J. Biochem. 127, 813–819 (2000)

Rescuing Activity of Galactoglycerolipids from Cellular Lesions Induced by 5-Aminolevulinic Acid

Kuniho Nakata[1]

Central Research Laboratories, Mercian Corporation, 4-9-1 Johnan, Fujisawa 251-0057

Received January 7, 2000; accepted February 16, 2000

An anti-oxygen radical reagent of a bacterial metabolite, M874 monogalactoglycerolipid (di-*O*-12-methyl-tetradecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol), was tested for its ability to protect two organisms against cellular lesions induced by 5-aminolevulinic acid (ALA) and light. In *Corynebacterium flavescens* ATCC 10340, extracellular uroporphyrin and coproporphyrin were the main porphyrin products. Although less than 2 mM ALA increased porphyrin synthesis, ALA levels above 3 mM inhibited the synthesis. Depending on the light intensity, the amount of porphyrin decreased and ALA-induced cytotoxicity increased. The lesion was more severe in the case of coproporphyrin than uroporphyrin. The porphyrin lesion produced in low intensity light (300 lx) was considerably reduced by 100μM M874 glycolipid, although the reduction in intense light (3,000 lx) was restricted to a lower level. Similar results were obtained with radish (*Raphanus sativus*). The ALA concentration that inhibited porphyrin synthesis and stem growth was similar to that seen with *C. flavescens*. Although the exogenous addition of M874 glycolipid to the radish did not prevent ALA-induced cellular injury, the co-culture of radish and a glycolipid producing bacterium (*Microbacterium* sp. M874) resulted in a significant prevention of cellular injury. This was true only under enforced adhesion conditions through the action of a polysaccharide flocculant H12. Some species of monogalactoglycerolipids were found in *Corynebacterium* and radish that showed prominent oxygen radical-protecting activities similar to that of M874 glycolipid. These monogalactoglycerolipids might function *in vivo* as agents to prevent ALA-induced cytological lesions, although the concentrations were low in *Corynebacterium* and radish.

Key words: aminolevulinic acid, galactoglycerolipid, glycoglycerolipid, oxygen radical, porphyrin.

5-Aminolevulinic acid (ALA) is the first common precursor in the tetrapyrrole biosynthetic pathway. Promotive effects of ALA on growth or photosynthesis have been reported in some organisms (*1–3*). The intracellular level of ALA, however, is maintained at low concentrations in normal cells, while the exogenous addition of higher concentrations of ALA is toxic to many organisms. The mechanism of this toxicity has been studied extensively and it has been demonstrated that ALA-induced injury is caused by oxygen radicals (*4*, *5*). Oteiza and Bechara (*6*) reported that ALA induces lipid peroxidation and increased membrane permeability by a similar mechanism with various prooxidants.

The addition of exogenous ALA inhibits porphyrin biosynthesis with a resulting accumulation of the porphyrin precursor molecules. It must be noted that in a limited number of cases no porphyrin precursors were found (*7*). In *Pseudomonas aeruginosa*, which produces extracellular uroporphyrin and coproporphyrin, ALA levels above 2 mM are toxic to the cells, resulting predominantly in the secretion of uroporphyrin (*3*).

Some scavengers of oxygen radicals have been found to prevent the ALA-induced accumulation of porphyrin precursors. For example, in hepatocyte cultures, ascorbic acid inhibits ALA-induced uroporphyrin accumulation (*8*). In cucumber, ALA reduces the photosynthetic activity through the accumulation of porphyrin intermediates such as protochlorophylide, an effect that can be alleviated by the introduction of singlet oxygen-scavenger molecules (*9*). Although the use of radical scavengers alleviates ALA toxicity to some extent, large numbers of scavenger molecules are consumed, making this process energy inefficient.

Lately, Matsufuji *et al.* (*10*) described two strains of bacteria, *Microbacterium* sp. M874 and *Corynebacterium aquaticum* S365, which produce substantial amounts of galactoglycerolipids. The lipids have a (2′S),2′,3′-di-*O*-acylglycerol-β-D-galactopyranoside structure and high anti-oxygen radical activity. As galactoglycerolipids have already been reported to be present at significant levels in plant chloroplast membranes (*11*), the effectiveness of glycolipids in protecting against ALA-induced cellular lesions is an interesting possibility. In this paper, ALA-induced lesions were analyzed in two organisms, a bacterium (*Corynebacterium flavescens*) and a plant (*Raphanus sativus*).

Recently, a flocculant-enhanced-adhesion-technique of a bacterium to a plant was performed in which, through a polysaccharide flocculant, H12, a symbiotic system was achieved between kaiware radish and an antibiotic pro-

[1] For correspondence: Tel: +81-3-5541-2731, Fax. +81-3-5541-2737, E-mail: knakata@mb.infoweb ne jp

Abbreviations: ALA, 5-aminolevulinic acid, DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; *t*BHP, *tert*-butyl hydroperoxide.

ducer, *Pseudomonas fluorescens*. In this report, to ensure a consistent administration of M874 glycolipid to radish, a similar symbiotic system with minor modifications was employed.

MATERIALS AND METHODS

Microorganisms and Plants—*C. flavescens* ATCC 10340 was used for the analysis of bacterial porphyrins. *Bacillus subtilis* IFO 3027 was used for tests of anti-oxygen-radical activity, as *C. flavescens* produces small amounts of anti-oxygen radical glycolipids. Seeds of kaiware radish (*Raphanus sativus*) were commercially supplied. Leaves of *R. sativus*, *Chrysanthemum frutescens*, and *Begonia semperflorens* were gathered from a private garden during the flowering season.

Materials—A galactoglycerolipid, M874, di-*O*-12-methyltetradecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, was prepared from the culture broth of *Microbacterium* sp. M874 as described previously (*10*). The preparative method of a polysaccharide flocculant from *Klebsiella pnuemoniae* H12 has been described previously (*12*). MGDG and DGDG were supplied by Wako Pure Chemical Industries. WST-1 [2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] was supplied by SIGMA Chemical. All other reagents and constituents for all media were of commercial grade quality.

Cultivation Conditions of Corynebacterium—*C. flavescens* were cultivated overnight in 250-ml Erlenmeyer flasks containing 30 ml of YMPG medium [1% glucose, 0.3% yeast extract (Difco), 0.3% malt extract (Difco), 0.5% polypepton (Nihon Pharmaceutical), pH 7.0]. An aliquot (0.3 ml) of this culture was added to a 30 ml fresh YG medium (pH 7.0) [yeast extract (Difco) 0.5%, glycerol 2%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.1%, pH 7.0] and the cells were cultured for 3 days at 28°C on a rotary shaker (220 rpm with a 50-mm stroke). For porphyrin production, ALA solution was filtered through a 0.45 μm cellulose acetate filter and added to the medium at the start of cultivation. Glycolipids (1% methanol solution) were then added to achieve the correct concentration. A blank of methanol was prepared in a similar manner. In the scavenger molecule tests, the glycolipids were replaced by an aqueous solution (10%) of ascorbic acid, a reduced form of glutathione, or by a methanol solution of α-tocopherol.

Analysis of Bacterial Porphyrins—Culture filtrate (400 μl) was freeze-dried and suspended in a solution of 95% methanol and 5% sulfuric acid. The mixture was maintained at room temperature overnight in the presence of dim light, after which an equal volume of chloroform was added. The resulting solution was sequentially washed with water to remove both the sulfuric acid and any unreacted porphyrins. The concentration of each porphyrin was determined by HPLC [mobile phase; 75% acetonitrile, column; super ODS (15 cm, Toso), oven temperature; 35°C and photodiode array detector].

Preparation of Plant Monogalactosyldiacylglycerols—Leaves (100 g) from *R. sativus*, *C. frutescens*, and *B. semperflorens* were gathered and immersed in 200 ml water. The material was mechanically blended for 3 min and the resulting suspension was extracted with 300 ml of chloroform. The aqueous phase was extracted with three 300 ml volumes of solvent (chloroform:methanol = 1:1). The solvent washings were combined and the solvent was removed under reduced pressure (rotary evaporated). The following method was the same as that described previously for *Microbacterium* sp. M874 (*10*). In two TLC-separation systems [silica gel thin layer (Merck 5715) chromatography developed with chloroform:methanol:water = 80:15:1, and reversed phase silica gel thin layer (Whatman LKC18F) chromatography developed with 90% methanol], the R_f-values of all glycolipids were identical with those of MGDG, and an orcinol–sulfuric acid reagent gave an identical brown-purple color. The ^{13}C-NMR spectra exhibited characteristic signals due to diacylglycoglycerolipid. The GLC analysis of hexitol acetate derivatives of the sugar, obtained by hydrolysis, reduction, and acetylation, showed the existence of galactose.

Rescue of Colony-Forming Ability from tBHP-Treated Cells—*B. subtilis* were cultivated at 28°C overnight (rotary shaken) in a 250-ml Erlenmeyer flask containing 30 ml of YMPG medium. An aliquot (0.3 ml) of this culture was added to 30 ml fresh YMPG medium and 1 ml aliquots were transferred into test tubes. After 2 h of constant shaking at 28°C, a 1% volume of glycolipid methanol solution was added to each test tube to achieve 100 mg/liter. As a blank, methanol was similarly added. After 1 h incubation, a 1% volume of 100 g/liter aqueous solution of *t*BHP was added, and the mixtures were incubated for 30 min. Following centrifugation, the cells were suspended in an equal volume of fresh YMPG medium and further incubated. A portion was withdrawn intermittently and the colony-forming number was measured by plating on a YMPG plate.

Anti-Oxygen Radical Activity Assessment by the WST-1 Method—WST-1, an artificial electron receptor molecule, is freely incorporated into bacterial cells and readily converted to formazan by bacterial glycolytic dehydrogenase (*13*). Because of this activity it has frequently been used as an indicator of cellular metabolic activity. The mechanism of its incorporation into bacterial cells was previously described in *B. subtilis*, which was used as an assay strain (*10*).

Addition of Fatty Acids—*C. flavescens* were cultivated overnight in 250-ml Erlenmeyer flasks containing 30 ml of YMPG medium. An aliquot (0.3 ml) of this culture was added to 30 ml of fresh YG medium (pH 7.0) containing 100 mg/liter of a fatty acid and 100 mg/liter of the M874 glycolipid in 250-ml Erlenmeyer flasks. After 2 h of cultivation, a filter-sterilized ALA solution (5 mM) was added to the medium. After a further 3 days of cultivation, the culture was centrifuged at 15,000 rpm for 10 min and the OD_{400} of the filtrate was measured.

Radish Cultivation Conditions—Ten fresh radish seeds were sown on wet absorbent cotton in a transparent hole (35 mm Φ × 18 mm) on a microtiter plate, which was then placed in a transparent box filled with a small amount of water to maintain moisture. The seeds were incubated at 25°C under a 1,000 lx fluorescent light lamp. The initial volume of tap water (including 1 g/liter of $MgCl_2 \cdot 2H_2O$) was 10 ml and this level was maintained throughout the cultivation by topping up with tap water. At the sprouting phase (48 h later), several concentrations of aqueous ALA solutions were added. After a further 48 h incubation, stem length was measured. The longest and shortest stems were omitted from the analysis and the average of the remaining eight scores was calculated. Experiments were carried out

6 times and the average stem length was calculated.

Radish Cultivation with Adherent Microbacterium sp. M874 Cells—Radish seeds were incubated as described above. Twenty-four hours later, several volumes of 2-day culture broth of M874 in YSG medium [0.3% yeast extract, 0.3% soy flour, 2% glucose, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, 0.2% $CaCO_3$ at pH 7], 1 g/liter aqueous solution of H12 polysaccharide (500 μl), and a 3% solution of $CaCl_2 \cdot 2H_2O$ (100 μl) were added in this order. After a further 24 h incubation, several concentrations of ALA solution were added. After a further 48 h incubation, stem length was measured as described above.

Analysis of Plant Chlorophyll—On day 4 of cultivation, 40 radish leaves were gathered and immersed in 3 ml of acetone in light-shielded screwed-capped glass tubes. Following a 30 min sonication, the mixtures were centrifuged at 15,000 rpm for 10 min. The supernatants were filtered through 0.2 μm pore diameter syringe filters, and the absorbance at 430 nm was measured.

RESULTS

Effects of ALA on Porphyrin Production by Corynebacterium—Corynebacterium flavescens ATCC 10340 secreted roughly 50 mg/liter porphyrin compounds in response to the exogenous addition of 300 mg/liter (2.29 mM) ALA. In the absence of ALA addition, porphyrins were scarcely detected. The two main porphyrin products were coproporphyrin III and uroporphyrin III. The initial concentration of ALA was changed from 0 to 6 mM and the formation of porphyrin derivatives was analyzed (Fig. 1). At ALA concentrations less than 3 mM, porphyrin productivity increased proportionally with ALA concentration, while ALA concentrations in excess of 3 mM inhibited porphyrin production. ALA more severely inhibited the production of coproporphyrin more severely than that of uroporphyrin.

The effect of illumination strength on ALA-containing cultures was investigated using three different light intensities (0, 300, 3,000 lx). Continuous illumination decreased the production of both porphyrin derivatives as did increased light intensity. Illumination enhanced the sensitivity of the cultures to ALA, with a 6 mM ALA concentration proving cytotoxic to the bacterium at 3,000 lx.

Effects of M874 Glycolipid on the ALA-Induced Porphyrin Lesions in Corynebacterium—M874 glycolipid (142 μM) was added to the ALA-containing bacterial culture under

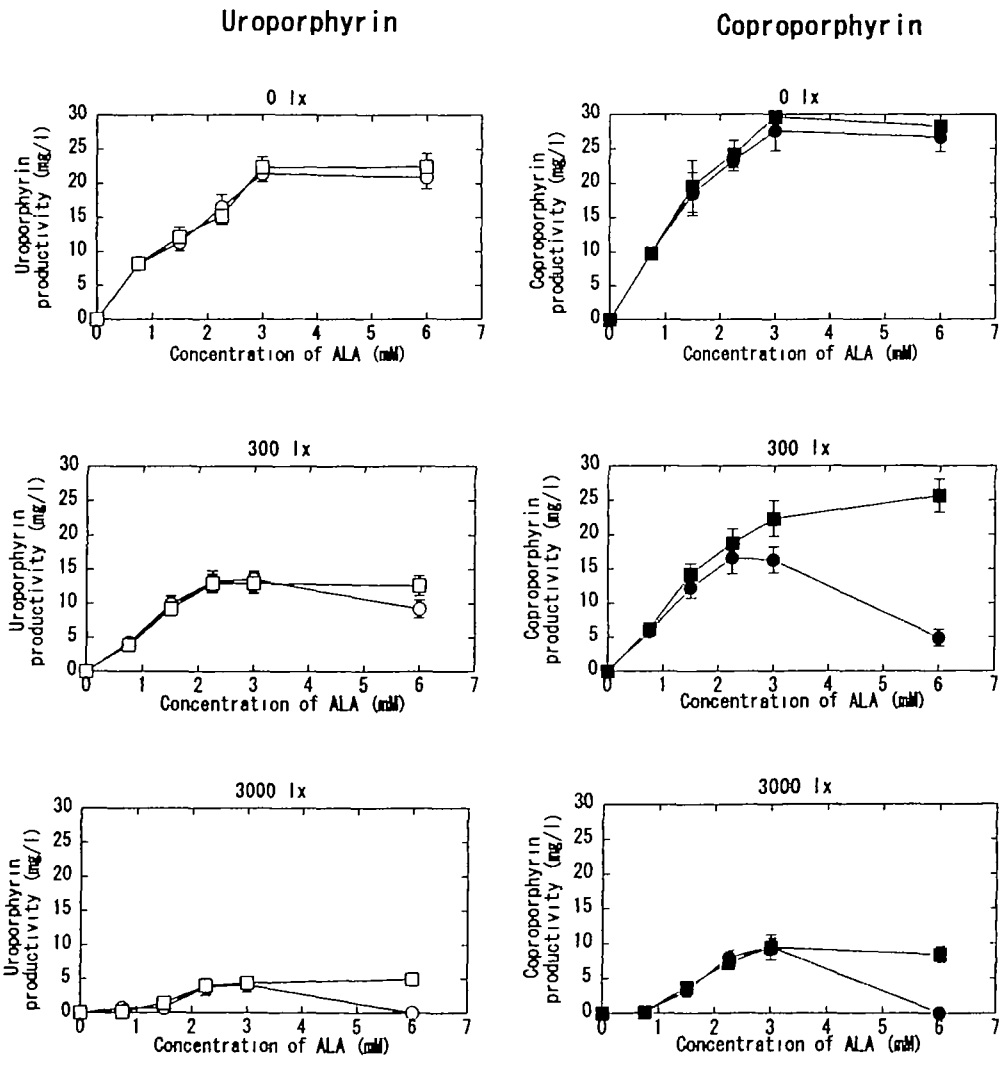

Fig 1. **Effect of ALA concentration and light intensity on bacterial porphyrin productivity.** The extracellular productivity of porphyrin derivatives was measured in a shaking culture of *C. flavescens* under several conditions of ALA concentration and light intensity. Initial concentrations of ALA were changed between 0 and 6 mM. Light intensity was varied (0, 300, and 3,000 lx). Uroporphyrin and coproporphyrin were measured by an HPLC method after methyl esterification. Data represents mean for three times experiment and error bars represents the SD-value. Symbols: □ and ■, in the addition of 100 μM M874 glycolipid, ○ and ●; in a blank condition.

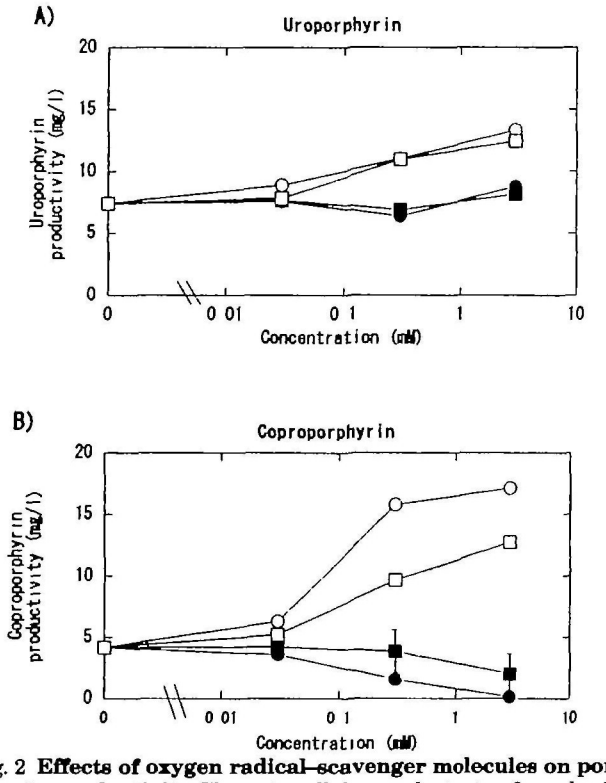

Fig. 2 **Effects of oxygen radical-scavenger molecules on porphyrin productivity.** The extracellular productivity of porphyrin derivatives was measured in a shaking culture of *C. flavescens* with several concentrations of scavenger molecules. ALA-concentration was fixed at 6 mM and light intensity was 300 lx. Symbols ○, M874 glycolipid, ●; ascorbic acid sodium salt, □, α-tocopherol, ■, reduced form glutathione.

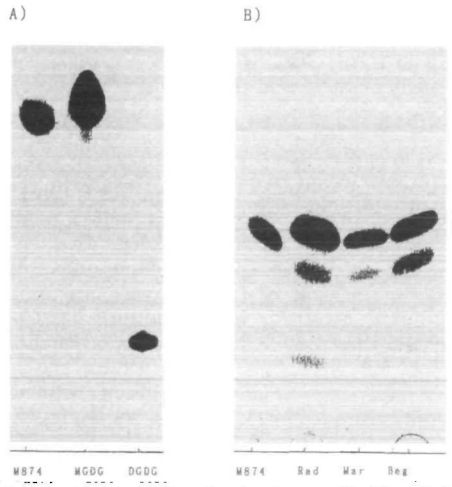

Fig. 3 **TLC-patterns of galactoglycerolipids.** Galactoglycerolipid samples, which were newly prepared from three plants, were analyzed on TLC, as well as M874 glycolipid and two commercially supplied galactoglycerolipids. TLC conditions: silica gel thin layer; Merck 5715, developing solution; chloroform.methanol·water = 80:15:1, detection; spraying an orcinol reagent [(sulfuric acid:water = 11.4:88.6) containing 2% orcinol] followed by heating at 110°C for 10 min. Abbreviations: Rad, Kaiware radish (*Raphanus sativus*); Mar, margaret (*Chrysanthemum frurescens*); Beg, begonia (*Begonia semperflorens*).

several illumination conditions, and porphyrin productivity was analyzed (Fig. 1). In the absence of light, the addition of the glycolipid caused a slight increase in uroporphyrin and coproporphyrin productivity. Under illuminated conditions, M874 glycolipid promoted the production of porphyrin, especially coproporphyrin. This enhancement was somewhat restricted under 3,000 lx conditions. The glycolipid also increased the resistance to ALA under illuminated conditions. These results indicate an eminent ability of the M874 glycolipid to protect against ALA- and light-induced porphyrin lesions.

Comparison of the M874 Glycolipid with Three Scavenger Molecules—Several concentrations of ascorbic acid, glutathione, and α-tocopherol were tested for their abilities to protect against ALA (6 mM)- and light (300 lx)-induced cellular lesions in *C. flavescens* (Fig. 2). Ascorbic acid and glutathione prevented both uroporphyrin and coproporphyrin synthesis. In contrast, α-tocopherol and M874 glycolipid enhanced production.

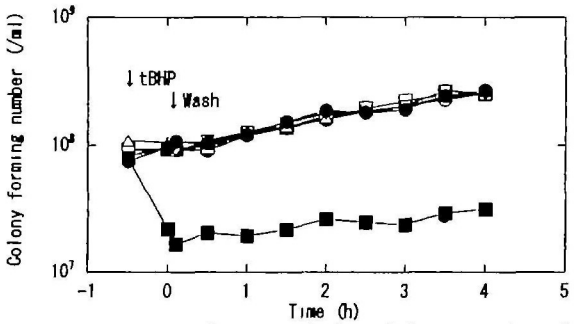

Fig 4 **Prevention of the *t*BHP-induced decrease in colony-formation by M874 glycolipid.** *B. subtilis* IFO 3027 was cultivated in a YMPG medium containing the tested glycolipid samples (100 mg/liter). One hour later, 11 2 mM *t*BHP was added to the culture, which was washed for a further 30 min The number of colonies was calculated by periodical plating on YMPG agar medium. Symbols ●, without *t*BHP and glycolipid, ○, with *t*BHP and M874 glycolipid, □, with *t*BHP and glycolipid from kaiware radish, △, with *t*BHP and glycolipid from margaret; ▽, with *t*BHP and glycolipid from begonia; ■, with *t*BHP but without glycolipid.

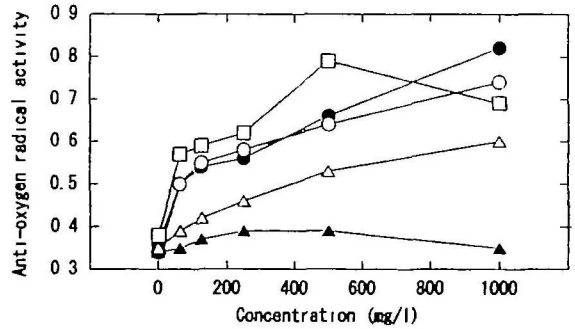

Fig 5 **Anti-oxygen radical activity of glycolipids measured by the WST-1 method.** *B. subtilis* IFO 3027 were cultivated in YMPG medium containing 0–1,000 mg/liter of the tested glycolipid sample and 5 mM *t*BHP After 1 h incubation, the WST-1 reagent was added as described previously. After a further 4 h incubation, the absorbance at 450 and 630 nm of the culture filtrate was measured by a photometer. A_{450}–A_{630} was regarded as the anti-oxygen radical activity. Symbols: ●, M874 glycolipid; □, glycolipid from radish, ○, glycolipid from margaret; △, glycolipid from begonia, and ▼, DGDG from spinach.

Galactoglycerolipids in Plants—Monogalactodiacylglycerolipids were partially purified from 100 g of leaves from three plant species as described in "MATERIALS AND METHODS." The TLC-pattern on silica gel is shown in Fig. 3. The yield was relatively low, *i.e.*, 2.3 mg (*C. frutescens*), 3.1 mg (*B. semperflorens*), and 3.9 mg (*R. sativus*), as compared to the bacterium M874, as 100 g of wet M874 cells contain 150 mg of glycolipids.

Anti-Oxygen Radical Activity of Galactoglycerolipids—The anti–oxygen radical activity of galactoglycerolipids was tested using two methods. The colony forming number of *B. subtilis* was significantly reduced upon the addition of 1,000 mg/liter (11.2 mM) *t*BHP to the culture. The addition of 100 μM of M874 glycolipid reversed this effect (Fig. 4). Similarly, this decrease in *B. subtilis* was completely reversed by the galactoglycolipids from each of the three plant species at a concentration of 100 μM. This suggests that the tested plants have their own protector molecules against ALA-induced cellular lesions. Commercially supplied MGDG from spinach also showed anti–oxygen activity, while DGDG did not (data not shown).

The newly isolated glycolipids were next assessed for anti–oxygen radical activity by the WST-1 method (Fig. 5). The *t*BHP-induced-decrease in absorbance at 450 nm was significantly inhibited with the anti–oxygen radical effect dependent on the concentration of the glycolipids. Commercially supplied MGDG from spinach also exhibited anti-oxygen activity (data not shown), while DGDG did not.

Effects of Plants Galactoglycerolipids on ALA-Induced Porphyrin Lesions in Corynebacterium—Plant galactoglycerolipids (100 or 1,000 mg/liter) were tested for their ability to reverse the ALA (6 mM)- and light (300 lx)-induced cellular lesions activity of *C. flavescens* (Fig. 6). The results indicated a protecting activity of MGDG against ALA- and light-induced defects in porphyrin synthesis.

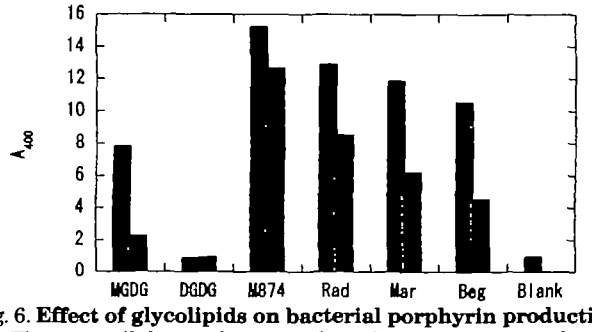

Fig. 6. **Effect of glycolipids on bacterial porphyrin productivity.** The extracellular productivity of porphyrin was measured in a shaking culture of *C flavescens* as the absorbance at 400 nm of the culture filtrate when 1,000 mg/liter (striped bars) or 100 mg/liter (solid bars) of test sample was initially added to the culture. The concentration of ALA was fixed at 6 mM and the light intensity was 300 lx Abbreviations: Rad, kaiware radish; Mar, margaret; Beg, begonia.

Effects of Fatty Acids on ALA-Induced Porphyrin Lesions in Corynebacterium—To investigate the function of MGDG, fatty acids (100 mg/liter) were tested for their ability to change the ALA (5 mM)-induced cellular lesions activity of *C. flavescens* (Table I). Exogenously added saturated fatty acids of lower molecular weight than myristic acid increased ALA-induced lesions, which were reversed by the co-addition of M874 glycolipid. C_{16}-unsaturated fatty acids having more unsaturated bonds than linoleic acid showed similar effects.

Effects of ALA on the Porphyrin Productivity of Radish—Kaiware radish (*R. sativus*) was water-cultivated and ALA-induced cellular disorder was analyzed. When more than 2.5 mM ALA was added to the culture medium 24 h after seed-sowing, roots and leaves gradually changed to a slightly reddish color and root hair formation was considerably inhibited. Stem length on the 4th-day of cultivation was much less than that observed under control conditions (Table II).

The radish leaves were gathered and the amounts of porphyrin were measured on day-4 (Fig. 7). At less than 2 mM ALA, porphyrin productivity increased proportionally with the addition of exogenous ALA. However, the productivity decreased at ALA concentrations greater than 2.5 mM ALA.

TABLE II. **Rescue from ALA-induced decrease in stem length in an adherent system of radish.**

M874 culture broth (%)	H12 bioflocculant (mg/liter)	Stem length (mm) at ALA (mM)		
		0	2.5	4.5
0	0	56.53±6.28	50.07±4.88	37.90±4.23
0	50	57.43±6.56	52.53±4.87	38.63±4.35
0.1	50	56.05±5.83	55.40±6.23	48.05±4.85
1	0	59.55±6.54	53.15±5.85	42.70±4.85
1	50	57.60±4.52	59.67±6.21	49.23±5.23

Data represent mean ± SD for 30 samples.

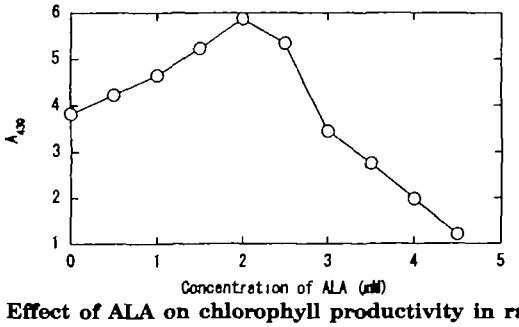

Fig. 7. **Effect of ALA on chlorophyll productivity in radish.** Radish was water-cultivated with the addition of 0–4.5 mM ALA, and chlorophyll productivity was measured as the absorbance at 430 nm as described in "MATERIALS AND METHODS."

TABLE I. **Effects of fatty acids on porphyrin synthesis by *C. flavescens*.**

ALA (mM)	M874 glycolipid (mg/liter)	Fatty acids (100 mg/liter)							
		Stearic acid	Palmitic acid	Myristic acid	Lauric acid	Oleic acid	Linoleic acid	Linolenic acid	Blank
0	0	0.34	0.28	0.35	0.21	0.27	0.27	0.32	0.31
5	0	3.90	5.10	0.46	0.31	5.10	0.26	0.33	4.48
5	100	8.75	8.56	7.85	5.31	9.23	7.93	2.22	8.22

Analytical methods are described in "MATERIALS AND METHODS."

TABLE III **Amount of MGDG in radish leaves.**

Exogenously added			Intracellular MGDG (mg/100 g leaves)
M874 culture broth (%)	H12 bioflocculant (mg/liter)	M874 glycolipid (mg/liter)	
0	0	0	3 1
1	0	0	3.9
1	50	0	10 2
0	0	500	3 5

MGDG was purified from 100 g of radish leaves on day-5 and the amount was measured.

Several concentrations of M874 glycolipid were administered to the water-cultures or onto the leaves, but neither treatment prevented growth inhibition or the porphyrin lesions induced by higher concentrations of ALA (data not shown).

Rescue from ALA-Induced Radish Lesions in an Adhesion-Enforced System with M874-Producer Bacteria—The co-addition of 1%- or 0.1%-volume of M874 culture broth and 50 mg/liter of a H12 flocculant polysaccharide to a radish water culture caused a considerable regeneration of 4.5 mM ALA-induced growth inhibition (Table II) to 85% of its pre-ALA level. The level of porphyrin in an adherent system also increased about twofold compared to non-adherent conditions (Fig. 8). In this case, the restored level was as much as 60% of that without bacterium and ALA. The level of MGDG in radish leaves in an adherent system increased about threefold compared to non-adherent conditions, although the addition of purified M874 glycolipid did not increase the amount, suggesting that increased intracellular MGDG causes the protection from ALA-induced cellular lesions (Table III).

DISCUSSION

The main goal of this study was to investigate how plants protect themselves from oxygen radicals produced during photosynthesis. The use of radical-scavenging systems implies high energy consumption, so an alternative is highly desirable. It is possible that some other low energy consuming systems might be used *in vivo*. Even though ALA is the principal precursor of porphyrin, ALA synthesis is held strictly at a low level. ALA is cytotoxic in many organisms and its mechanism of toxicity has been found to depend on oxygen radical levels (*4, 5*). In this paper, the decrease in bacterial porphyrin at 6 mM ALA under intense light was significantly rescued by 100 μM M874 glycolipid, indicating the high rescuing activity of the lipid from ALA-induced cellular lesions.

Exogenously added M874 glycolipid was immediately incorporated into the cells of *C. flavescens* (data not shown). Farr and Kogoma (*11*) reported that when fatty-acid chains become shortened or gain charges through peroxidation, their ability to rotate within the membrane increases and the membrane becomes more fluid. The enforced ALA-induced injury of *C. flavescens* by short-length fatty acids (*e.g.* lauric acid and myristic acid) and highly unsaturated fatty acids (*e.g.*, linoleic acid and linolenic acid) was degenerated by M874 glycolipid (Table I). The results suggest that the role of membrane-incorporated MGDG is to increase membrane rigidity, although some other possibilities can not be excluded.

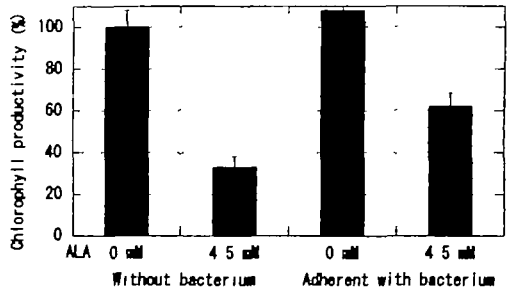

Fig 8 **Prevention of ALA-induced chlorophyll deficiency in radish adherent with a glycolipid producer bacterium.** Radish adhered with *Microbacterium* sp M874 cells was water-cultivated in the presence of 4.5 mM ALA. Chlorophyll productivity was measured as described in "MATERIALS AND METHODS." Three control experiments were also carried out and the amount of chlorophyll at 0 mM ALA without bacterial adhesion was regarded as 100%. Data represents means of three experiments and error bars represent the SD-values

Galactoglycerolipids found in plants were found in the present study actively to protect the plants against oxygen radicals and ALA-induced porphyrin lesions in *C. flavescens*. Galactoglycerolipids are ubiquitous in Gram-positive bacteria and plant chloroplasts, but their physiological role has never been elucidated. Although MGDG is one of the main plant galactoglycerolipids species, it is unevenly distributed throughout the plant body and is abundant in plastid membranes. M874 glycolipid has two C15:0 anteiso fatty acid moieties, and the main fatty acids in spinach are predominantly C18:3 and C16:0 (*12*). The anteiso type is rare, so plant galactoglycerolipids might differ structurally from M874 glycolipid. As the quantity of galactoglycerolipids is relatively low in plants tissues, compared with bacterium M874, it is probable that plants MGDG might be functional at low concentration.

The exogenous addition of M874 glycolipid to radish cultures did not prevent ALA-induced cellular lesions. In the experiment, the amount of MGDG was never changed in the extracts of root tissues with organic solvent (Table III) and the extracellular level of M874 glycolipid did not change significantly. H12 polysaccharide flocculant has recently been reported to be effective in enforcing an adhesion enforcement of a bacterium to a plant in a water culture, and enforced adhesion of an antibiotic-producing bacterium to radish roots is exemplified there (*13, 14*). In the present report, coculture of radish and M874 glycolipid producer bacterium (*Microbacterium* sp. M874) resulted in a significant prevention of ALA-induced plant cellular injury only under enforced adhesion conditions through the action of H12 polysaccharide. In this system, physiologically active products of a bacterium might be incorporated effectively into a plant vessel and transported to the distal plant tissues. Although the intracellular MGDG level in plant leaves increased about threefold, the level (10.2 mg/100 g tissue) was about 1/10 of the level in *Microbacterium* sp. M874 or *C. flavescens*, including exogenously added M874 glycolipid. More efficient methods to increase the intracellular level would be desirable.

It is possible that MGDG plays an important role in porphyrin synthesis and photosynthesis in plants. Additionally, it might be useful in the enforcement of photosynthesis under the oxygen radicals–rich conditions.

REFERENCES

1 Sasaki, K., Marquez, F.J., Nishio, N., and Nagai, S. (1995) Promotive effect of 5-aminolevulinic acid on the growth and photosynthesis of *Spirulina platensis*. *J Ferment. Bioeng.* **79**, 453–457

2 Tanaka, T, Takahashi, K., Hotta, Y, Takeuchi, Y., and Konnnai, M (1992) 5-Aminolevulinic acid as plant growth stimulator. *Proc. Plant Growth Regul Soc. Am.* **19**, 237–242

3 Harris, W.F III, Burkhalter, R S., Lin, W., and Timkovich, R. (1993) Enhancement of bacterial porphyrin biosynthesis by exogenous aminolevulinic acid and isomer specificity of the products. *Bioorg. Che.* **21**, 209–220

4 Vercesi, A.E , Castilho, R.F., Meinicke, A.R., Ualle, V.G R , Hermes-Lima, M., and Bachara, E.J.H (1994) Oxidative damage of mitochondria induced by 5-aminolevulinic acid, role of Ca^{2+} and membrane protein thiols. *Biochim. Biophys. Acta* **1188**, 86–92

5 Onuki, J., Medeiros, M.H G , Bechara, E.J.H., and Mascio, P D (1994) 5-Aminolevulinic acid induces single-strand breaks in plasmid pBR322 DNA in the presence of Fe^{2+} ions *Biochim. Biophys. Acta* **1225**, 259–263

6. Oteiza, P.I. and Bechara, E.J H (1993) 5-Aminolevulinic acid induces lipid peroxidation in cardiolipin-rich liposomes. *Arch Biochem. Biophys.* **305**, 282–287

7 Shioi, Y , Doi, M , Tanabe, K., and Shimokawa, K. (1988) Inhibition of porphyrin biosynthesis by exogenous 5-aminolevulinic acid in an aerobic photosynthetic bacterium, *Erythrobacter* sp OCh 114 *Arch. Biochem. Biophys.* **266**, 478–485

8 Sinclair, P.R., Gorman, N., Walton, H S , Berment, W.J., Jacobs, J.M., and Sinclair, J.F (1993) Ascorbic acid inhibition of cytochrome P450-catalyzed uroporphyrin accumulation. *Arch. Biochem. Biophys.* **304**, 464–470

9. Chakraborty, N and Tripathy, B.C (1992) Involvement of singlet oxygen in 5-aminolevulinic acid-induced photodynamic damage of cucumber (*Curumis Sativus* L) chloroplasts. *Plant Physiol.* **98**, 7–11

10. Matsufuji, M., Nakata, K., and Yoshimoto, A. (2000) Glycoreglycolipids preventing *tert*-butylhydroperoxide-induced cell death from *Microbacterium* sp and *Corynebacterium aquaticum* strains. *J. Biosci. Bioeng* in press

11 Farr, S B and Kogoma, T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55**, 561–585

12. Harwood, J L and Russell, N.J (1984) *Lipids in Plants and Microbes*, p. 42, George Allen Unwin, London

13. Nakata, K. and Kurane, R (1999) Production of an extracellular polysaccharide bioflocculant by *Klebsiella pneumoniae*. *Biosci. Biotechnol. Biochem.* **63**, 2064–2068

14 Nakata, K., Harada, N., Sumitomo, K., and Yoneda, K. (2000) Enhancement of plant stem growth by flocculation of the antibiotic-producing bacterium, *Pseudomonas fluorescens* S272, on the roots. *Biosci. Biotechnol Biochem.* **64**, 459–465