

Characterization of Glycolipids in Light-Harvesting Chlorosomes from the Green Photosynthetic Bacterium *Chlorobium tepidum*

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Glycolipids were isolated from extramembraneous light-harvesting complexes, called "chlorosomes," in a thermophilic green photosynthetic bacterium *Chlorobium tepidum*. The lipids were first characterized in terms of their precise structures of both hydrophilic (saccharides) and hydrophobic (fatty acids) moieties by means of ^{1}H and ^{13}C NMR spectroscopy as well as high-performance liquid chromatography coupled with an evaporative light-scattering detector and an electron spray ionization mass spectrometer. The results clearly demonstrated that glycolipids having a disaccharide group, rhamnosylgalactosyldiacylglycerides, were the majority in the total glycolipid component of the chlorosomes, in addition to the second major glycolipids with a monosaccharide group, monogalactosyldiacylglycerides, which had been believed to be predominant before the report by Miller and her co-workers (*Photosynth. Res.* **2008**, *95*, 191–196). We also found that these glycolipids had methylene-bridged palmitoleyl (=(*Z*)-9-hexadecenoyl) (17:1) and palmitoyl groups (16:0) as their predominant acyl chains on the glycerol moiety. By using enzymatically site-specific hydrolysis of the acyl chain at the sn-1 position on the glycerol, the modified palmitoleyl group was determined to be esterified at the sn-1 position. These unique glycolipids might play an important role in maintaining fluidization of chlorosomal envelopes and achieving thermal stability of chlorosomes at 45 °C.

The basic unit of biomembranes is a bilayer formed by (glycero)phospholipids and sphingolipids. In the bilayer membranes, the hydrophilic moieties of lipids are facing the outside of the membranes and their acyl chains constitute the hydrophobic domain. In most photosynthetic organisms, functional pigment–protein complexes including light-harvesting and reaction-center complexes are embedded in the lipid bilayer as integral membrane proteins, and they closely interact with each other for efficient conversion of light to chemical energy.[1]

In sharp contrast to the above pigment–protein complexes, green photosynthetic bacteria have unique extramembraneous light-harvesting complexes called "chlorosomes."[2–5] Chlorosomes are micelle-like nanoarchitectures attached to the cytoplasmic side of membranes. The core part is made up of light-absorbing bacteriochlorophyll(BChl)-*c*, -*d*, or -*e* molecules, depending on species, in their highly ordered self-aggregates, while the envelope is thought to be a monolayer of mostly glyco/phospholipids containing proteins.[6,7]

The extramembraneous chlorosomes are believed to be an excellent antenna system in terms of capturing photons with extremely low light irradiation, since a green sulfur bacterium *Chlorobium* (*Chl.*) *phaeobacteroides* species is photosynthetically alive in a deep sea (ca. 80 m).[8] In particular, another green bacterium found in a hydrothermal vent at a deeper sea (ca. 2000 m) is assumed to use dim light from geothermal illumination for photosynthesis.[9] Chlorosomes are also suitable for preparation of an artificial antenna system, since they are constructed on a simple structural principle, i.e., self-aggregation of antenna pigments (BChls-*c*/*d*/*e*) in the micelle-like lipid monolayer without any assistance of proteins.[10] Concerning the chlorosomal core, there are numerous studies on structures, functions, and applications of self-aggregation of pigments both in vivo and in vitro (see Refs. 11–14 for comprehensive reviews). However, the details of chlorosomal envelopes are lagging behind, although it is essential to produce hydrophobic environments for construction of the antenna core by self-assembly of photosynthetic pigments.

The envelopes of chlorosomes contain BChl-*a* molecules associated with a specific protein, CsmA.[15–17] The BChl-*a* molecules mediate excitation energy from chlorosomes to other antenna complexes including B808-866 antenna in non-sulfur bacteria and Fenna–Matthews–Olson proteins (referred to as FMO proteins) in sulfur bacteria. It had been believed that glycolipids having a monosaccharide group, monogalactosyldiacylglycerides (MGDGs), were predominant in the total glycolipids of the envelopes.[6] Miller and co-workers recently reported that a significant amount of glycolipids with a disaccharide group, rhamnosylgalactosyldiacylglycerides (RGDGs), were also present in the chlorosomes from *Chl. tepidum* using GC-MS after derivation of the extracted lipid component.[7] However, the molecular structures and compositions of the MGDG and RGDG having a variety of fatty acids were not fully characterized.

In this study, we extracted and purified glycolipids from the chlorosomes of a thermophilic green bacterium, *Chl. tepidum*, and analyzed them as the intact forms in detail by means of NMR spectroscopy as well as high-performance liquid chromatography (HPLC) coupled with an evaporative light-scattering detector (ELSD) and electron spray ionization (ESI) mass

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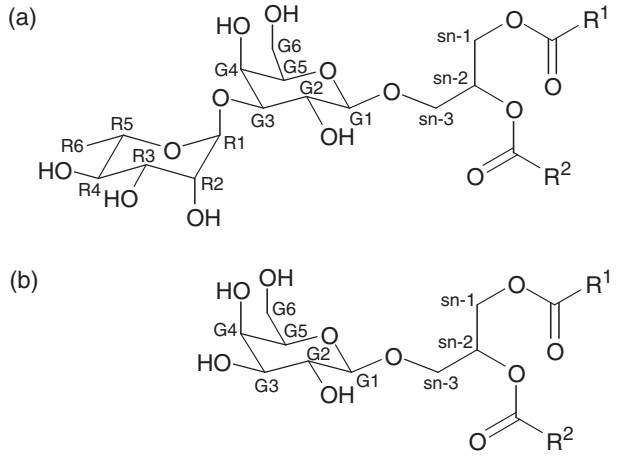


Figure 1. Molecular structures of RGDG (a) and MGDG (b) found in the chlorosomes from *Chl. tepidum* together with the numbering used in this study. Hydrocarbons in the acyl chains at the sn-1 and sn-2 positions are indicated by R^1 and R^2, respectively (the details are shown in Figure 2).

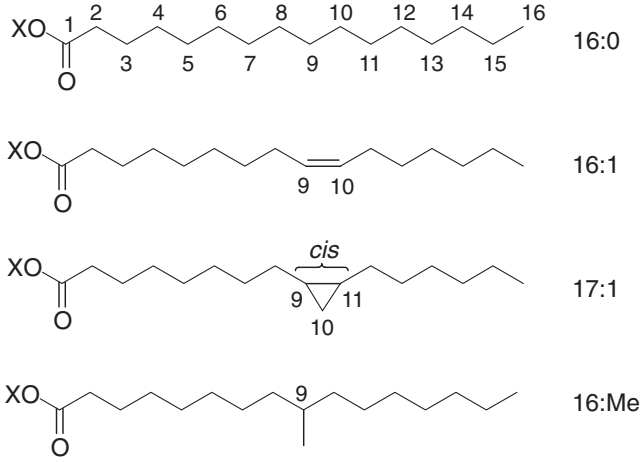


Figure 2. Structures of the acyl chains in glycolipids together with the numbering used in this study. The replacement of X with a hydrogen atom gives the fatty acids: palmitic, palmitoleic, methylene-bridged palmitoleic, and 9-methylated palmitic acids from top to bottom.

spectrometry. Knowledge of chlorosomal envelopes at the molecular level would enable us to understand both fundamental and applicable aspects of the unique light-harvesting properties of green photosynthetic bacteria.

Results and Discussion

Molecular Structures of Glycolipids, RGDG and MGDG. Structures of Saccharides: Figure 1 depicts molecular structures of glycolipids investigated here together with the carbon numbering: RGDG (a) and MGDG (b). Here, their two hydrocarbons in the acyl chains at the sn-1 and sn-2 positions on the glycerol moiety are indicated by R^1 and R^2, respectively. Before the report by Miller and her co-workers,[7] it had been believed that MGDG with a monosaccharide group is predominant in the total polar lipids of light-harvesting chlorosomes. Based on GC-MS analysis after derivation of the lipid component extracted from a thermophilic green photosynthetic bacterium *Chl. tepidum*, they suggested that a significant amount of RGDG with a disaccharide group was present in the chlorosomes in addition to MGDG. They also estimated that the glycolipids, MGDG and RGDG, comprised more than half of polar lipids (55%) in the chlorosomes (phospholipids made up about 30% of them). However, the details of the molecular structure of RGDG, especially for the linkage between rhamnosyl and galactosyl groups, were not mentioned. According to agglutination experiments of chlorosomes with a lectin,[18] the saccharide moieties in glycolipids are facing the outside of the envelopes and might assist the connection of chlorosomes with the cytoplasmic side of membranes.

Precise Structures of Fatty Acids: Figure 2 depicts molecular structures of acyl chains in chlorosomal RGDG and MGDG from *Chl. tepidum* investigated here together with the carbon numbering (replacement of X with a hydrogen atom corresponds to fatty acids). There are few reports on the precise structures and composition of hydrocarbons of the two acyl chains in chlorosomes; this is mostly due to difficulty in detecting lipids in their intact form on chromatographic separation. To overcome this problem, various derivation techniques have been used. For example, Miller and her co-workers reported the distribution of fatty acids in glycolipids from the chlorosomes of *Chl. tepidum* by derivation of the corresponding methyl esters.[7] The results showed that distribution in RGDG and MGDG was quite similar and they mainly have saturated palmitic acid (16:0) as shown in the top of Figure 2. Only a minor amount of the unsaturated fatty acids having 16 and 17 carbon atoms were reported: palmitoleic (=(*Z*)-9-hexadecenoic) (16:1), methylene-bridged palmitoleic (17:1), and 9-methylated palmitic (16:Me) acids.

Extraction and Purification of Glycolipids. In this study, we used an ELSD as a detector of HPLC-separated glycolipids. Various derivation techniques have been used to detect lipids, but such chemical modifications are likely to add complexity and additional preparation steps to the analysis. ELSD can be applied to detect all compounds including molecules lacking any chromophores in a molecule without their derivatization.[19] ELSD is based on the generation of particles from the eluent of the HPLC column via heated nebulization and light-scattering of the resulting particles to produce a signal. ELSD responds only to number of particles and gives a nonlinear response to analytes. Thus, this must be taken into account for their quantitative analysis, although it can be semiquantitatively used (vide infra).

Figure 3a shows a representative ELSD–HPLC profile of glycolipids, which were extracted from the chlorosomes of *Chl. tepidum* and purified by silica gel column chromatography. We can see clearly two sets of peaks denoted as #1–#4 and #1′–#4′. Peak #3 was not clearly seen in the profile, but it could be detected by a photodiode array (PDA) detector using benzoate derivatives (vide infra). These peaks were safely assigned to glycolipids having different hydrophilic and hydrophobic moieties (RGDG (#1–#4) and MGDG (#1′–#4′)) on the basis of online ESI-MS (vide infra). The profile also indicates that the glycolipids obtained by silica gel column chromatography as mentioned in *Experimental* were sufficiently pure and

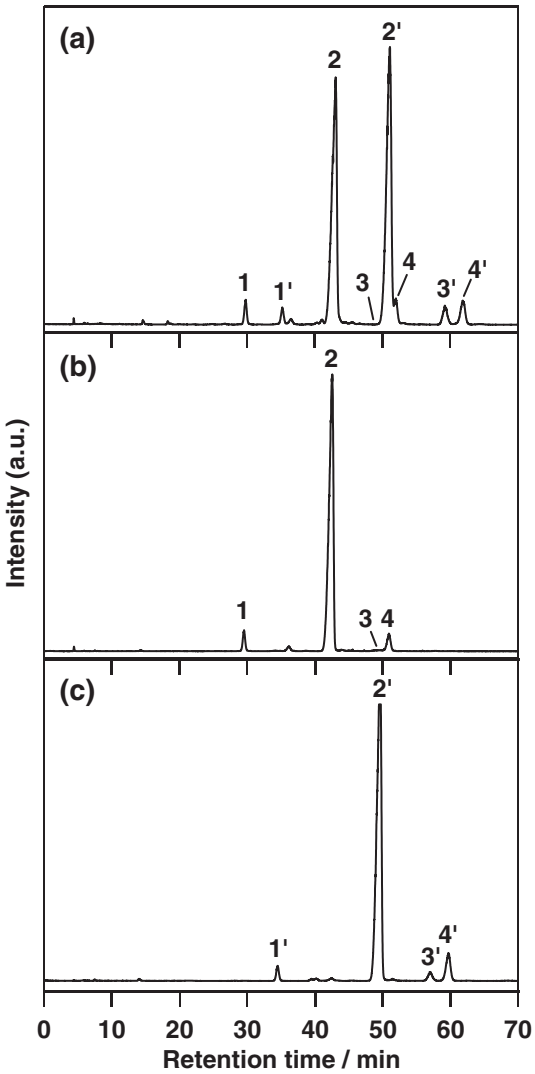


Figure 3. Representative HPLC profiles of the glycolipids extracted from chlorosomes detected by ELSD: a mixture of glycolipids before fractionation (a), and the isolated RGDG (b) and MGDG (c).

without contamination by phospholipids and/or waxes, and were adequate for further structural analysis (We also measured ^{31}P NMR spectra of the glycolipid fraction, but no ^{31}P signals originating from phospholipids were observed.).

Figures 3b and 3c show reverse-phase HPLC profiles of the isolated RGDG and MGDG by further silica gel column chromatographic separation of the above mixed glycolipids, respectively. As compared with the HPLC profile of their mixture (Figure 3a), RGDG and MGDG were well fractionated by the silica gel column chromatography, although the glycolipid components with different acyl chains were not isolated in the present separation on a silica gel column. The peak #2 (#2′) was seen to be dominant in a RGDG (MGDG) mixture, which was confirmed by its derivation. Using perbenzoate derivatives of the RGDG and MGDG, peak #2 (#2′) was estimated to be 75.8% (71.6%) of the total RGDG (MGDG) components (vide infra).

The present preparation method is suitable for isolation of RGDG, MGDG, and their mixtures from chlorosomes. We also examined the distribution of glycolipids extracted from the whole cells of *Chl. tepidum*, and found the results are compatible with those obtained from the isolated light-harvesting chlorosomes (data not shown). The observation indicates that their distribution in other membranes of green bacteria is almost the same as that of chlorosomes and/or that most of the glycolipids are present in chlorosomes.

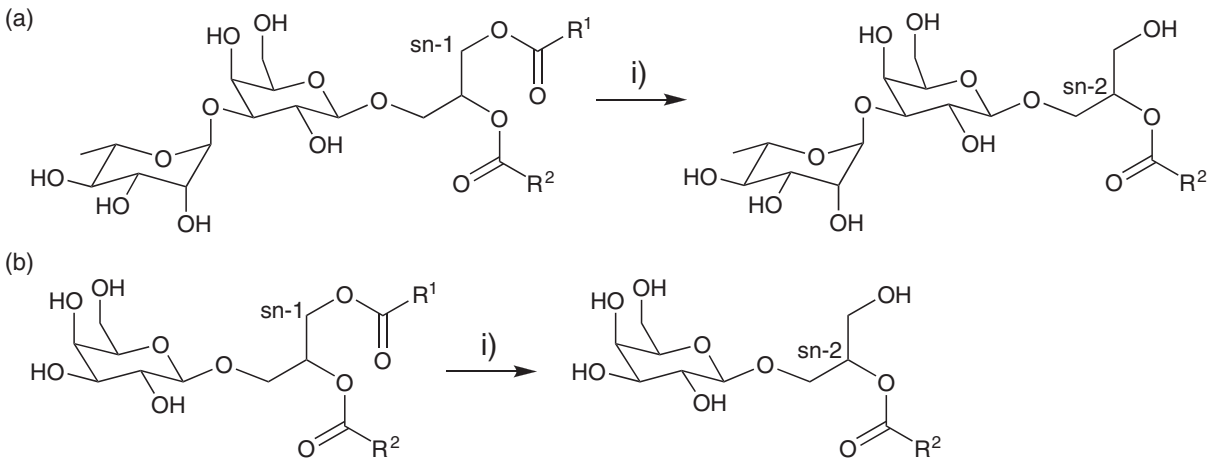
ESI-LCMS Analysis of Glycolipids. To identify the glycolipids resolved into eight components by HPLC, we used online ESI-MS. Table 1 lists the results of mass spectrometry of the two sets of glycolipids, #1–#4 and #1′–#4′. The molecular ion and fragment ions can be used to identify each glycolipid component in terms of the hydrophilic saccharide and hydrophobic acyl groups. Glycolipids #1–#4 gave peaks at $m/z = 878.7$, 906.5, 894.8, and 908.7 corresponding to their ammonium adducts ($[M + NH_4]^+$), and 535.5, 563.5, 551.4, and 565.5 corresponding to the removal of their saccharides ($[M1 + H]^+$): see also Figures S1 and S2 in Supporting Information for the fragmentation patterns of glycolipids and representative ESI-MS spectra of the two major peaks #2 and #2′. The differences between $[M + NH_4]^+$ and $[M1 + H]^+$ in peaks #1–#4 as shown in the parentheses of Table 1 gave identical values (343 Da), indicating that these glycolipids have a rhamnosylgalactosyl moiety ($326 + NH_3$) as their common saccharide. The further fragmented ions by the cleavage of an acyl chain at the sn-1 or sn-2 position were used to identify the fatty acids in the glycolipids. For example, peak #2 showed these ions at $m/z = 325.3$ and 313.3 denoted as $[M2 + H]^+$ and $[M2' + H]^+$, respectively. These observed values were consistent with those calculated for fragment species attached to methylene-bridged palmitoleyl (17:1) and palmitoyl (16:0) groups, respectively, although the hydrocarbon chain of the acyl groups at the sn-1 and sn-2 positions could not be identified due to there being two possible fragmentation patterns as shown in Figure S1. Glycolipids #1′–#4′ were similarly assigned to MGDG. These had a galactosyl moiety as the common saccharide because of the differences between $[M + NH_4]^+$ and $[M1 + H]^+$ ($180 + NH_3$). As the unsaturated structures in the acyl chains of peaks #2 and #2′, it could not be determined whether these glycolipids had a cyclopropane ring or an olefinic C=C double bond, since their molecular weight was identical. Similarly, concerning peaks #4 and #4′, it could not be characterized whether these had (16:Me, 16:0) or (17:0, 16:0) as their fatty acids; they were distinguished by the following NMR (vide infra). The MS analysis did not clarify the following regio(stereo)isomeric points: (1) the positions of two acyl chains at the sn-1 and sn-2 positions (R^1 at the sn-1 and R^2 at the sn-2 or R^2 at the sn-1 and R^1 at the sn-2), (2) the unsaturated structure and its position in the hydrocarbon chain, and (3) the linkage between rhamnosyl and galactosyl groups in RGDG. Therefore, we used site-specific hydrolysis reaction to solve (1) and NMR spectra for (2) and (3).

Structural Determination of the Isolated RGDG and MGDG. Site-Determination of Acyl Groups on Glycerol: To determine the acyl chains at the sn-1 and sn-2 positions on the glycerol moiety, we used site-specific hydrolysis with an enzyme, lipase. According to the method reported by Murakami et al.,[20] the hydrolysis was strongly dependent upon the solvent system used: in a buffer containing boric acid and

Table 1. Mass Spectrometric Data for the Intact Glycolipids Extracted from the Chlorosomes of *Chl. tepidum* and the Two Major Hydrolyzed Lyso Derivatives

HPLC peak #	Glycolipids	Observed peaks				Calculated values for $[M + X]^+$			
		$[M + NH_4]^+$	$[M1 + H]^+$ a)	$[M2 + H]^+$	$[M2' + H]^+$	$[M + NH_4]^+$	$[M1 + H]^+$	$[M2 + H]^+$	$[M2' + H]^+$
1	RGDG(16:1,15:0)	878.7	535.5 (343.2)	—b)	—b)	878.62	535.46	311.25	299.25
2	RGDG(17:1,16:0)	906.5	563.5 (343.0)	325.3	313.3	906.65	563.50	325.27	313.27
3	RGDG(16:0,16:0)	894.8	551.4 (343.4)	313.0		894.65	551.50	313.27	313.27
4	RGDG(16:Me,16:0)	908.7	565.5 (343.2)	327.1	313.1	908.63	565.51	327.28	313.27
1′	MGDG(16:1,15:0)	732.6	535.5 (197.1)	—b)	—b)	732.56	535.46	311.25	299.25
2′	MGDG(17:1,16:0)	760.5	563.5 (197.0)	325.3	313.3	760.59	563.50	325.27	313.27
3′	MGDG(16:0,16:0)	748.5	551.4 (197.1)	313.0		748.59	551.50	313.27	313.27
4′	MGDG(16:Me,16:0)	762.6	565.5 (197.1)	327.1	313.1	762.61	565.51	327.28	313.27
	Lyso-RGDG(16:0)	656.5			313.3	656.42			313.27
	Lyso-MGDG(16:0)	510.5			313.3	510.36			313.27

a) Values in parentheses indicate difference between $[M + NH_4]^+$ and $[M1 + H]^+$. b) Not detected.



Scheme 1. Site-selective hydrolysis of the isolated RGDG (a) and MGDG (b) at the sn-1 position using lipase: i) boric acid and tetraborate decahydrate (pH 7.7) at 38 °C.

tetraborate decahydrate (pH 7.7), the reaction occurred site-specifically at the sn-1 position, while, in a tris(hydroxymethyl)aminomethane (=2-amino-2-(hydroxymethyl)-1,3-propanediol)–HCl (Tris) buffer (pH 7.7), the reaction occurred at the sn-2 position. In this study, we used the former solvent system in order to obtain sn-2 acyl lyso glycolipids as shown in Scheme 1. A mixture of RGDG (MGDG) having different acyl chains was hydrolyzed at the sn-1 ester. The products containing mixed sn-2 acyl lyso-RGDG (MGDG) were directly analyzed by ESI-LCMS similarly to the intact RGDG (MGDG). The major lyso-RGDG (MGDG) prepared from the RGDG #2 (MGDG #2′) was identified as lyso-RGDG(16:0) (lyso-MGDG(16:0)) shown in Table 1. Based on the results, the methylene-bridged palmitoleyl (17:1) and palmitoyl (16:0) groups were unambiguously connected at the sn-1 and sn-2 positions, respectively, of both RGDG #2 and MGDG #2′ (see the Graphic Abstract).

Structural Determination of Hydrophilic Saccharides in RGDG: Figure 4 shows the NMR spectra of RGDG (a) and MGDG (b) in the two parts from 6.2 to 5.6 and from 4.9 to 3.9 ppm, where characteristic ^{1}H signals of the saccharide as well as glycerol moieties of the glycolipids appear. Each signal was assigned by 2D NMR experiments. The observed 2D-correlations between relevant atoms are summarized in Table 2 (see also Experimental). The sequential correlations among the rhamnosyl or galactosyl group in the COSY spectrum established the assignments. Typically, the signal of a methyl group in the disaccharide is also shown in the inset of Figure 4a, indicating that one of the two saccharide moieties is a 6-deoxoglucoside derivative.

Based on the vicinal couplings of saccharides,[21] we reconfirmed that the disaccharide moiety of the present isolated glycolipids unambiguously consisted of rhamnosyl and galactosyl groups. The coupling constants among the relevant atoms indicate their relative orientation. For examples, R2- and R4-protons in the rhamnosyl group gave the coupling constants $^3J_{HH} = 3.0$ and 10.2 Hz, respectively. These observations strongly supported that the R2- and R4-protons were situated at the equatorial and axial positions, respectively, since the corresponding axial- and equatorial-oriented epimer, fucose, gave the opposite values, $^3J_{HH} \approx 10$ and 1 Hz.[22,23] Similarly, the stereochemistry of G4-proton in RGDG ($^3J_{HH} = 3.0$ Hz) was confirmed to be equatorial: the corresponding axial epimer, glucose, gave a larger value ($^3J_{HH} \approx 10$ Hz).[24]

Furthermore, the relative orientation at the anomeric proton of the galactosyl group in RGDG was determined to be the β-

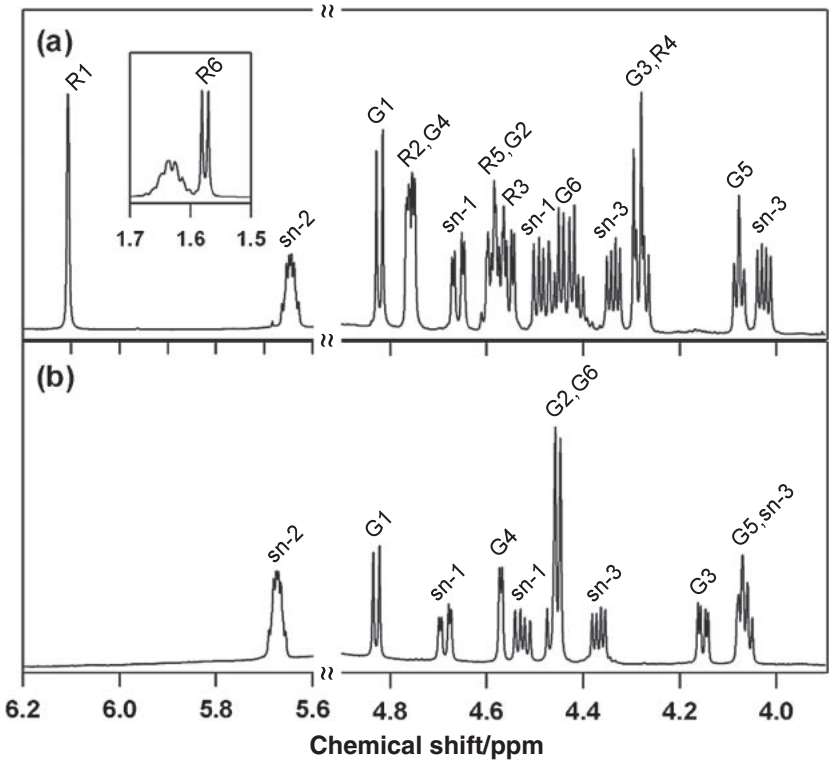
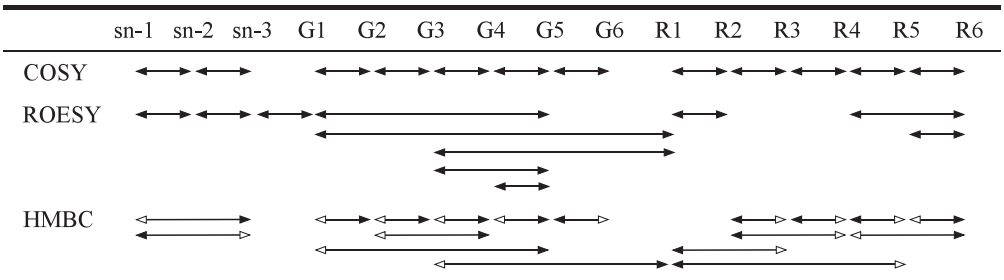


Figure 4. Partial ^{1}H NMR spectra of the isolated RGDG (a) and MGDG (b) in the range from 6.2 to 5.6 and from 4.9 to 3.9 ppm in pyridine-d_5 at room temperature. Insert of (a): the spectrum of RGDG from 1.7 to 1.5 ppm.

Table 2. 2D NMR Correlations among the Relevant ^{1}H and ^{13}C Signals of RGDG in Pyridine-d_5 at Room Temperature[a)]



a) ^{1}H and ^{13}C atoms are indicated by closed and open arrow-heads, repectively. See position numbers in Figure 1a.

configuration, based on the coupling constant between G1- and G2-protons ($^{3}J_{HH} = 7.8$ Hz).[24] The anomeric orientation of the rhamnosyl group was also determined to be the α-configuration based on the one-bond C–H coupling between R1-proton and R1-carbon ($^{1}J_{CH} = 169.8$ Hz, see also Figure S3), since the $\alpha(\beta)$-anomeric configuration gave it at $^{1}J_{CH} = 170$ (160) Hz.[21] Such a one-bond coupling constant ($^{1}J_{CH}$) is one of the reliable criteria to determine conclusively the anomeric configuration of a saccharide having almost identical three-bond ^{1}H–^{1}H coupling values for both anomers. ^{13}C-chemical shifts of saccharides are the other criterion to confirm their relative configuration. We compared the values of RGDG isolated in this study with those reported for other glycolipids. The rhamnosyl (galactosyl) group in RGDG exhibited the almost identical values with those reported for the lipids containing a rhamnosyl (galactosyl) group, except for the value of connected G3-carbon in the galactosyl group. The details were summarized in Table S1.

To determine the linkage between rhamnosyl and galactosyl groups in RGDG unambiguously, we used its ^{1}H–^{13}C HMBC spectrum. The HMBC correlation between G3-carbon and R1-proton established the linkage at the G3 and R1 positions (Figure S3 shows the partial HMBC spectrum of the isolated RGDG in pyridine-d_5 at room temperature). Compared with the spectra of the structurally well-defined MGDG as shown in Figure 4b,[24] the ^{1}H signals at the G2-, G3-, and G4-positions of RGDG were shifted to the lower field, whereas the other signals (G1, G5, and G6) exhibited almost identical values (a typical deshielding of glycosidation). The results also support the linkage between rhamnosyl and galactosyl groups at the R1

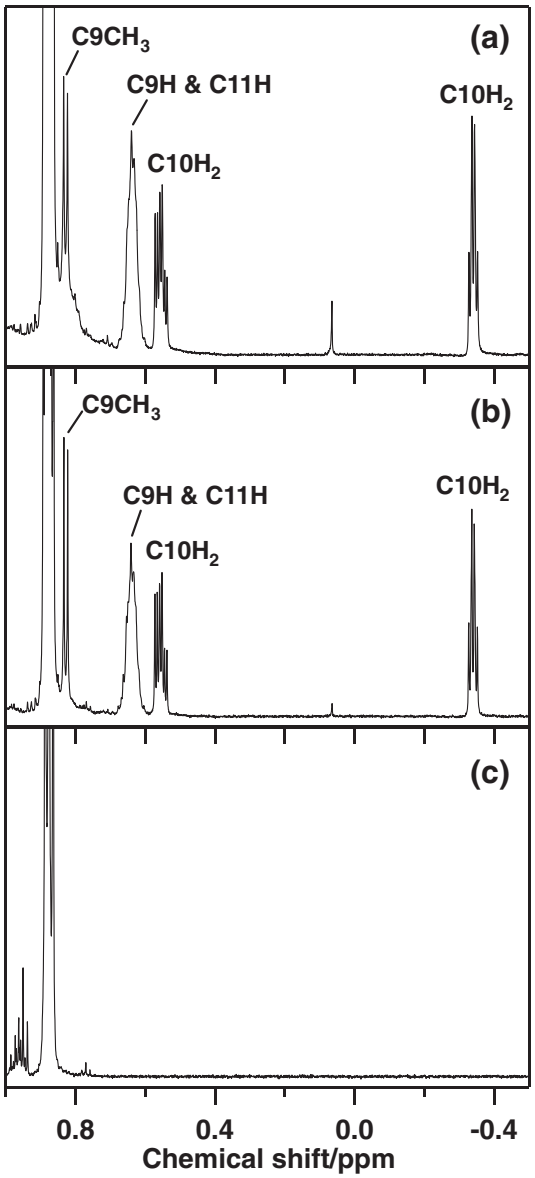


Figure 5. Partial ^{1}H NMR spectra of the isolated RGDG (a), MGDG (b), and the authentic saturated MGDG(18:0,16:0) (c) in the range from 1.0 to −0.5 ppm in pyridine-d_5 at room temperature.

and G3 positions. Consequently, the structure of the newly isolated disaccharide from *Chl. tepidum* was determined to be rhamnose-$\alpha(1 \rightarrow 3)$–galactose-β.

Determination of Unsaturated Structures of Acyl Chains: Figure 5 shows the ^{1}H NMR spectra of the isolated RGDG (a) and MGDG (b) in the range from 1.0 to −0.5 ppm, together with authentic saturated MGDG(18:0,16:0) having stearyl and palmitoyl moieties (c). Compared with the spectrum of the authentic MGDG, both RGDG and MGDG exhibited characteristic, high-field-shifted signals at −0.33, 0.55, and 0.64 ppm. According to the reported signals of lipids having a unique cyclopropane ring,[25] these high-field-shifted ^{1}H signals are assigned to cyclopropane protons. The sequential correlations among them were also confirmed by COSY spectra as C9H ↔ $C10H_2$ and $C10H_2$ ↔ C11H (Figure 2). Moreover, the chemical shift values of these protons indicated that C9H and C11H took *cis*-configuration (the corresponding *trans*-conformer gave more high-field-shifted values).[25] The position of the unique cyclopropane ring in the acyl chain at the sn-1 position could not be determined in the present study, but the position of a C=C double bond in the 16:1 acyl component was generally reported to be at the C9 position as the palmitoleyl group. The methylene group would add to the palmitoleyl C9=C10 to yield the modified palmitoleyl group, i.e., methylene-bridged palmitoleyl group as shown in Figure 2.

The presence of the branched C16 methyl structure (16:Me) in the acyl chains (the bottom of Figure 2) of each isolated RGDG and MGDG mixture was simultaneously confirmed on the basis of characteristic doublet methyl signals at 0.83 ppm in ^{1}H NMR spectra shown in Figures 5a and 5b.

Unique Glycolipid, RGDG(17:1,16:0), Was Predominant in a Thermophilic Green Photosynthetic Bacterium. HPLC profiles obtained by ELSD cannot exhibit the linear response between peak areas and concentrations of analytes. To characterize the glycolipids quantitatively, we first used the integration of the intrinsic signals of RGDG and MGDG in ^{1}H NMR. The spectrally separated R1 signal of RGDG and G3 signal of MGDG were used in order to estimate the relative ratio of RGDG to MGDG in the isolated glycolipid mixture as shown in Figure 3a. It gave the ratio of 1.3 to 1.0. Thus, RGDG was slightly more abundant in the total glycolipids of the chlorosomes from *Chl. tepidum*. This was almost consistent with the data reported by Miller and her co-workers by GC analysis after derivation of the lipids component (RGDG:MGDG = 1.9:1.0).[7]

To clarify the distribution of fatty acids in each class of glycolipids quantitatively, we modified the isolated RGDG and MGDG into their derivatives having perbenzoate substituents, and analyzed them using PDA based on the absorption of the benzoate. The composition of each peak obtained by PDA (ELSD) was estimated as follows: peaks #1–#4 = 8.0 (2.4):75.8 (92.7):1.9 (0.0):14.3 (4.9), and peaks #1′–#4′ = 3.1 (2.0):71.6 (90.5):4.0 (0.2):21.3 (7.3). The small peaks detected by ELSD were underestimated in the present analyses. The details are summarized in Figures S4 and S5 of Supporting Information. The two major peaks #2 in RGDG derivatives and #2′ in MGDG derivatives were estimated to be 75.8 and 71.6%, respectively. Comparing relative ELSD peak areas of intact and modified RGDG (Figures 3b and S4a) with those of the corresponding MGDG (Figures 3c and S5a), the composition of acyl chains in the intact RGDG and MGDG must be similar. This indicates that unique RGDG should be biosynthesized by transferring an α-rhamnosyl group to the G3 position of MGDG.

Concluding Remarks

We identified two sets of glycolipids from a green photosynthetic bacterium *Chl. tepidum* in their intact forms. The major RGDG and MGDG among them had methylene-bridged palmitoleyl (17:1) and palmitoyl (16:0) groups at the sn-1 and sn-2 positions, respectively, over 70% of each class of glycolipids. The disaccharide structure of the newly isolated RGDG was determined to be rhamnose-$\alpha(1 \rightarrow 3)$–galactose-β. The composition of fatty acids in RGDG and MGDG was

almost identical, so the biosynthesis of RGDG would occur via MGDG by the attachment of an α-rhamnosyl group at the G3 position. Consequently, a thermophilic green photosynthetic bacterium biosynthesized unique disaccharide-RGDG and monosaccharide-MGDG having a methylene-bridged palmitoleyl group containing a cyclopropane ring as the predominant glycolipids. The unique natural selection of glycolipids might regulate the fluidization of chlorosomal envelopes of *Chl. tepidum* at 45 °C (an optimized culturing temperature), thus efficiently achieving an excitation energy transfer from a chlorosome to a FMO protein via a baseplate protein embedded in the envelope.

Experimental

General. All solvents were used without further purification, except for dry pyridine for preparation of benzoate derivatives of the isolated RGDG and MGDG. Standard MGDG having stearic and palmitic acids (18:0, 16:0) was purchased from Matreya (Pennsylvania, USA). The details in preparation of the perbenzoate derivatives are shown in the Supporting Information.

Culturing of a Green Photosynthetic Bacterium and Preparation of Its Main Light-Harvesting Complexes, Chlorosomes. *Chl. tepidum* strain ATCC49652 was cultured at 45 °C under anaerobic-light conditions (30 μmol m^{-2} s^{-1}) as described previously,[26] after its single colony isolation. The extramembraneous light-harvesting complexes called chlorosomes were prepared from the harvested cells in a mixture of 2 M sodium thiocyanate, 10 mM potassium phosphate (pH 7.4), and 10 mM sodium ascorbate by the method of Gerola and Olson.[27] The fraction, which banded at 15% (wt/vol) sucrose after ultracentrifugation, was used without further purification.

Extraction and Purification of Glycolipids from Chlorosomes. Glycolipids were extracted from the isolated chlorosomes by the method of Bligh and Dyer with slight modification.[28] Briefly, the aqueous chlorosomes in 50 mM Tris buffer (pH 8.0) were homogenized with a mixture of chloroform, methanol, and 100 mM acetic acid (1:2:0.8 (v/v/v)). The suspension was filtered and then centrifuged for 10 min at 1880 × *g*. The bottom layer was collected and concentrated in vacuo. The extract including glycolipids as well as phospholipids, waxes, and photosynthetic pigments was purified by silica gel chromatography (Wakogel C-300, Wako Pure Chemical Industries, Osaka, Japan). To remove photosynthetic pigments (carotenoids and chlorophylls) and waxes, chloroform was used as an eluent. Then, acetone was used in order to elute the glycolipid component. The resultant glycolipids, a mixture of MGDG and RGDG, were fractionated into each class of glycolipids by the second run of chromatography with a mixture of acetone and chloroform (3:7 (v/v)).

Analysis of Glycolipids in Chlorosomes by LCMS. LCMS of the extracted and/or fractionated glycolipids was performed using a Shimadzu LCMS-2010EV system (Shimadzu, Kyoto, Japan) comprising a liquid chromatograph (SCL-10Avp system controller and LC-10ADvp pump) and a quadrupole mass spectrometer equipped with an ESI probe as described previously.[29] To detect and characterize glycolipids, we used the flow separator at the ratio of 3:7 (v/v): one (30%) is connected to an ESI probe and the other (70%) to an ELSD (ELSD-LTs, Shimadzu). HPLC was performed under the isocratic reverse-phase conditions: column, Cosmosil 5C18-AR-II (4.6 × 250 mm, Nacalai Tesque, Kyoto); eluent, a mixture of acetone and aqueous 25 mM ammonium acetate (pH 6.3) = 85:15 (v/v); flow rate, 0.45 mL min^{-1}. ESI-MS spectrometry was carried out at the positive ion mode.

Site-Specific Hydrolysis of Glycolipids by Lipase. The acyl chain at the sn-1 position of the glycerol moiety was site-specifically hydrolyzed using a commercially available lipase (*Rhizopus arrhizus*, Sigma-Aldrich, St. Louis, USA) as described previously.[20] Briefly, the isolated RGDG (MGDG) fraction and lipase were stirred at 38 °C in a mixture of boric acid and sodium tetraborate decahydrate (pH 7.7) for 30 min. After addition of acetonitrile, the mixture was concentrated in vacuo. The resultant sn-2 acyl lyso RGDG (sn-2 acyl lyso MGDG) was directly characterized by the above LCMS.

NMR Measurements of the Isolated RGDG and MGDG. ^{1}H and ^{13}C NMR spectra of the isolated RGDG and MGDG fractions were measured by a JEOL ECA-600 NMR spectrometer in pyridine-d_5 (Euriso-top, Saclay, France) at room temperature: as internal references, undeuterated *meta*-proton and deuterated *meta*-carbon-13 of the solvent molecule were used at 7.19 and 123.5 ppm, respectively. ^{1}H signals of glycolipids were assigned using ^{1}H–^{1}H COSY and ROESY (τ_m = 300 ms) spectra, except for methylene protons in acyl chains. The ^{13}C signals were also assigned using DEPT as well as ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC ($^{n}J_{CH}$ = 12 Hz) spectra, except for methylene carbons in acyl chains. Hydroxy groups in both RGDG and MGDG were not identified due to the fast exchange with trace contaminant H_2O.

Rhamnosylgalactosyldiacylglyceride Having Methylene-Bridged Palmitoleyl and Palmitoyl Groups, RGDG (17:1, 16:0): ^{1}H NMR (600 MHz, pyridine-d_5): *saccharide and glycerol moieties*: δ 6.10 (1H, br s, R1), 5.65 (1H, m, sn-2), 4.82 (1H, d, J = 7.8 Hz, G1), 4.76 (1H, dd, J = 1.2 and 3.0 Hz, R2), 4.75 (1H, br d, J = 3.0 Hz, G4), 4.66 (1H, dd, J = 3.3 and 12.0 Hz, sn-1), 4.59 (1H, m, R5), 4.57 (1H, dd, J = 3.6 and 9.6 Hz, G2), 4.55 (1H, dd, J = 3.0 and 9.6 Hz, R3), 4.49 (1H, dd, J = 6.6 and 12.0 Hz, sn-1), 4.43 (2H, m, G6), 4.34 (1H, dd, J = 5.4 and 10.8 Hz, sn-3), 4.29 (1H, d, J = 9.6 Hz, G3), 4.28 (1H, dd, J = 6.0 and 10.2 Hz, R4), 4.07 (1H, br t, J = 6.0 Hz, G5), 4.03 (1H, dd, J = 5.4 and 10.8 Hz, sn-3), 1.58 (3H, d, J = 6.0 Hz, R6), *acyl chains*: δ 2.34 (4H, t, J = 7.2 Hz, $C2H_2$ × 2), 1.63 (4H, m, $C3H_2$ × 2), 1.41–1.26 (44H, m, CH_2 × 22), 0.86, 0.85 (each 3H, t, J = 6.6 Hz, $C16H_3$ and $C17H_3$), 0.64 (2H, m, cyclopropyl C9H and C11H), 0.55, −0.33 (each 1H, m, cyclopropyl $C10H_2$); ^{13}C NMR (150 MHz, pyridine-d_5): *saccharide and glycerol moieties*: δ 105.6 (G1), 104.0 (R1), 81.2 (G3), 76.9 (G5), 74.0 (R4), 72.5 (R3), 72.2 (R2), 71.4 (G2), 70.9 (sn-2), 69.9 (R5), 69.6 (G4), 68.1 (sn-3), 63.2 (sn-1), 61.9 (G6), 18.5 (R6), *acyl chains*: δ 173.1, 173.0 (C1 × 2), 34.3, 34.1 (C2 × 2), 32.1–27.2 (methylene-C × 22), 25.14, 25.12 (C3 × 2), 16.1 (cyclopropyl C9 and C11), 14.2 (C16 and C17), 11.2 (cyclopropyl C10).

Monogalactosyldiacylglyceride Having Methylene-Bridged Palmitoleyl and Palmitoyl Groups, MGDG (17:1, 16:0): ^{1}H NMR (600 MHz, pyridine-d_5): *saccharide and glycerol moieties*: δ 5.67 (1H, m, sn-2), 4.83 (1H, d, J =

7.2 Hz, G1), 4.68 (1H, dd, $J = 3.0$ and 12.0 Hz, sn-1), 4.57 (1H, br d, $J = 3.0$ Hz, G4), 4.52 (1H, dd, $J = 6.6$ and 12.0 Hz, sn-1), 4.46 (1H, dd, $J = 6.0$ and 10.8 Hz, G2), 4.45 (2H, d, $J = 6.0$ Hz, G6), 4.37 (1H, dd, $J = 5.4$ and 10.8 Hz, sn-3), 4.16 (1H, dd, $J = 3.6$ and 9.0 Hz, G3), 4.07 (1H, t, $J = 5.4$ Hz, G5), 4.06 (1H, dd, $J = 5.4$ and 11.4 Hz, sn-3), *acyl chains*: δ 2.36, 2.34 (each 2H, t, $J = 7.2$ Hz, $C2H_2 \times 2$), 1.64 (4H, m, $C3H_2 \times 2$), 1.41–1.26 (44H, m, $CH_2 \times 22$), 0.86, 0.85 (each 3H, t, $J = 6.6$ Hz, $C16H_3$ and $C17H_3$), 0.64 (2H, m, cyclopropyl C9H and C11H), 0.55, −0.33 (each 1H, m, cyclopropyl $C10H_2$); ^{13}C NMR (150 MHz, pyridine-d_5): *saccharide and glycerol moieties*: δ 105.7 (G1), 77.1 (G5), 75.2 (G3), 72.2 (G2), 71.0 (sn-2), 70.0 (G4), 68.0 (sn-3), 63.2 (sn-1), 62.3 (G6), *acyl chains*: δ 173.2, 173.0 (C1 × 2), 34.4, 34.1 (C2 × 2), 32.1–22.8 (methylene-C × 22), 25.2, 25.1 (C3 × 2), 16.1, 16.0 (cyclopropyl C9 and C11), 14.2 (C16 and C17), 11.2 (cyclopropyl C10).

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Supporting Information

Preparation of benzoate derivatives of the isolated RGDG and MGDG; fragmentation patterns on mass spectrometry of RGDG, MGDG, sn-2 acyl lyso-RGDG, and sn-2 acyl lyso-MGDG; the representative ESI-MS spectra of RGDG, MGDG and the corresponding sn-2 acyl lyso-RGDG and sn-2 acyl lyso-MGDG; the partial HMBC and expanded ^{1}H-1D spectra of RGDG in pyridine-d_5; reverse-phase HPLC profiles of perbenzoate derivatives of the isolated RGDG (MGDG) mixture obtained by ELSD and PDA. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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