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Temperature- and Time-Dependent Changes in the Structure and Composition of Glycolipids during the Growth of the Green Sulfur Photosynthetic Bacterium *Chlorobaculum tepidum*

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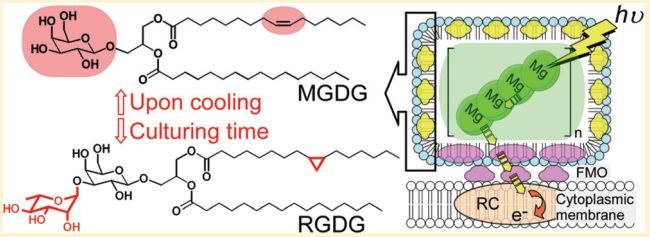
Supporting Information

ABSTRACT: The green sulfur photosynthetic bacterium *Chlorobaculum* (*Cba.*) *tepidum* (previously known as *Chlorobium tepidum*), which grows at an optimal temperature of around 45 °C, biosynthesized unique disaccharide rhamnosylgalactosyldiacylglyceride (RGDG) having a methylene-bridged palmitoleyl (17:Cyc) and a palmitoyl group (16:0) as the two acyl chains in a molecule [RGDG(17:Cyc,16:0)], together with the corresponding monosaccharide monogalactosyldiacylglyceride (MGDG). Here, we report changes in the structure and composition of the glycolipids that are dependent upon the temperature and period of cultivation. With a decrease in temperature to 25 °C, the two major glycolipids were almost completely eliminated, and MGDG with a palmitoleyl (16:1) and a (16:0) group concomitantly became the major glycolipid. MGDG(16:1,16:0) corresponded to the removal of an α-rhamnosyl and a cyclopropyl methylene group from RGDG(17:Cyc,16:0) and the lack of the CH_2 group in MGDG(17:Cyc,16:0). The structural conversion was almost reversible when the *Cba. tepidum* adapted to low and high temperatures was cultured again at 45 and 25 °C, respectively. Moreover, during this cultivation, the structure and composition of glycolipids were sequentially changed: MGDG(16:1,16:0), MGDG(17:Cyc,16:0), and RGDG(17:Cyc,16:0) predominated in the exponential, stationary and late phases of the cultivation, respectively. On the basis of these time-dependent changes, the unique disaccharide RGDG(17:Cyc,16:0) was thought to be created by the site-specific transfer of an α-rhamnosyl group to MGDG(17:Cyc,16:0) after insertion of a methylene group into the precursor MGDG(16:1,16:0). These culturing temperature- and time-dependent changes in glycolipids at the molecular level allow us to discuss their biosynthesis as well as physiological function in green photosynthetic bacteria.



Green photosynthetic bacteria have main, unique, and extramembranous light-harvesting (LH) systems called "chlorosomes".[1−4] Compared to usual LH systems embedded in cytoplasmic membranes of various classes of photosynthetic organisms, chlorosomes are characterized as follows. They are large, micellelike nanoarchitectures with dimensions of 10−25, 20−100, and 70−260 nm.[5−8] In the architectures, a huge number of photosynthetic pigments, estimated to be 150000−215000 chlorophyll pigments per single chlorosome, are encapsulated as well-ordered self-aggregates.[9−12] This is in sharp contrast to the number of other LHs in (an)oxygenic photosynthetic organisms (24−96 chlorophyll molecules).[13−15] Moreover, no proteins are required for the arrangement of a huge number of pigments inside chlorosomes,[16] although other organisms construct their LHs by stoichiometric pigment−protein interactions.[13,14]

The envelopes of chlorosomes consist of glyco- and phospholipid-containing proteins.[17−19] Recently, Miller and co-workers demonstrated that glycolipids, mostly monogalactosyldiacylglyceride (MGDG), in the isolated chlorosomes from *Chlorobaculum* (*Cba. tepidum* (previously known as *Chlorobium tepidum*) comprised more than half of the polar lipids (55%) and phospholipids made up ∼30% of them, based on gas chromatography (GC) and mass spectrometry (MS) analysis after derivation of the lipid extract.[18] They also estimated that the lipids, including glycolipids, covered only 10% of the chlorosomal surfaces,[18] the remainder being covered by 10 different proteins, including specific pigment−protein complexes that are often termed baseplate proteins.[19,20] The proteins (CsmA) play a crucial role in forming mature chlorosomes as well as in transferring excitation energy to photochemical reaction center complexes embedded in cytoplasmic membranes via other LHs, i.e., B808−866 antenna in nonsulfur bacteria and Fenna−Matthews−Olson proteins (FMO proteins) in sulfur bacteria.[8,20]

The 10 proteins, especially for baseplate proteins, in chlorosomal envelopes have been well characterized by molecular

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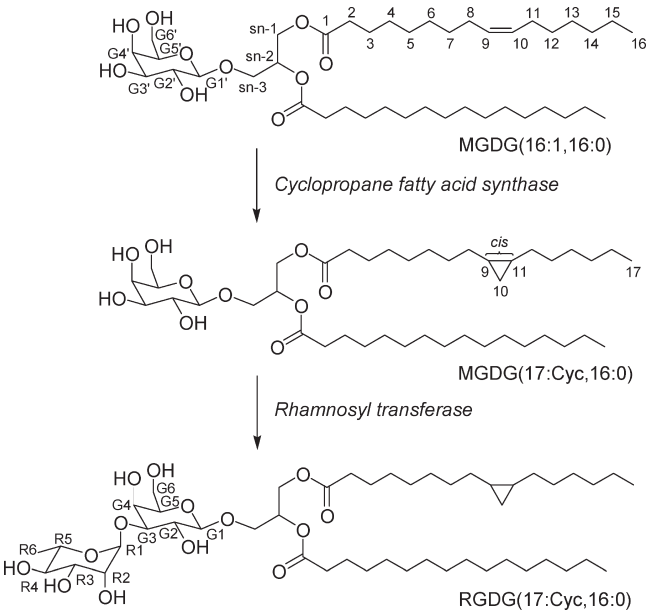
Scheme 1. Proposed Biosynthetic Pathway of Disaccharide RGDG Predominantly Found in *Cba. tepidum* and Abbreviations of the Glycolipids Used in This Study[a]



[a] The suggested enzymes for catalyzing the reactions are also indicated.

genetic techniques,[20,21] while the chlorosomal lipids are less studied, probably because of difficulties in separation and detection of lipids by chromatography as intact forms. Thus, the composition, biosynthesis, and physiological function of glycolipids in chlorosomes have still not been unraveled at the molecular level. To the best of our knowledge, no genetic approaches to glycolipids in chlorosomal envelopes are available, although whole genome analyses of many green photosynthetic bacteria, including *Cba. tepidum* investigated, here have been reported.[22,23] MGDG, however, one of the most abundant lipids on the earth, produced by plant, algae, cyanobacteria, and some photosynthetic bacteria, has been widely investigated using sophisticated molecular genetic techniques.[24,25]

In our previous report, we found that the green sulfur bacterium *Cba. tepidum* biosynthesized unique disaccharide {rhamnosylgalactosyldiacylglyceride [rhamnose-α(1→3)-galactose-β] (RGDG)} and monosaccharide glycolipids (MGDG), which had a methylene-bridged palmitoleyl group at the *sn*-1 position (see the structures in Scheme 1), which made up more than 70% of all the glycolipids.[26] To efficiently detect and analyze glycolipids as intact forms, we used an evaporative light scattering detector (ELSD) for high-performance liquid chromatography (HPLC). ELSD can be applied for detecting all compounds, including molecules that lack chromophores in a molecule without their derivatization. On the basis of HPLC coupled with ELSD (hereafter simply ELSD−HPLC), we were able to investigate glycolipids in green photosynthetic bacteria at the molecular level.

In this study, we demonstrated changes in the structure and composition of glycolipids in *Cba. tepidum* dependent upon its growth conditions, especially the temperature and period of cultivation. Using thermophilic *Cba. tepidum* (grown at around 45 °C), the bacterium was cultured at an ambient temperature (25 °C). Changes in the structure and composition of the glycolipids were then evaluated by isolating two types of chlorosomes and examining their thermal stability using electronic absorption and dynamic light scattering (DLS); one type was isolated from the cells of *Cba. tepidum* grown under normal cultivation conditions (at 45 °C) and the other from cells grown at a lower temperature (25 °C). During the growth of *Cba. tepidum*, time-dependent changes in the structure and composition of the glycolipids were determined to gain knowledge of their biosynthesis.

MATERIALS AND METHODS

General. All solvents were used without further purification. Electronic absorption and DLS measurements of isolated chlorosomes were performed in 50 mM tris(hydroxymethyl) aminomethane [2-amino-2-(hydroxymethyl)-1,3-propanediol] buffer (pH 8.0) (Tris-HCl) using Hitachi U-3500 (Hitachi, Ltd., Tokyo, Japan) and Malvern Zetasizer Nano-ZS (Malvern Instruments, Ltd., Worcestershire, U.K.) instruments, respectively. The optical density was ∼1.0/10 mm at the Q_y absorption maximum for spectroscopic measurements.

Culturing of Green Photosynthetic Bacteria and Preparation of Their Main Light-Harvesting Systems, Chlorosomes. A green sulfur bacterium, *Cba. tepidum* strain ATCC49652, was cultured at 45 and 25 °C under anaerobic light conditions (30 μmol m^{-2} s^{-1}) after its single-colony isolation as described previously.[27] The light-harvesting chlorosomes from both the harvested cells were prepared as "normal" and "low-temperature" chlorosomes 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 without further purification.[28]

Measurement of the Growth Profile. *Cba. tepidum* was precultured by being allowed to grow to its stationary phase at 45 °C (or 25 °C) under anaerobic light conditions (30 μmol m^{-2} s^{-1}) in 185 mL bottles. A 9.25 mL volume (0.5% per bottle) of the preculture was inoculated in freshly prepared medium in 185 mL bottles, and the cultures were then grown under the conditions described above. The cell density was measured at 660 nm with a Hitachi U-3500 spectrophotometer. The grown cells were harvested and their glycolipid components analyzed by ELSD−HPLC and ^{1}H nuclear magnetic resonance (NMR) (two independent experiments were performed to confirm the reproducibility of the data).

Extraction and Purification of Glycolipids. Glycolipids were extracted from the harvested cells of *Cba. tepidum* or the isolated chlorosomes by the method of Bligh and Dyer with slight modification as described previously.[26,29] The extracts, including glycolipids as well as phospholipids, waxes, and photosynthetic pigments, were purified by silica gel chromatography as described previously.[26]

Analysis of Glycolipids by ELSD−HPLC Coupled with ESI-MS. Liquid chromatography (LC) and MS of the extracted glycolipid component were performed using a Shimadzu LCMS-2010EV system (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) probe as described previously.[30] To detect and characterize glycolipids simultaneously, we used the flow separator at a ratio of 3:7 (v/v): one (30%) connected to an ESI probe and the other (70%) to an ELSD (ELSD-LTs, Shimadzu). HPLC was performed under isocratic reverse-phase conditions: column, Cosmosil 5C18-AR-II (4.6 mm × 250 mm, Nacalai Tesque, Kyoto, Japan); eluent, an 85:15 (v/v) acetone/aqueous 25 mM ammonium acetate (pH 6.3) mixture or a 75:5:20

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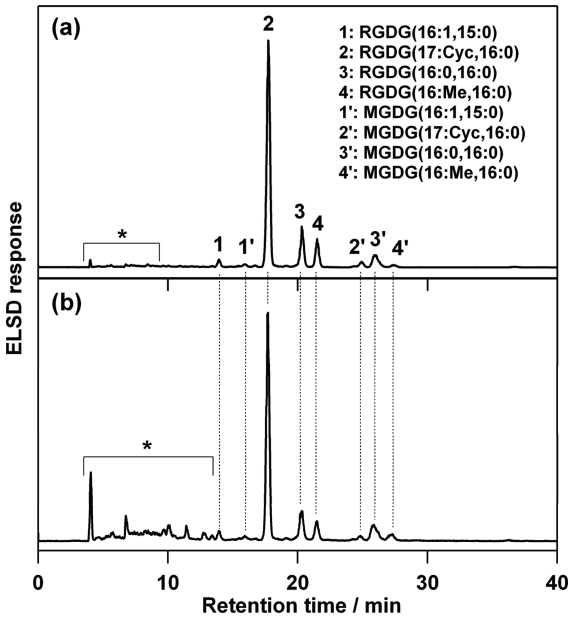

Figure 1. ELSD−HPLC profiles of the glycolipid components extracted from the cells of *Cba. tepidum* grown at 45 °C (a) and the isolated chlorosomes (b). The cells were harvested in the stationary phase of the cultivation. The assignment of each peak is shown in panel a. Impurities during handling of the materials are denoted with asterisks. An 85:15 (v/v) acetone/aqueous 25 mM ammonium acetate (pH 6.3) mixture was used as the HPLC eluent.

(v/v/v) acetone/chloroform/aqueous 25 mM ammonium acetate (pH 6.3) mixture; flow rate, 0.5 mL/min.

NMR Measurements of Glycolipids. ^{1}H NMR spectra of the isolated glycolipids were recorded with a JEOL ECA-600 NMR spectrometer (JEOL, Ltd., Akishima, Japan) in pyridine-d_5 (Euriso-top, Saclay, France) at room temperature; as an internal reference, undeuterated *meta* protons of the solvent molecule were used at 7.19 ppm. To determine the relative content of MGDGs and RGDGs in the isolated mixture of glycolipids, we used the integration of the intrinsic signals of MGDG and RGDG in ^{1}H NMR: the G3′ proton for MGDG and the R1 proton for RGDG (see the numbering in Scheme 1).

RESULTS

Distribution of Glycolipids in the Cells of *Cba. tepidum* and in the Corresponding Light-Harvesting Chlorosomes. To investigate the composition of glycolipids in green photosynthetic bacteria under various culturing conditions, first we compared the composition of the cells with that of the isolated chlorosomes. Figure 1 shows ELSD−HPLC profiles of the glycolipid components extracted from the cells of *Cba. tepidum* cultured at its optimal temperature of 45 °C (a) and the isolated chlorosomes (b). The cells were harvested in the late phase of cultivation (vide infra), and the same harvested cells were used for the isolation of chlorosomes. We can identify two sets of glycolipids labeled as peaks 1 (1′) through 4 (4′): peaks 1−4 were assigned to the glycolipids with a disaccharide group, RGDGs, and the other set of peaks (1′−4′) to the corresponding monosaccharide glycolipids, MGDGs. The assignment of each peak is shown in panel a of Figure 1, and the results of online ESI-MS spectrometry are summarized in Table 1. In this study, we used the following abbreviations for saturated and unsaturated fatty acids having 16 and 17 carbon atoms at the main chain: palmitic (16:0), palmitoleic (16:1), methylene-bridged palmitoleic (17:Cyc), and methylated palmitic (16:Me) acids (the structures of the former three fatty acids are given in Scheme 1).

More details of the characterization of each peak found in the ELSD−HPLC profile by ^{1}H and ^{13}C NMR as well as site-specific hydrolysis of the acyl group at the *sn*-1 position were reported in our previous paper.[26] Briefly, the molecular ion and fragment ions on ESI-MS could be used to identify each glycolipid in terms of the saccharide as well as the two acyl groups in a molecule. Glycolipids 1−4 gave peaks at m/z 878.7, 906.5, 894.8, and 908.7, respectively, corresponding to their ammonium adducts ($[M + NH_4]^+$), and at m/z 535.5, 563.5, 551.4, and 565.5, respectively, corresponding to the removal of their saccharides ($[M1 + H]^+$). The differences between $[M + NH_4]^+$ and $[M1 + H]^+$ in peaks 1−4 shown in parentheses in the third column of Table 1 gave identical values (343 Da = 326 Da + NH_3), indicating that these glycolipids had a rhamnosylgalactosyl moiety (326 Da) as their common saccharide. The ions fragmented 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, the dominant peak 2 in the profiles 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:Cyc) and palmitoyl (16:0) groups, respectively. Glycolipids 1′−4′ were similarly assigned to MGDG. These had a galactosyl moiety as the common saccharide because of the identical differences between $[M + NH_4]^+$ and $[M1 + H]^+$ (197 Da = 180 Da + NH_3).

Comparison of the two profiles in Figure 1 shows that the distribution of glycolipids having different saccharide and acyl groups was almost identical. Thus, in this study, we extracted and analyzed glycolipids not from the isolated chlorosomes but from the cells of green sulfur bacteria. The MGDG:RGDG ratios were determined to be 21:79 for the cells and 20:80 for the chlorosomes, using the spectrally separated proton signals at the G3′ H for MGDGs and the R1 H for RGDGs, because the integration of peak areas in ^{1}H NMR is one of the reliable criteria for evaluating the relative ratio of analytes (see the numbering in Scheme 1 and the representative ^{1}H NMR spectra in Figure S1 of the Supporting Information). The contents thus determined were almost consistent with the reported data that were obtained by GC−MS analysis after derivation of the lipid component extracted from the chlorosomes of *Cba. tepidum* (34:66 MGDG:RGDG).[18] Most of the glycolipids in *Cba. tepidum* was disaccharide-type RGDG. The relative contents of glycolipids with different acyl groups were also estimated by peak areas in the ELSD−HPLC profiles: 2.1 (1.2):65.7 (2.5):12.0 (6.7):8.4 (1.2) peak 1 (1′):peak 2 (2′):peak 3 (3′):peak4 (4′) ratio for the cells and 2.9 (1.9):62.8 (1.8):11.3 (9.3):6.6 (3.3) peak 1 (1′):peak 2 (2′):peak 3 (3′):peak4 (4′) ratio for the chlorosomes. In both the cells and isolated chlorosomes, the majority was found to be RGDG(17:Cyc,16:0) (peak 2).

Temperature-Dependent Changes in the Structure and Composition of Glycolipids. Figure 2a shows an ELSD−HPLC profile of the glycolipid component extracted from the bacterium grown at 45 °C. The cells were harvested in the stationary phase of cultivation (vide infra). In comparison with the profile shown in Figure 1a obtained from the late phase of cultivation (21:79

Table 1. Mass Spectrometric Data for the Intact Glycolipids Extracted from the Cells of *Cba. tepidum* Grown at 45 °C (peaks 1−4 and 1′−4′) and 25 °C (peaks a, b, a′, and b′)

		observed peaks				calculated values for [M + X]			
HPLC peak	glycolipid	$[M + NH_4]^{+a}$	$[M1 + H]^+$	$[M2 + H]^+$	$[M2' + H]^+$	$[M + NH_4]^+$	$[M1 + H]^+$	$[M2 + H]^+$	$[M2' + H]^+$
1	RGDG(16:1,15:0)	878.7 (343.2)	535.5	–[b]	–[b]	878.6_2	535.4_6	311.2_5	299.2_5
2	RGDG(17:Cyc,16:0)	906.5 (343.0)	563.5	325.3	313.3	906.6_5	563.5_0	325.2_7	313.2_7
3	RGDG(16:0,16:0)	894.8 (343.4)	551.4	313.0	–	894.6_5	551.5_0	313.2_7	–
4	RGDG(16:Me,16:0)	908.7 (343.2)	565.5	327.1	313.1	908.6_3	565.5_1	327.2_8	313.2_7
1′	MGDG(16:l,15:0)	732.6 (197.1)	535.5	–[b]	–[b]	732.5_6	535.4_6	311.2_5	299.2_5
2′	MGDG(17:Cyc,16:0)	760.5 (197.0)	563.5	325.3	313.1	760.5_9	563.5_0	325.2_7	313.2_7
3′	MGDG(16:0,16:0)	748.5 (197.1)	551.4	313.0	–	748.5_9	551.5_0	313.2_7	–
4′	MGDG(16:Me,16:0)	762.6 (197.0)	565.5	327.1	313.1	762.6_1	565.5_1	327.2_8	313.2_7
a	RGDG(16:1,16:1)	890.7 (343.2)	547.5	311.3	–	890.6_2	547.4_6	311.2_5	–
b	RGDG(16:l,16:0)	892.5 (343.0)	549.5	313.3	311.3	892.62	549.52	313.2_7	311.2_5
a′	MGDG(16:1,16:1)	744.6 (197.1)	547.5	311.3	–	744.5_6	547.4_6	311.2_5	–
b′	MGDG(16:l,16:0)	746.5 (197.0)	549.5	313.3	311.3	746.5_9	549.5_0	313.2_7	311.2_5

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

MGDG:RGDG), the total content of MGDGs, denoted as dashed labels in the profile (peaks 1′−4′), increased (43:57 MGDG:RGDG, determined by ^{1}H NMR). The results indicated that the biosynthesis of RGDGs was strongly dependent upon the duration of cultivation; this is discussed in the next section. Peak 2 (2′) having 17:Cyc and 16:0 as the two acyl groups and peak 4 (4′) having 16:Me and 16:0 were dominant in the profile, and only a trace of glycolipids having olefinic C═C double bonds in the acyl groups was detected. This was unequivocally confirmed by the ^{1}H NMR spectrum shown in Figure S1 of the Supporting Information, because no signals of olefinic protons were found around 5.45 ppm.

Figure 2b shows the ELSD−HPLC profile of the glycolipid component extracted from the cells grown with a decrease in the culturing temperature to 25 °C. Here, the preculture of *Cba. tepidum* growing to its stationary phase at 45 °C was first prepared. The culture was inoculated in a freshly prepared medium (0.5% per 185 mL bottle) and was then grown to the stationary phase. Interestingly, the composition of glycolipids was drastically changed in comparison with that obtained from the cells grown at the optimal temperature of 45 °C shown in Figure 2a. The dominant glycolipids found at 45 °C, peaks 2, 2′, 4, and 4′, were almost completely eliminated, and concomitantly, new components denoted as a, a′, b, and b′ appeared. Peaks b and b′ was assigned to RGDG(16:1,16:0) and MGDG(16:1,16:0), respectively, by online ESI-MS and off-line ^{1}H NMR spectra. The other peaks (a and a′) were also assigned to RGDG(16:1,16:1) and MGDG(16:1,16:1), respectively. The results are summarized in Table 1. Here, the MGDG:RGDG ratio was determined to be 89:11 by ^{1}H NMR. The major glycolipid (b′) was isolated and was fully characterized by ^{1}H NMR and site-specific hydrolysis of the *sn*-1 acyl group. These results apparently showed the exclusive presence of an olefinic C═C bond, not a cyclopropane ring, in the *sn*-1 acyl group (see Figure S1 and Scheme S1 of the Supporting Information). Details of the characterization are given in the Supporting Information. When a thermophilic green sulfur bacterium adapted to a "low" temperature, the glycolipids thus produced were drastically changed in terms of the structure and composition. The biosynthesis of MGDG(16:1,16:0) in *Cba. tepidum* adapted to low temperatures as the dominant glycolipid was consistent with that in other green sulfur bacteria grown around 30 °C.[31−33]

We also investigated the reversibility of changes in the structure and composition of glycolipids dependent upon cultivation temperature. Figure 2c shows the ELSD−HPLC profile of the glycolipids extracted from *Cba. tepidum* adapted to low temperatures that was directly recultured at 45 °C (in the stationary phase). In the profile, the major peaks found in the bacteria adapted to low temperatures (Figure 2b) were completely eliminated, and again the intensities of the major peaks (2 and 2′) found in the normal cultivation (Figure 2a) increased. These results appeared to demonstrate the reversibility of the temperature-dependent changes in the structure and composition of glycolipids in *Cba. tepidum*. These temperature-dependent changes support the idea that unique glycolipids with a cyclopropane ring in the *sn*-1 acyl group play an important role in the growth of this bacterium at a higher optimal temperature.

Time-Dependent Changes in the Structure and Composition of Glycolipids. Figure 3 shows time-dependent changes in glycolipids during the growth of *Cba. tepidum* at 45 °C (the preculture growing to its stationary phase at 45 °C was used): panel a, the growth profile based on the absorption at 660 nm; panel b, the relative content of the total RGDGs and MGDGs determined by ^{1}H NMR; panel c, the relative content of glycolipids with different acyl groups determined by ELSD−HPLC (the time-dependent changes at 25 °C are shown in Figure S2 of the Supporting Information). In the exponential phase (12−42 h), the RGDG (●) and MGDG (○) contents shown in Figure 3b were almost constant and MGDGs were dominant (≈20:80 RGDG:MGDG). After the exponential phase, the level of MGDGs gradually decreased and the level of RGDGs concomitantly increased in the initial stationary phase (∼42−90 h), the content of the two types becoming almost the same. In the late phase (after ∼90 h), RGDGs became dominant, finally accounting for ∼80% of all glycolipids. These results support the idea that RGDGs are biosynthesized from MGDGs by site-specific transfer of an α-rhamnosyl group at the G3′ position of the galactosyl group in MGDGs during the growth of *Cba. tepidum*.

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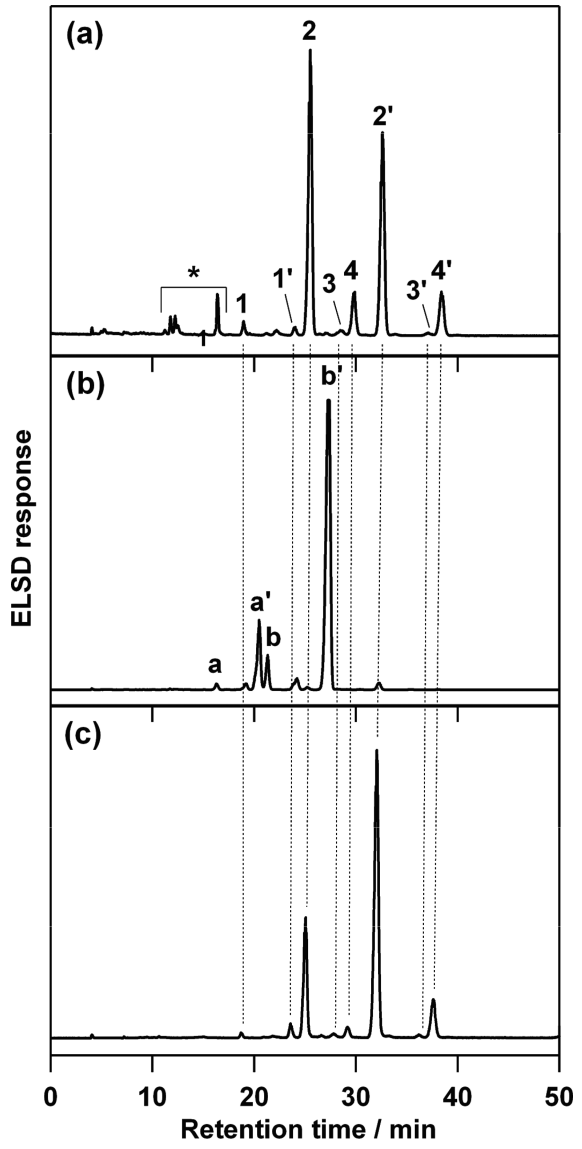

Figure 2. ELSD−HPLC profiles of the glycolipid components extracted from the cells of *Cba. tepidum* grown at 45 °C (a), 25 °C (b, cooled), and 45 °C (c, rewarmed). Impurities are denoted with asterisks. The numbering of each peak corresponds to that in Table 1. A 75:5:20 (v/v/v) acetone/chloroform/aqueous 25 mM ammonium acetate (pH 6.3) mixture was used as the HPLC eluent.

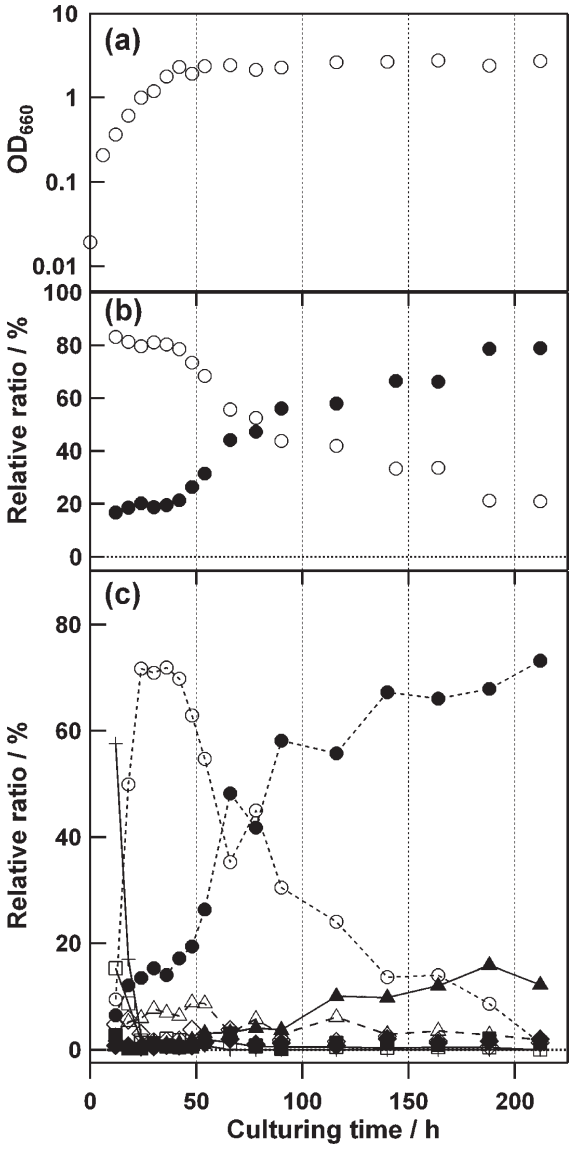

Figure 3. Time-dependent changes in the composition of glycolipids: growth profile at 45 °C (a) and relative contents of the total RGDGs (●) and MGDGs (○) determined by ^{1}H NMR (b) and those of glycolipids having various acyl groups determined by ELSD−HPLC (c). Symbols in panel c: (○) MGDG(17:Cyc,16:0), (●) RGDG(17:Cyc,16:0), (△) MGDG(16:Me,16:0), (▲) RGDG(16:Me,16:0), (◇) MGDG-(16:0,16:0), (◆) RGDG(16:0,16:0), (□) MGDG(16:1,15:0), (■) RGDG(16:1,15:0), and (+) MGDG(16:1,16:0).

With regard to changes in glycolipids with various acyl groups shown in Figure 3c, in the exponential phase where MGDGs were dominant, the levels of the major MGDG(16:1,16:0) (+) and the second major MGDG(16:1,15:0) (□) decreased and the levels of MGDG(17:Cyc,16:0) (○) rapidly increased to become ∼70% of all glycolipids. The conversion indicates that the cyclopropane group is created by the insertion of a methylene group into an olefinic C═C double bond. The methylene group would add to the palmitoleyl C9═C10 group to yield the modified palmitoleyl group, i.e., the methylene-bridged palmitoleyl group shown in Scheme 1. The corresponding MGDG-(17:Cyc,15:0) could not be identified in this study. Changes in glycolipids with other acyl groups were negligible. In the stationary phase where the biosynthesis of RGDGs proceeded, the level of the major MGDG(17:Cyc,16:0) gradually decreased and concomitantly the level of RGDG(17:Cyc,16:0) (●) that was found to be the majority in *Cba. tepidum* increased to ∼50% of all glycolipids. MGDG(16:Me,16:0) (△) was also converted to RGDG(16:Me,16:0) (▲). This conversion also supports the idea that RGDGs are biosynthesized from the corresponding MGDGs. In the late phase, the monosaccharide glycolipids [MGDG(16:1,16:0) and MGDG(16:Me,16:0)] were further consumed. These time-dependent changes in the structure and composition of glycolipids during the growth of *Cba. tepidum* thus strongly indicate that MGDG(16:1,16:0) is the precursor of unique RGDG(17:Cyc,16:0) in the bacterium. This unique glycolipid is essential for the formation of mature chlorosomes.

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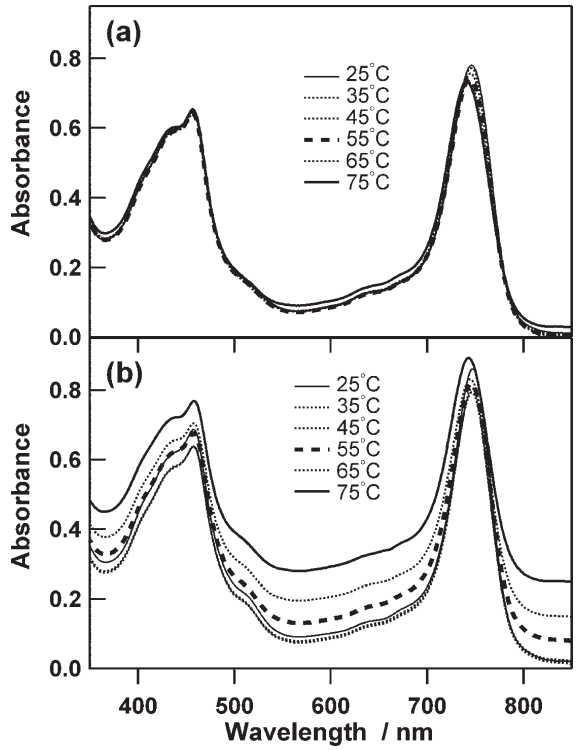

Figure 4. Absorption spectra of chlorosomes isolated from normal (a) and low-temperature (b) chlorosomes in 50 mM Tris-HCl buffer (pH 8.0) with an increase in temperature from 25 to 75 °C at each 10 °C interval. Spectra recorded at 25, 55, and 75 °C are represented by thin solid, thick dotted, and thick solid lines, respectively, and those recorded at the other temperatures are represented by thin dotted lines.

Thermal Stability of Light-Harvesting Chlorosomes with Different Glycolipid Contents. As mentioned above, *Cba. tepidum* had been thought to regulate the biosynthesis of glycolipids for its optimal growth, especially the construction of light-harvesting systems (chlorosomes). To clarify how the regulation was governed, the thermal stability of two kinds of chlorosomes was examined: one from cells grown at the optimal temperature of 45 °C and the other from cells adapted to low temperatures (25 °C) (hereafter termed "normal" and "low-temperature" chlorosomes, respectively).

Figure 4 shows changes in the absorption spectra with an increase in temperature from 25 to 75 °C at 10 °C intervals in an aqueous 50 mM Tris-HCl buffer: panel a, normal chlorosomes; panel b, low-temperature chlorosomes. With an increase in temperature, low-temperature chlorosomes exhibited extremely intense scattering (an increase in the baseline) even at 55 °C (thick dotted line in Figure 4b), whereas normal chlorosomes had a slight scattering at 75 °C (thick solid line in Figure 4a). These changes were not reversible after the samples had cooled (data not shown).

Figure 5 shows changes in the hydrodynamic diameters with an increase in temperature. No clear changes in the diameters were found for normal chlorosomes (○); however, changes for low-temperature chlorosomes were apparent at 55 °C, those 90−145 nm in diameter (●). These observations were consistent with those made with the absorption spectra. Thus, the changes in the absorption spectra with an increase in temperature were thought to be due to agglutination of the isolated chlorosomes in an aqueous suspension, because monomerization of a huge number of chlorophyll pigments as self-aggregates inside

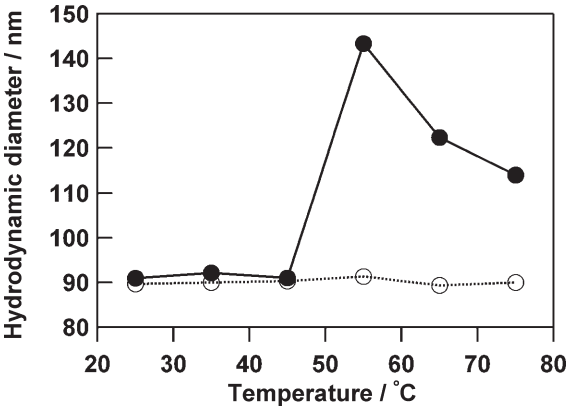

Figure 5. Temperature-dependent hydrodynamic diameters of normal (○) and low-temperature (●) chlorosomes in an aqueous 50 mM Tris-HCl buffer (pH 8.0) being heated from 25 to 75 °C.

chlorosomes (showing an absorbance at around 670 nm) was not observed for either type of chlorosome even at 75 °C, and most of the normal chlorosomal surface was estimated to be covered with proteins.[18]

DISCUSSION

Normal chlorosomes consisted mostly of disaccharide RGDG-(17:Cyc,16:0) and monosaccharide MGDG(17:Cyc,16:0) as their glycolipids, while low-temperature chlorosomes contained MGDG(16:1,16:0) and MGDG(16:1,16:1). The latter composition is consistent with that reported for other green sulfur bacteria that are grown around an optimal temperature of 30 °C.[31−33] On the basis of our ELSD−HPLC work coupled with ESI-MS, we reconfirmed the glycolipid composition of the five following species of green sulfur bacteria grown at 30 °C: *Chlorobium limicola* strains Larsen and DSM245, *Chlorobaculum parvum* strain NCIB8327 (previously known as *Chlorobium vibrioforme*), *Chlorobaculum limnaeun* strain 1549 (previously known as *Chlorobium phaeobacteroides*), and *Chlorobium phaeovibrioides* strain DSM269. All biosynthesized MGDG(16:1,16:0) and MGDG(16:1,16:1) as their major glycolipids (data not shown). These glycolipids were reported to be localized in light-harvesting chlorosomes, and the content was ∼3 times larger than that of purified cytoplasmic membranes using the anthrone method.[31,32] This distribution of glycolipids in the cells and the isolated chlorosomes of *Cba. tepidum* is consistent with the reported data, and most glycolipids are thought to be localized in chlorosomes. In contrast, some different hypotheses concerning the biogenesis of unique light-harvesting chlorosomes in green photosynthetic bacteria have recently been reported:[20,34] chlorosomes are regarded as specialized lipid bodies with a monolayer and are derived from a leaflet of cytoplasmic membranes. Thus, knowledge of the precise structures and the composition of glycolipids in isolated chlorosomes and purified cytoplasmic membranes might be useful for understanding and discussing the biogenesis as well as the entire structure of chlorosomes. Furthermore, we performed silver-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis with the proteins expressed in the envelopes of both normal and low-temperature chlorosomes to confirm the relative ratio of the proteins. No large differences between them could be confirmed (data not shown). As the two chlorosomes had almost identical diameters

determined by DLS, their surface areas were judged to be almost the same. Thus, the differences in thermal stability observed for the two types of chlorosomes might be ascribed to differences in the structure and composition of the composite glycolipids.

The presence of lipids (phospholipids, as an example) having a cyclopropyl group in the acyl chain has been reported in many bacterial species.[35−37] The cyclopropane ring is formed by the addition of a methylene group to the C═C bond of mostly palmitoleate or oleate.[35,36] The methylene group was inserted not into the free fatty acids but into mature (phospho)lipid molecules already present in membranes at the onset of the stationary phase of bacterial cultivation. Therefore, the formation of a cyclopropyl group in the acyl chain of a lipid molecule can be regarded as a direct regulation of the properties of bacterial membranes.

When organisms are grown below their optimal temperatures, lipids having lower-melting point fatty acids should be synthesized and included in membranes to maintain the membrane fluidity required for bacterial growth. In some bacteria, this can be accomplished via the modification of acyl groups in a lipid molecule: desaturation of fatty acids (dehydrogenation of a CH−CH bond to a C═C bond), *trans*-to-*cis* isomerization of unsaturated fatty acids, and introduction of a cyclopropyl group at fatty acids as well as a decrease in the length of fatty acids.[38,39] These modifications in the acyl groups lead to an increase in membrane fluidity, i.e., lowering the phase transition temperature from the liquid crystalline state (disordered) to the gel state (ordered).[37] Upon cooling, the flexible membranes thus modified exhibit stronger resistance to a low-temperature stress for bacterial survival.

Conversely, increasing the cultivation temperature destabilizes membranes because of their excess fluidization. To prevent this heating effect, lipids having saturated or *trans*-type fatty acids are required in addition to an increase in the length of the fatty acids. Using fluorescence anisotropy measurements, it was demonstrated that lipids with saturated fatty acids in thylakoid membranes of a cyanobacterium played a heat-protective role[40] and *trans*-type fatty acids had a similar function as saturated fatty acids,[37] as expected from the discussion in the preceding paragraph. In contrast, the formation of a cyclopropyl group in fatty acids at a temperature higher than those found under typical cultivation conditions correlated with a decrease in membrane fluidity, although the detailed effect of its formation upon this fluidity was not clarified.[37] On the basis of the observations, the thermophilic green sulfur bacterium *Cba. tepidum* might prefer the formation of a cyclopropyl group in the glycolipids to the saturation and *cis*-to-*trans* isomerization of their palmitoleyl group during its growth at 45 °C, to maintain the properties (mostly fluidity) of chlorosomal membranes containing various functional proteins under optimal thermal conditions. The result would be that the efficient transfer of excitation energy from light-harvesting chlorosomal bacteriochlorophyll *c* aggregates to photochemical reaction center complexes is achieved via the mediating baseplate/FMO proteins.

According to agglutination experiments with chlorosomes with a lectin, the saccharide moieties in glycolipids face the outside of the envelopes and might assist in the connection of chlorosomes with cytoplasmic membranes.[41] Therefore, glycosidation of a rhamnosyl to a galactosyl group in MGDG [rhamnose-α(1→3)-galactose-β] after the formation of a cyclopropyl group in acyl chains would serve to enhance the connection under thermal conditions. The deficiency of disaccharide glycolipids (digalactosyldiacylglyceride) in an *Arabidopsis* mutant caused a decrease in the thermal stability of thylakoid membranes.[25] The mutant hampered the formation of these well-ordered membranes containing the main light-harvesting complexes. Thus, one more glycosidation to produce lipids having a dissacharide moiety would subordinately serve to enhance the thermal stability of chlorosomes. For clarification of these ideas, some molecular genetic approaches targeting the enzymes catalyzing the formation of a cyclopropyl group and the transfer of an α-rhamnosyl group to MGDG are essential (see Scheme 1).

■ ASSOCIATED CONTENT

Supporting Information. Characterization of glycolipids isolated from *Cba. tepidum* adapted to low temperatures (25 °C) together with ^{1}H NMR spectra of the glycolipid components extracted from the cells grown at 25 and 45 °C, site-selective hydrolysis of MGDG(16:1,16:0) at the *sn*-1 position using a lipase, and time-dependent changes in glycolipids during the growth of *Cba. tepidum* at 25 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

Cba., *Chlorobaculum*; DLS, dynamic light scattering; ELSD, evaporative light scattering detector; ESI, electrospray ionization; FMO, Fenna−Matthews−Olson; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LH, light-harvesting; MGDG, monogalactosyldiacylglyceride; MS, mass spectrometry; NMR, nuclear magnetic resonance; RGDG, rhamnosylgalactosyldiacylglyceride; Tris, tris(hydroxymethyl)aminomethane.

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