Biochemistry and Biophysics Reports 9 (2017) 42–46

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Biosynthesis of unnatural glycolipids possessing diyne moiety in the acyl chain in the green sulfur photosynthetic bacterium *Chlorobaculum tepidum* grown by supplementation of 10,12-heptadecadiynic acid

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ARTICLE INFO

Keywords:
Biosynthesis
Chlorosome
Fatty acid
Glycolipid
Green sulfur bacteria

ABSTRACT

Unnatural glycolipids possessing the diyne moiety in their acyl groups were successfully biosynthesized in the green sulfur photosynthetic bacterium *Chlorobaculum* (*Cba.*) *tepidum* by cultivation with supplementation of 10,12-heptadecadiynic acid. Monogalactosyldiacylglycerol (MGDG) and rhamnosylgalactosyldiacylglycerol (RGDG) esterified with one 10,12-heptadecadiynic acid were primarily formed in the cells, and small amounts of glycolipids esterified with the two unnatural fatty acids can also be detected. The relative ratio of these unnatural glycolipids occupied in the total glycolipids was estimated to be 49% based on HPLC analysis using a evaporative light scattering detector. These results indicate that the acyl groups in glycolipids, which play important roles in the formation of extramembranous antenna complexes called chlorosomes, can be modified *in vivo* by cultivation of green sulfur photosynthetic bacteria with exogenous synthetic fatty acids. Visible absorption and circular dichroism spectra of *Cba. tepidum* containing the unnatural glycolipids demonstrated the formation of chlorosomes, indicating that the unnatural glycolipids in this study did not interfere with the biogenesis of chlorosomes.

1. Introduction

Photosynthetic light-harvesting complexes capture the sunlight energy and transfer it to the reaction center complexes in the early stage of photosynthetic events. In most light-harvesting complexes, photosynthetic pigments such as chlorophylls (Chls), bacteriochlorophylls (BChls), and carotenoids are embedded in the protein matrix [1]. The only exception is extramembranous antenna complexes of green photosynthetic bacteria called chlorosomes [2–5]. Chlorosomes are ellipsoidal particles with the dimensions of approximately 100–150 nm length, 30–50 nm width, and 10–25 nm height. The huge number of BChls *c*, *d*, *e*, and *f* are densely packed and assembled with no help of proteins in the interior of chlorosomes [6,7]. The BChl self-aggregates are surrounded by a lipid layer on chlorosomes. The specific interactions among chlorosomal BChl pigments, namely the coordination bond of the 3^1-hydroxy group of one BChl with the central magnesium in another BChl and the hydrogen bond of the coordinated 3^1-hydroxy group with the 13-keto group in a third BChl, play crucial roles in the pigment self-assemblies in chlorosomes [8,9]. Such unique architecture has attracted considerable attentions in photobiology and photobiophysics.

The major components of the envelop of chlorosomes are glycolipids and membranous proteins called Csm proteins [3,5,10–13]. The green sulfur photosynthetic bacterium *Chlorobaculum* (*Cba.*) *tepidum* has two types of glycolipids, namely monogalactosyldiacylglycerol (MGDG) and disaccharide-type rhamnosylgalactosyldiacylglycerol (RGDG), both of which are attached with various acyl groups (Fig. 1). In addition, *Cba. tepidum* possesses ten kinds of Csm proteins, whose roles in chlorosomes have been studied by means of biochemical and molecular genetic techniques [14–17]. In contrast to the extensive researches on the Csm proteins, detailed contributions of glycolipids for chlorosomes have not been unraveled yet. One possible reason for little information of glycolipids in chlorosomes comes from difficulties in separation and characterization of glycolipids from green photosynthetic bacteria. Recently, Tamiaki and coworkers have performed precise analysis of glycolipids in green photosynthetic bacteria to

Abbreviations: BChl, bacteriochlorophyll; BChl c_F, bacteriochlorophyll *c* esterified with farnesol; *Cba.*, *Chlorobaculum*; ELSD, evaporative light scattering detector; ESI, electrospray ionization; MGDG, monogalactosyldiacylglycerol; RGDG, rhamnosylgalactosyldiacylglycerol

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http://dx.doi.org/10.1016/j.bbrep.2016.11.007
Received 26 July 2016; Received in revised form 9 November 2016; Accepted 12 November 2016
Available online 19 November 2016

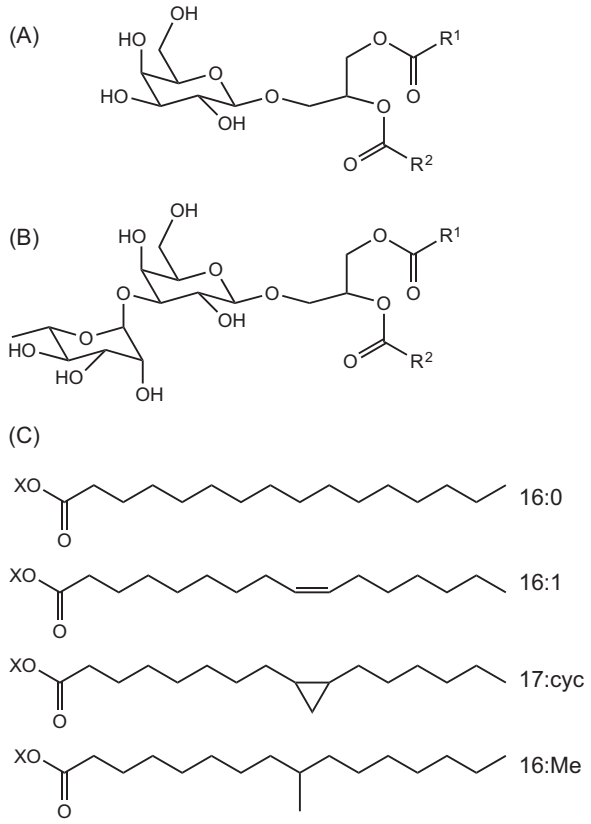

Fig. 1. The molecular structures of MGDG (A) and RGDG (B) found in chlorosomes of *Cba. tepidum*. Hydrocarbons in the acyl chains are indicated by R^1 and R^2 in A and B. The structures of major acyl chains (R^1 and R^2) in glycolipids are shown in C, where the esterifying alcoholic parts are indicated by X.

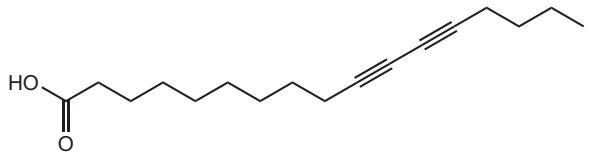

Fig. 2. Molecular structure of 10,12-heptadecadiynic acid supplemented in the liquid cultures of *Cba. tepidum* in this study.

overcome these problems [18–22]. Another problem would originate from difficulties in alteration of glycolipids in green photosynthetic bacteria. There is little information, to our best knowledge, on the genetic approach to glycolipids in green photosynthetic bacteria [23]. The methodology for *in vivo* modification of glycolipids in green photosynthetic bacteria will be a clue to investigation of the supramolecular structure and biogenesis of chlorosomes. In this study, we first report modification of the acyl moieties in glycolipids of *Cba. tepidum* using their biosynthetic reactions by supplementation of a commercially available fatty acid 10,12-heptadecadiynic acid, which has a diyne moiety in the middle of the hydrocarbon chain (the molecular structure is shown in Fig. 2).

2. Experimental

2.1. Apparatus

Analysis of glycolipids by a HPLC system equipped with an evaporative light scattering detector (ELSD) was performed with a Shimadzu LC-20AT pump and an ELSD-LT II detector by the control of column temperature with a Shimadzu CTO-20AC column oven. HPLC analysis of BChl *c* was carried out with a Shimadzu LC-20AT pump and an SPD-M20A detector. Liquid chromatography-mass spectrometry (LC-MS) was done with a Shimadzu LCMS-2020 system equipped with an electrospray ionization probe (ESI). Visible absorption and circular dichroism (CD) spectra were measured with a Shimadzu UV-2450 spectrophotometer and a JASCO J-820 spectropolarimeter, respectively.

2.2. Cultivation

Pre-cultured cells (1 mL) of the green sulfur photosynthetic bacterium *Cba. tepidum* ATCC 49652 were inoculated into a freshly prepared liquid medium (*ca.* 650 mL), in which 10 mg of 10,12-heptadecadiynic acid (Tokyo Chemical Industry, Co., Ltd.) was supplemented. Then, *Cba. tepidum* was grown in the liquid medium by continuous irradiation with fluorescence lamps (13 μmol s^{-1} m^{-2}) at 42 °C for 3 days [24–26].

2.3. Extraction and analysis of glycolipids

Glycolipids were extracted from the harvested cells with a mixture of 0.1 M acetic acid, methanol, and chloroform, and purified from the crude extracts by silica-gel chromatography according to the previous reports [18–22]. Glycolipids obtained were analyzed by ELSD-HPLC using a reverse-phase column Cosmosil $5C_{18}$-AR-II (4.6 mm i.d.×250 mm) with acetone/25 mM ammonium acetate (pH 6.7) (85/15, vol/vol) at the flow rate of 0.5 mL min^{-1}. Glycolipids were assigned by LC-MS as well as the elution patterns reported previously [18–22].

2.4. Extraction and analysis of BChl c

BChl *c* was extracted from the harvested cells with methanol/acetone (1/1, vol/vol), followed by filtration. The organic solutions containing the extracted pigments were diluted with diethyl ether, washed with NaCl-saturated water, and dried over anhydrous Na_2SO_4, followed by evaporation. BChl *c* obtained was analyzed by HPLC using a reverse-phase column Cosmosil $5C_{18}$-AR-II (6 mm i.d.×250 mm) with methanol/water (95/5, vol/vol) at the flow rate of 1.0 mL min^{-1} [24–27].

3. Results and discussion

3.1. Compositions of glycolipids

Glycolipids were extracted from *Cba. tepidum* cells, which were grown under the normal conditions and by supplementation of 10,12-heptadecadiynic acid, and analyzed by ELSD-HPLC. Hereafter the following abbreviations are used for naturally occurring fatty acids at the main chain of glycolipids: palmitic (16:0), palmitoleic (16:1), methylated palmitic (16:Me), and methylene-bridged palmitoleic (17:cyc) acids. Fig. 3 shows typical elution patterns of glycolipids extracted from *Cba. tepidum* grown under the normal conditions and by supplementation of 10,12-heptadecadiynic acid. *Cba. tepidum* grown under the normal conditions possessed six major glycolipids, which eluted from 18 to 30 min under the present HPLC conditions (fractions 1–6 in Fig. 3A). These natural glycolipids were analyzed by LC-MS: the results of their online ESI-MS spectrometry are summarized in Table 1. The present LC-MS analysis and the elution order in the previous reports [18–22] allowed us to assign the naturally occurring glycolipids (fractions 1–6) to be RGDG (16:1,16:0), MGDG (16:1,16:0), RGDG (17:cyc,16:0), MGDG (17:cyc,16:0), MGDG (16:0,16:0), and MGDG (16:Me,16:0), respectively.

Supplementation of 10,12-heptadecadiynic acid in the liquid medium of *Cba. tepidum* produced novel glycolipids, which were observed from 7 to 13 min in the ELSD-HPLC chromatogram (fractions 1′–6′ in Fig. 3B). The molecular ion peaks of these novel glycolipids were observed at *m/z* 906.6, 760.5, 912.6, 766.6, 900.7, and 754.6,

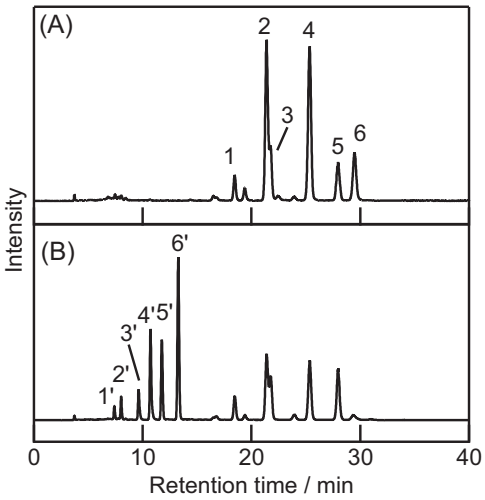

Fig. 3. HPLC elution patterns of glycolipids in *Cba. tepidum* grown under the normal conditions (A) and by supplementation of 10,12-heptadecadiynic acid (B). The glycolipids were eluted on a reverse-phase column $5C_{18}$-AR-II (4.6 mm i.d.×250 mm) with acetone/25 mM ammonium acetate (pH 6.7) (85/15, vol/vol) at a flow rate of 0.5 mL min^{-1}. The chromatograms were normalized at the highest peaks in the chromatograms.

Table 1
LC-MS results of natural (fractions 1–6 in Fig. 3A) and unnatural glycolipids (fractions 1′–6′ in Fig. 3B) in *Cba. tepidum*.

Fraction	Glycolipids	Observed ions (m/z)		Calculated values for [M+X]$^+$	
		[M+NH_4]$^+$	[M1+H]$^+$	[M+NH_4]$^+$	[M1+H]$^+$
1′	RGDG (diyne, diyne)	906.6	563.3	906.56	563.40
2′	MGDG (diyne, diyne)	760.5	563.5	760.50	563.40
3′	RGDG (diyne, 17:cyc)	912.6	569.4	912.60	569.45
4′	MGDG (diyne, 17:cyc)	766.6	n.d.	766.55	569.45
5′	RGDG (diyne, 16:0)	900.7	557.5	900.60	557.45
6′	MGDG (diyne, 16:0)	754.6	557.6	754.55	557.45
1	RGDG (16:1, 16:0)	892.6	549.6	892.64	549.48
2	MGDG (16:1, 16:0)	746.6	549.6	746.58	549.48
3	RGDG (17:cyc, 16:0)	906.4	563.0	906.65	563.50
4	MGDG (17:cyc, 16:0)	760.6	563.6	760.59	563.50
5	MGDG (16:0, 16:0)	748.6	551.5	748.59	551.48
6	MGDG (16:Me, 16:0)	762.7	565.6	762.61	565.51

respectively, in the elution order by LC-MS analysis (Table 1). All the values corresponded to the calculated values of ammonium adducts [M+NH_4]$^+$ of glycolipids possessing one or two 10,12-heptadecadiynoyl moieties. In addition, the fragment ions that lacked the saccharide moiety were detected as reported earlier [18–22], and these values were almost the same as the calculated values of the protonated forms [M1+H]$^+$(Table 1). These results indicate that the exogenous 10,12-heptadecadiynic acid was attached to glycolipids in *Cba. tepidum* in place of natural fatty acids.

The relative ratio of the unnatural glycolipids esterified with 10,12-heptadecadiynic acid over the total glycolipids in *Cba. tepidum* cells, judged from the fraction area in the ELSD-HPLC chromatograms, was estimated to be 49 ± 5.0% (the average and standard deviation of four cultures). Surprisingly, approximately half amounts of the unnatural glycolipids were *in vivo* synthesized *via* the biosynthetic pathway of glycolipids in *Cba. tepidum*.

Generally, glycolipids are biosynthesized as follows: acyl groups are attached to a glycerol 3-phosphate by two acyltransferases, namely glycero-3-phosphate and monoacylglycerol-3-phosphate acyltransferases, followed by the removal of a phosphate and the subsequent transfer of galactose to the resulting diacylglycerol [28–30]. The large amounts of the unnatural glycolipids demonstrated here suggest loose recognition of fatty acids in the enzymes participating in the biosynthesis of glycolipids in *Cba. tepidum*. The similar chain length of 10,12-heptadecadiynic acid (17 carbon atoms) to major fatty acids possessing 16 and 17 carbon atoms in glycolipid in *Cba. tepidum* would result in smooth recognition in the biosynthetic reactions of glycolipids and accumulation in cells.

3.2. *BChl* c *composition*

BChl *c* homologs in *Cba. tepidum* cells grown by supplementation of 10,12-heptadecadiynic acid as well as those grown under the normal conditions were analyzed by reverse-phase HPLC (Fig. 4). Four BChl *c* fractions were observed in these chromatograms. The fractions were assigned as 8-ethyl-12-methyl, 8-ethyl-12-ethyl, 8-propyl-12-ethyl, and 8-isobutyl-12-ethyl homologs of BChl *c* esterified with farnesol (denoted as BChl c_F) in the elution order [24–27]. The composition of four BChl c_F homologs in cells grown with 10,12-heptadecadiynic acid was almost the same as that in cells grown under the normal conditions. No other fraction exhibiting on-line absorption spectra characteristic of BChl *c* (λ_{max}=435 and 669 nm in this eluent) was detected, indicating that no modification of the esterifying group in BChl *c* occurred even if *Cba. tepidum* was grown with exogenous 10,12-heptadecadiynic acid (Fig. 4B). Therefore, cultivation of *Cba. tepidum* with 10,12-heptadecadiynic acid did not affect the biosynthesis of BChl *c* in *Cba. tepidum*.

3.3. *Spectral properties*

Fig. 5 shows visible absorption spectra of *Cba. tepidum* cells grown by supplementation of 10,12-heptadecadiynic acid and under the normal conditions. *Cba. tepidum* cells grown in the normal culture

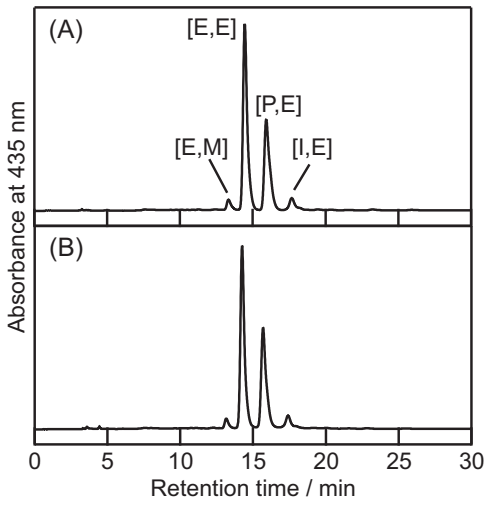

Fig. 4. HPLC elution patterns of BChl *c* in *Cba. tepidum* grown under the normal conditions (A) and by supplementation of 10,12-heptadecadiynic acid (B). The pigments were eluted on a reverse-phase column $5C_{18}$-AR-II (6 mm i.d.×250 mm) with methanol/water (95/5, vol/vol) at a flow rate of 1.0 mL min^{-1}. The chromatograms were recorded at 435 nm and normalized at the peaks of the fractions of [E,E]BChl c_F.

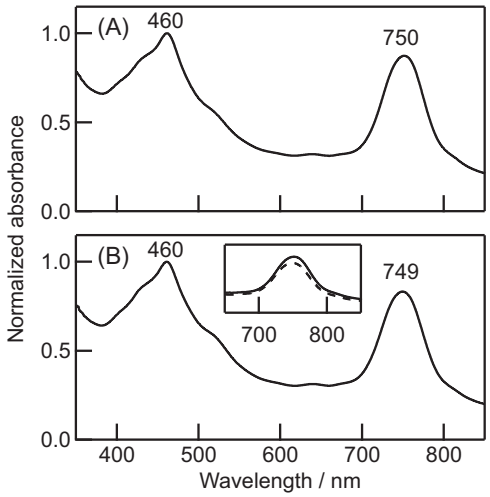

Fig. 5. Visible absorption spectra of *Cba. tepidum* cells grown under the normal conditions (A) and by supplementation of 10,12-heptadecadiynic acid (B). The spectra were measured after dilution of cell cultures with 50 mM Tris–HCl buffer (pH 8.0) and normalized at the Soret peaks. Insert: overlapped spectra of the Q_y absorption bands of the cells grown under the normal conditions (solid curve) and by supplementation of 10,12-heptadecadiynic acid (broken curve) after 10-fold dilution of cell cultures.

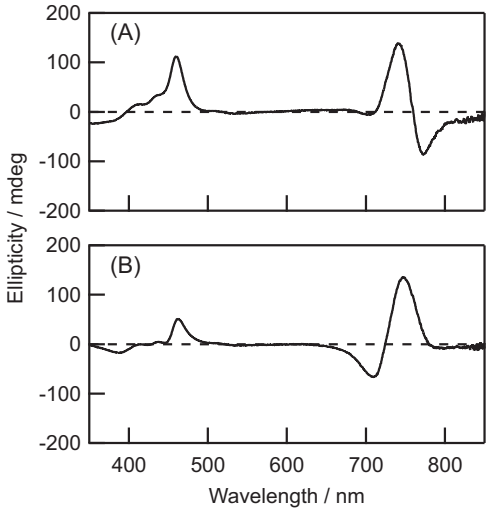

Fig. 6. CD spectra of *Cba. tepidum* cells grown under the normal (A) and by supplementation of 10,12-heptadecadiynic acid (B). The spectra were measured after 10-fold dilution of cell cultures with 50 mM Tris–HCl buffer (pH 8.0).

exhibited the Soret and Q_y bands at 460 nm and 750 nm, respectively (Fig. 5A). In a while, bacterial cells containing the unnatural glycolipids esterified with 10,12-heptadecadiynic acid exhibited the Soret and Q_y absorption bands at 460 and 749 nm, respectively (Fig. 5B). The peak positions and the spectral shapes of both the absorption bands, which were characteristic of BChl *c* self-aggregates, in *Cba. tepidum* containing the unnatural glycolipids were analogous to those in cells grown in the normal culture. It is worthy noting that no Q_y absorption band of BChl *c* monomers around 670 nm were detected in the absorption spectrum of cells containing the unnatural glycolipids (Fig. 5B).

Fig. 6 shows CD spectra of cells grown with 10,12-heptadecadiynic acid and under the normal conditions. *Cba. tepidum* grown under the normal conditions exhibited a reverse S-shaped CD signal around the Q_y region accompanying a slight negative signal at the shorter wavelength side as well as relatively less intense positive signals around the Soret region (Fig. 6A). The intense CD signals were characteristic of the ordered self-assembly of BChl *c* pigments inside chlorosomes [31–33]. This CD spectrum in the Q_y region is mainly interpreted as type I, in which type II is mixed [31]. In contrast, the Q_y CD signal of *Cba. tepidum* grown with this fatty acid was S-shaped (Fig. 6B). This spectrum is classified into type II [31]. The relative intensity of the CD signal in the Soret region against the Q_y region became smaller than that of cells grown under the normal conditions. These indicate that the attachment of the exogenous fatty acid to chlorosomal glycolipids do not interfere with the formation of chlorosomes in this bacterium, but some perturbation to mesoscopic structures of the BChl *c* self-aggregates might occur by interactions of the farnesyl moieties in BChl *c* with the acyl groups in glycolipids. These hydrophobic interactions would play important roles in the biogenesis of chlorosomes, but no information is so far available because of the spectral silence of both the esterifying groups in chlorosomal BChl pigments and the acyl groups in glycolipids. Therefore, the present methodology will be helpful to introduce the probes in the unclear region in chlorosomes by metabolic reactions of green photosynthetic bacteria for elucidation of the unclear regions in chlorosomes. The changes in the Q_y CD signals observed here might originate from changes in chlorosome sizes by substitution of the acyl groups in glycolipids, since the sizes of chlorosomes are also responsible for their CD spectra [34–36]. The effects of unnatural glycolipids on the sizes and shapes of chlorosomes will be also useful information to understand the chlorosome biogenesis in green photosynthetic bacteria.

4. Conclusion

This study first demonstrates *in vivo* modification of the acyl group in glycolipids in the green sulfur photosynthetic bacterium *Cba. tepidum* grown by supplementation with the synthetic fatty acid 10,12-heptadecadiynic acid. Accumulation of large amounts of the unnatural glycolipids in *Cba. tepidum* suggests loose recognition of fatty acid substrates by enzymes that function in the biosynthetic pathway of glycolipids in this bacterium. The diyne moiety in the middle of the acyl group in glycolipid will be useful as probes in vibrational spectroscopy and reactive groups for polymerization to study the supramolecular structures and biogenesis of chlorosomes.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

We thank Mrs. Hayato Yamashita, Keisuke Hayashi, and Keiya Hirota, Kindai University, and Dr. Taichi Yoshitomi, Ritsumeikan University, for their experimental assistance. This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas ‘Artificial Photoshynthesis (AnApple)’ (No. 15H00887) from the Japan Society for the Promotion of Science (JSPS).

Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.11.007.

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