**Separation and quantitation of individual polar lipids.** Cells were harvested at the
indicated times for the extraction of total lipids, which were then used for separation of
individual polar lipids by two-dimensional TLC⁵⁰. A novel lipid found in this study was
identified through mass spectrometric analyses. Samples were diluted in IPA/MeOH/H₂O
(5:4:1, v/v/v) containing 10 mM ammonium acetate, and then directly infused at 10
µL/min flow into a triple quadrupole linear ion trap mass spectrometer (MS) equipped
with an electro spray ionization (ESI) source (3200Q with a Trap Turbo V ion source;
Sciex, CA). The optimized parameters for DGTS under positive and negative ionization
conditions were as follows: Ion spray voltage, 5500 V (positive) and -4500 V (negative);
declustering voltage, 100 V (positive) and -55 V (negative); temperature, ambient (both
positive and negative). The collision energies for product ion scanning were 60 V
(positive) and -45 V (negative). The mass range was scanned m/z 600–900 in the Enhanced
Mass Scan (EMS) mode for precursor ions and m/z 50–900 in the Enhanced
Product Ion Scan (EPI) mode for product ions. The mass spectrum data were analyzed with reference to DGTS of *Chlamydomonas reinhardtii* 137c and that of chlorarachniophytes\(^{28}\). DGTS from *C. kessleri* was used for the preparation of *sn*-2 monoacyl lysoDGTS through TLC after treatment of it with *Rhizomucor miehei* lipase (Sigma-Aldrich)\(^{17}\). Fatty acid methyl esters derived from total lipids, individual polar lipids or *sn*-2 monoacyl lysoDGTS were quantified by capillary GLC based on their constituent fatty acids\(^{17}\).

**Cloning of cDNA for BTA1, determination of its nucleotide sequence, and its expression in *E. coli*.** A blast search was performed with the amino acid sequence of BTA1 of *C. reinhardtii* (CrBTA1) as a query in the genomic DNA database of *C. kessleri* (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=Assembly&LINK_LOC=blasttab&ASSEMBLY_NAME=GCA_00159875.1). Sixteen regions that aligned closely on the genome were hit, and therefore, as a whole, were postulated to encode a BTA1 homolog. One of these regions was chosen for primer setting for 5’- and 3’-race PCR with a SMARTer RACE 5’/3’ kit (Takara, Tokyo; primer set 1, Supplementary Table 1), the nucleotide sequence of the
amplified DNA being determined with a BigDye Terminator v3.1 cycle sequencing kit on a 3500 genetic analyzer (Thermofisher, Tokyo).

For heterologous expression of BTA1 in E. coli, the ORF of the BTA1 cDNA was amplified by PCR with primer set 2 (Supplementary Table 1), and then ligated to a pMAL-c5X vector (New England Biolabs Japan, Tokyo), for generation of the vector designated as pMAL-BTA1. The pMAL-BTA1 or empty pMAL-c5X vector was introduced into NEB express competent E. coli cells (New England Biolabs Japan, Tokyo). Transformed cells were subjected to the induction of gene expression with IPTG, and then to SDS-PAGE analysis of total cellular proteins, as described in the manufacture’s manual, and to TLC analysis of lipids.

Phylogenetic tree. For phylogenetic analysis, the amino acid sequences of BTA1 homologs were searched for in available databases with CrBTA1 (CHLREDRAFT_77062), and BtaA (RSP_0856) and BtaB (RSP_0857) of Rodobacter sphaeroides 2.4.1, as queries. The homolog sequences of CrBTA1 obtained are summarized in Supplementary Table 2. The sequences were aligned after edition including the deletion of lowly conserved regions among these sequences with Geneious 9.1.8 (Tomy Digital Biology, Tokyo), and thereafter, the aligned sequences were
subjected to phylogenetic analysis with IQ-tree 1.6.12 by the maximum-likelihood method\textsuperscript{31}.

Semi-quantitative PCR analysis. Total RNA was extracted from \textit{C. kessleri} cells by phenol–chloroform extraction for cDNA synthesis by random-primer based reverse-transcription\textsuperscript{24}. Subsequently, the synthesized cDNA was used as a template for semi-quantitative RT-PCR. Specific forward (F) and reverse (R) primer sets 3-6 were designed for the respective genes (Supplementary Table 1), on the basis of the information on above described genomic DNA sequences of \textit{C. kessleri}. The amplified DNA fragments were separated by agarose gel electrophoresis, and then subjected to staining with ethidium bromide to obtain a fluorescent image by photography\textsuperscript{52}. The fluorescence intensities of DNA bands for the individual genes were estimated with ImageJ (http://rsbweb.nih.gov/ij/), relative to that for the \(\beta\)-actin gene (\textit{ACT}), as an internal control.

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**Author contributions**

Y.O., R.O., Y.I., E.K., M.A. and N.S. performed research; Y.O., R.O., Y.I., E.K., M.A., M.T., S.F. and N.S. analyzed data; N.S. provided supervision, and drafted the manuscript, and all authors read and approved the final version.

**Additional information**

Supplementary information accompanies this paper.

Competing interests: The authors declare no competing interests.
Fig. 1 DGTS and PC contents in green algae grown under +P conditions. (a) Respective contents of DGTS and PC relative to that of total polar lipids. (b) The proportion of DGTS and PC contents. White and black bars indicate the contents of DGTS and PC, respectively. The values were obtained from previous reports (10-15,17).
Fig. 2 Effects of -P stress on physiological behavior in *C. kessleri* cells. (a) Cell growth on the basis of the OD$_{730}$ value of the culture. (b) Chl content in the culture. (c) The cellular Chl content obtained through estimation of the relative ratios of the values in (b) to those in (a). White and black circles indicate cells grown under +P and -P conditions, respectively. (d) Pi contents measured in cells grown under +P conditions (0 h), and in ones grown further for 48 h under + P and -P conditions. (e) Images of the cells emitting red auto-fluorescence of Chl and/or the green fluorescence of SYTOX bound to chromosomal DNA. The survival ratio of the cells was determined through estimation of the proportion of the number of viable cells emitting only red fluorescence, relative to that of total cells, including non-viable cells with emission of green fluorescence only and with that of both red and green fluorescence. The values shown are averages ± SEM for two technical replicates for each of four biological replicates (a-c), and those for three biological replicates (d, e). Data were analyzed by one-tailed Student's *t*-test.
Fig. 3 Lipid remodeling in C. kessleri cells in response to -P stress. (a) Qualitative TLC analysis of individual polar lipids prepared from cells grown under +P conditions (0 h), and from ones shifted to -P conditions for further growth for 24, 48, and 72 h. (b) Two-dimensional TLC profile of polar lipids in the cells grown for 48 h under +P or -P conditions. (c) Polar lipid composition in the cells grown for 48 h under +P (white bars) or -P conditions (black bars). (d) The contents of Pi included in individual phospholipids, relative to that in total cellular fraction. The values shown are averages ± SEM for two technical replicates for each of two biological replicates (c), and those estimated on the basis of the data in Fig. 2d and Fig. 3c (d). Data were analyzed by one-tailed Student's t-test.
Fig. 4  Identification of a -P-induced novel lipid as DGTS through LC/MS² analysis. ESI mass spectra as to a novel lipid of *C. kessleri* (a) and DGTS of *C. reinharditii* (b). Product ion spectra (c), (d), and (e), as to m/z 737 in (a), m/z 735 in (b), and m/z 761 in (a), respectively.
Fig. 5  Fatty acid composition of individual polar lipids. The fatty acid compositions of MGDG (a), DGDG (b), SQDG (c), and PG (d) are shown for 48-h grown +P (black bars) and -P cells (white bars), whereas those of PC (e) and PE (f), and DGTS (g) are for +P and -P cells, respectively. The fatty acid composition of sn-2 monoacyl lysoDGTS is shown in (h). The values shown are averages ± SEM for two technical replicates for each of two biological replicates. Data were analyzed by one-tailed Student's t-test.
Fig. 6  Metabolic mechanism of DGTS accumulation under -P conditions. Changes in the contents of DGTS (white), PC (grey), and PE (black) in cells grown for 12 h after a shift from +P to -P conditions, which were estimated in cells on an OD_{730} ml culture basis (a), and in the culture (b). (c) Effects of metabolic inhibitors or light conditions on the accumulation of DGTS in the cells shifted to -P conditions. The values were estimated as the proportion of the DGTS content in the cells with application of chloramphenicol (CAP), cycloheximide (CHI), cerulenin, and DCMU, and in those shifted to the dark conditions, relative to that in non-treated control cells. The values shown are averages ± SEM for three biological replicates. Data were analyzed by one-tailed Student's t-test.
Fig. 7 Structural and functional identification of a BTA1 homolog in *C. kessleri*. (a) Alignment of the postulated amino acid sequence of a BTA1 homolog with that of CrBTA1. Identical and similar amino acid residues are shadowed in black and grey. The vertical red line indicates the boundary of the N-terminal BtaB- and C-terminal BtaA-like domains, whereas two black boxes shows the conserved dipeptide VD for the binding of SAM, the substrate in the respective catalytic actions of BtaA and BtaB. (b) The structure of the of the *BTA1* homolog of *C. kessleri*. The *BTA1* homolog is composed of 18 exons.
on the nuclear genome. (c) Expression of cDNA of the BTA1 homolog in E. coli. SDS-PAGE of total cellular proteins shows induction of the expression of the homolog protein in pMAL-CkBTA1 introduced E. coli cells, but not in empty-vector introduced ones. TLC of total cellular lipids shows the appearance of a novel lipid specifically in pMAL-CkBTA1 introduced E. coli cells. (d) The ESI mass spectrum of the novel lipid in the transformant of E. coli. (e) The product ion spectrum of m/z 739 in (d). Note that the product ion spectrum exhibited three signals (m/z 144, 162 and 236) for identification of the novel lipid as DGTS.
Fig. 8  Phylogenetic trees of the BtaA and BtaB domains in BTA1 proteins. (a) Bacterial BtaA and eukaryotic BtaA-domains. (b) Bacterial BtaB and eukaryotic BtaB-domains. Two linked boxes, AB and BA, indicate eukaryotic A and B type BTA1 proteins, respectively, whereas single boxes, A and B, demonstrate bacterial BtaA and BtaB proteins, respectively.
Fig. 9 Regulatory expression of the genes for -P induced lipid remodeling in C. kessleri. The expression levels of the BTA1, PLC\(_{C1}\), PLC\(_{C2}\), and PLP genes were investigated through semi-quantitative RT-PCR analysis in C. kessleri cells before and after a shift to -P conditions. The intensities of the DNA bands that correspond to mRNAs of the individual genes were used for determination of the values, relative to that of ACT. The values shown are averages ± SEM for three biological replicates. Data were analyzed by one-tailed Student's t-test.
Supplementary Fig. 1  Mass spectrometric identification of DGTS. (a) ESI mass spectra of DGTS expressed in *E. coli*. (b) Product ion spectrum of m/z 739 at (a). (c) Estimated fragmentation pattern for DGTS (16:0/18:1) from the product ion spectrum, in which the *sn* position of fatty acids is provisional.
### Supplementary Table 1  
Primer sets

| set | gene   | Forward                                      | Reverse                                      | Query              |
|-----|--------|----------------------------------------------|----------------------------------------------|--------------------|
| 1   | 5' race cDNA | ctaattaacactatatagggcaagcagtgttatcaacgcagag | gattagaeccaacgcttcagctcaactgggtctcacaagcgac    |                     |
| 2   | 3' race cDNA | gattaacaagcttttcccgccaccttctcgttcac         | ctaattaacactatatagggcaagcagtgttatcaacgcagag |                     |
| 3   | BTA1   | gcgcgaachacatgagggcggcagagggaggtgagat      | tgtgttgttgttgttgtggatgtgcaggtggcctcacaagcgac |                     |
| 4   | BTAI   | tacctctagtagaaccctcc                       | tccgaacattgtcagagaecccc                      | Cre.07.g324200.t1.2 |
| 5   | PLCC1  | cctccttcctgcccgac                      | ttagctctctgccacccctga                      | Cre03.g203600.t1.2 |
| 6   | PLCC2  | acaacccctccctgacacac                      | cctctctgatcatgctcgatct                          | Cre16.g683850.t1.3 |
| 7   | PLP    | acaaccccctccctgacacac                      | gccgacacactggacgccgtgcag                      | AT1G17710           |
| 8   | ACT    | ccggtgacacactctgtgcac                      | ccggtgacacactctgtgcac                       | AB046457.1          |
| BtaA | Organisms | gene ID |
|------|-----------|---------|
| **Bacteria** | | |
| *Rhodobacter sphaeroides* | ATCC 17025 (Rsph17025_3617) | |
| *Rhizobium* sp S41 | BA939_05055 | |
| *Rhizobium leguminosarum* bv. *trifolii* WSM2304 | Rleg2_2613 | |
| *Rhizobium jaguaris* | CCGE525_12990 | |
| *Rhizobium tropici* | RTCIAT899_CH11455 | |
| *Rhizobium etli* bv. *mimosae* Mim1 | REMIM1_CH02915 | |
| *Sinorhizobium meliloti* | NP_386300 | |
| **BtaB** | | |
| *Rhodobacter sphaeroides* ATCC | Rsph17025_3616 | |
| *Rhizobium* sp NT-26 | NT26_2169 | |
| *Rhizobium leguminosarum* bv. *trifolii* WSM2304 | Rleg2_2614 | |
| *Rhizobium jaguaris* | CCGE525_12995 | |
| *Rhizobium etli* bv. *phaseoli* | IE4803_CH03093 | |
| **BTA1** | | |
| **Green algae** | | |
| *Chlamydomonas reinhardtii* | CHLREDRAFT_77062 | |
| *Coccomyxa subellipsoidea* | COCSUDRAFT_28345 | |
| *Auxenochlorella protothecoides* | F751_5248 | |
| *Volvox carteri* f. *nagariensis* | VOLCADRAFT_83033 | |
| *Ostreococcus lucimarinus* | OSTLU_27669 | |
| *Ostreococcus tauri* | OT_ostta15g02860 | |
| *Micromonas pusilla* | MICPUDRAFT_48920 | |
| *Bathycoccus prasinus* | Bathy08g00260 | |
| *Monoraphidium neglectum* | MNEG_12083 | |

| **Fern** | | |
| *Selaginella moellendorffii* | SELMODRAFT_429378 | |

| **Moss** | | |
| *Physcomitrella patens* subsp. *Patens* | 112274025 | |

| **Secondary endosymbiotic algae** | | |
| *Emiliania huxleyi* | CCMP1516 | |
| *Nannochloropsis oceanica* | LC375792 | |

| **Fungi** | | |
| *Metarhizium robertsii* | MAA_07461 | |
| *Purpureocillium lilacinum* | VFPFJ_09846 | |
| *Colletotrichum fioriniae* | CFIO01_08357 | |
| *Nectria haematococc a* | NECHADRAFT_31251 | |
| *Fusarium graminearum* | FGSG_00742 | |
| *Flammulina velutipes* | KM668875 | |
| *Neurospora crassa* | NCU03032 | |
Figure 1

DGTS and PC contents in green algae grown under +P conditions. (a) Respective contents of DGTS and PC relative to that of total polar lipids. (b) The proportion of DGTS and PC contents. White and black bars...
indicate the contents of DGTS and PC, respectively. The values were obtained from previous reports (10-15,17).

Figure 2

Effects of -P stress on physiological behavior in C. kessleri cells. (a) Cell growth on the basis of the OD730 value of the culture. (b) Chl content in the culture. (c) The cellular Chl content obtained through estimation of the relative ratios of the values in (b) to those in (a). White and black circles indicate cells grown under +P and -P conditions, respectively. (d) Pi contents measured in cells grown under +P conditions (0 h), and in ones grown further for 48 h under +P and -P conditions. (e) Images of the cells emitting red auto-fluorescence of Chl and/or the green fluorescence of SYTOX bound to chromosomal
DNA. The survival ratio of the cells was determined through estimation of the proportion of the number of viable cells emitting only red fluorescence, relative to that of total cells, including non-viable cells with emission of green fluorescence only and with that of both red and green fluorescence. The values shown are averages ± SEM for two technical replicates for each of four biological replicates (a-c), and those for three biological replicates (d, e). Data were analyzed by one-tailed Student’s t-test.

![Figure 3](image)

**Figure 3**

Lipid remodeling in C. kessleri cells in response to -P stress. (a) Qualitative TLC analysis of individual polar lipids prepared from cells grown under +P conditions (0 h), and from ones shifted to -P conditions for further growth for 24, 48, and 72 h. (b) Two-dimensional TLC profile of polar lipids in the cells grown for 48 h under +P or -P conditions. (c) Polar lipid composition in the cells grown for 48 h under +P (white bars) or -P conditions (black bars). (d) The contents of Pi included in individual phospholipids, relative to that in total cellular fraction. The values shown are averages ± SEM for two technical replicates for each of two biological replicates (c), and those estimated on the basis of the data in Fig. 2d and Fig. 3c (d). Data were analyzed by one-tailed Student’s t-test.
Figure 4

Identification of a -P-induced novel lipid as DGTS through LC/MS2 analysis. ESI mass spectra as to a novel lipid of C. kessleri (a) and DGTS of C. reinhardtii (b). Product ion spectra (c), (d), and (e), as to m/z 737 in (a), m/z 735 in (b), and m/z 761 in (a), respectively.
Figure 5

Fatty acid composition of individual polar lipids. The fatty acid compositions of MGDG (a), DGDG (b), SQDG (c), and PG (d) are shown for 48-h grown +P (black bars) and -P cells (white bars), whereas those of PC (e) and PE (f), and DGTS (g) are for +P and -P cells, respectively. The fatty acid composition of sn-2 monoacyl lysoDGTS is shown in (h). The values shown are averages ± SEM for two technical replicates for each of two biological replicates. Data were analyzed by one-tailed Student's t-test.
Figure 6

Metabolic mechanism of DGTS accumulation under -P conditions. Changes in the contents of DGTS (white), PC (grey), and PE (black) in cells grown for 12 h after a shift from +P to -P conditions, which were estimated in cells on an OD730 ml culture basis (a), and in the culture (b). (c) Effects of metabolic inhibitors or light conditions on the accumulation of DGTS in the cells shifted to -P conditions. The values were estimated as the proportion of the DGTS content in the cells with application of chloramphenicol (CAP), cycloheximide (CHI), cerulenin, and DCMU, and in those shifted to the dark conditions, relative to that in non-treated control cells. The values shown are averages ± SEM for three biological replicates. Data were analyzed by one-tailed Student's t-test.
Figure 7

Structural and functional identification of a BTA1 homolog in C. kessleri. (a) Alignment of the postulated amino acid sequence of a BTA1 homolog with that of CrBTA1. Identical and similar amino acid residues are shadowed in black and grey. The vertical red line indicates the boundary of the N-terminal BtaB- and C-terminal BtaA-like domains, whereas two black boxes shows the conserved dipeptide VD for the binding of SAM, the substrate in the respective catalytic actions of BtaA and BtaB. (b) The structure of the of the
BTA1 homolog of C. kessleri. The BTA1 homolog is composed of 18 exons on the nuclear genome. (c) Expression of cDNA of the BTA1 726 homolog in E. coli. SDS PAGE of total cellular proteins shows induction of the expression of the homolog protein in pMAL-CkBTA1 introduced E. coli cells, but not in empty-vector introduced ones. TLC of total cellular lipids shows the appearance of a novel lipid specifically in pMAL CkBTA1 introduced E. coli cells. (d) The ESI mass spectrum of the novel lipid in the transformant of E. coli. (e) The product ion spectrum of m/z 739 in (d). Note that the product ion spectrum exhibited three signals (m/z 144, 162 and 236) for identification of the novel lipid as DGTS.
Figure 8

Phylogenetic trees of the BtaA and BtaB domains in BTA1 proteins. (a) Bacterial BtaA and eukaryotic BtaA-domains. (b) Bacterial BtaB and eukaryotic BtaB-domains. Two linked boxes, AB and BA, indicate eukaryotic A and B type BTA1 proteins, respectively, whereas single boxes, A and B, demonstrate bacterial BtaA and BtaB proteins, respectively.

Figure 9
Regulatory expression of the genes for -P induced lipid remodeling in C. kessleri. The expression levels of the BTA1, PLCC1, PLCC2, and PLP genes were investigated through semi-quantitative RT-PCR analysis in C. kessleri cells before and after a shift to -P conditions. The intensities of the DNA bands that correspond to mRNAs of the individual genes were used for determination of the values, relative to that of ACT. The values shown are averages ± SEM for three biological replicates. Data were analyzed by one-tailed Student’s t-test.

**Supplementary Files**

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