

## Growth of the Cyanobacterium *Synechococcus leopoliensis* CCAP1405/1 on Agar Media in the Presence of Heterotrophic Bacteria

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The cyanobacterium *Synechococcus leopoliensis* CCAP1405/1 does not grow on common solid media made of agar, agarose HT, noble agar, gelrite and gelatin, although it grows in liquid media with the same components. The inoculation of *S. leopoliensis* CCAP1405/1 at a high initial cell density allowed it to grow on the agar media, and co-inoculation with one of the heterotrophic bacterial strains belonging to a wide range of phylogeny, showed the same effects even at a low initial cell density of *S. leopoliensis* CCAP1405/1. The addition of thiosulfate and high concentrations of vitamin B<sub>12</sub>, biotin and thiamine also supported growth on solid media, but catalase had no effect. On inorganic solid media, the autotrophic cyanobacterial strain supported the growth of heterotrophic bacteria, suggesting mutual interaction.

**Key words:** cyanobacteria, *Synechococcus*, solid media, mutual interaction

Agar, which consists of agarose and agarpectin, is a valuable material in microbiology and has been used for preparing solid media to grow microorganisms. However, certain cyanobacterial strains, including *Anabaena* sp. (1, 36), *Anacystis nidulans* (2), *Prochlorococcus* sp. (18), *Synechococcus* sp. (36), and *Synechocystis* sp. (36), do not grow on agar though they grow in liquid media with the same components.

Marler and Baalen (16) reported that the H<sub>2</sub>O<sub>2</sub> arising from the reaction of citrate with Mn<sup>2+</sup> in a medium following autoclaving inhibited the growth of single cell of *A. nidulans* on the surface of, or in, agar. Allen (2) suggested that toxic products formed when agar was autoclaved with a mineral medium. Allen and Gorham (1) also reported inhibitory effects of products of agar following autoclaving but excluded the involvement of H<sub>2</sub>O<sub>2</sub> because they observed no adverse effect on cyanobacterial strains exposed to H<sub>2</sub>O<sub>2</sub> at 176 mM for 24 hours. However, the growth of *A. nidulans* and *Anabaena variabilis* was reported to be inhibited by H<sub>2</sub>O<sub>2</sub> at 0.11–1.1 mM in batch cultures (26). In the case of bacterial or fungal strains, a fatty acid in agar (14), acid-hydrolyzed agar (24, 42), and H<sub>2</sub>O<sub>2</sub> (3, 13) have been reported as inhibitory substances. Thus, different mechanisms seem to be involved in the inhibition of growth on agar media. In addition, drying stress might inhibit growth on agar, and the quality of the agar used in studies in the 1960s might be a factor.

Several attempts have been made to grow cyanobacterial strains on agar media. Allen and Gorham (1) and Waterbury and Willey (40) showed that washing agar enabled several cyanobacterial strains to grow. Thiel *et al.* (36) reported that cyanobacterial strains efficiently formed colonies when agar was autoclaved separately from mineral medium, and thiosulfate was added to the medium. Marler and Baalen (16) and Morris *et al.* (19) added catalase to agar to breakdown

H<sub>2</sub>O<sub>2</sub>. Use of a solidifying agent at higher purity or a lower concentration was reported to be another method of culturing cyanobacteria (18, 30, 38).

Recently, Morris *et al.* (19) showed that the co-culture of several heterotrophic bacterial strains improved the colony-forming ability of *Prochlorococcus* sp. strains on 0.42% noble agar medium. They also showed that a catalase-deficient mutant of *Vibrio fischeri* ES114 lost the ability to grow *Prochlorococcus* sp. MIT 9215 on the agar medium, and suggested that H<sub>2</sub>O<sub>2</sub> in the medium inhibited the cyanobacterial growth and the heterotrophic bacterial strain helped it by producing catalase. Intimate interactions between cyanobacteria and heterotrophic bacteria have been reported in several ecosystems, including a river biofilm (15), on the surface of concrete (5), in an oil-degrading consortium (27), and in lichen (7). In these cases, cyanobacteria and heterotrophic bacteria are assumed to exchange nutrients such as fixed nitrogen, organic matter, and/or substances essential for their growth. However, there has been only one report (19) dealing with the mutual interaction of cyanobacteria and heterotrophic bacteria on solid media.

*Synechococcus* has been reported as the dominant cyanobacterium in aquatic environments, and contributes to primary production in ecosystems (22, 33). We have been cultivating the cyanobacterium *Synechococcus leopoliensis* CCAP1405/1 in liquid media, and found that the strain was originally contaminated with several kinds of bacteria. In the process of purifying this strain, we found that it does not grow on agar media with the same composition as the corresponding liquid media, but does grow on agar when the bacterial strain C-Pink1, which is most homologous with *Porphyrobacter* sp., is co-cultured on the same plate.

In this study, we isolated a number of heterotrophic bacteria from soil and river biofilms, and examined their ability to support growth of *S. leopoliensis* CCAP1405/1 on agar media. Second, we investigated the effects of the composition of the medium, concentration of agar, and initial cell density of *S. leopoliensis* CCAP1405/1 on growth in the

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co-cultures. Third, we examined the effects of additives such as thiosulfate, vitamins, and catalase on the growth of *S. leopoliensis* CCAP1405/1. Finally, we showed that H<sub>2</sub>O<sub>2</sub> is not involved in the growth inhibition of *S. leopoliensis* CCAP1405/1 on agar media.

## Materials and Methods

### Microorganisms

The bacterial strains used are listed in Table 1. *S. leopoliensis* CCAP1405/1 was purchased from the Culture Collection of Algae and Protozoa (Oban, UK) in 2005, and has been subcultured in CT liquid medium (Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O 0.150 g, KNO<sub>3</sub> 0.1 g, β-Na<sub>2</sub> glycerophosphate-nH<sub>2</sub>O 0.05 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.04 g, vitamin B<sub>12</sub> 0.1 μg, biotin 0.1 μg, thiamine HCl 10 μg, PIV metal solution 3 mL, *N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) 0.4 g L<sup>-1</sup> [pH 8.2], PIV metal solution: FeCl<sub>3</sub>·6H<sub>2</sub>O 0.196 g, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.036 g, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.022 g, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.004 g, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.0025 g, Na<sub>2</sub>EDTA·2H<sub>2</sub>O 1 g L<sup>-1</sup>) with standing at 20°C under a 12-hour light-dark cycle at 4000 lux. *S. leopoliensis* CCAP1405/1 was originally contaminated with several strains of bacteria checked by a plate culturing method. We attempted to eliminate these bacteria by repeating the culture on CT agar and 1/16PYG+Sp agar (glucose 0.031 g, yeast extract 0.063 g, polypeptone 0.125 g, sodium pyruvate 0.15 g, agar 15 g L<sup>-1</sup> [pH 7.0]) (34, 35) under the conditions described above, but pink colonies appeared with the green colonies of *S. leopoliensis* CCAP1405/1 on the plates at all times (Fig. 1). The bacterial strain C-Pink1 was isolated, and DNA extraction, PCR amplification of 16S rRNA genes, and determination of a partial nucleotide sequence (1397 bp) and of its most homologous sequence were performed according to procedures described previously (9). The 16S rRNA gene sequence of this strain was most homologous (100%) with that of *Porphyrobacter* sp. KK351 (accession number: AB033326). The culture of *S. leopoliensis* CCAP1405/1 was removed with a toothpick from an independent green colony which was separated from the colony of C-Pink1 on 1/16PYG+Sp agar (Fig. 1) and inoculated into CT liquid medium because it could not make any colony on the corresponding agar medium by itself. Axenic status was confirmed by the absence of heterotrophic growth on 1/16PYG+Sp, 1/10PTYG (peptone 0.25 g, tryptone 0.25 g, yeast extract 0.5 g, glucose 0.5 g, Mg SO<sub>4</sub>·7H<sub>2</sub>O 0.03 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.003 g L<sup>-1</sup> [pH 6.8]) (12), and LB (tryptone 10 g, yeast extract 5 g, NaCl 10 g L<sup>-1</sup>

[pH 7.0]) agar media, other cells in epifluorescence microscopic observation (BHT-RFK-A1 equipped with PM-10ADS-1, OLYMPUS, Tokyo, Japan) with ethidium bromide-staining (32), and other bands in 16S rRNA gene PCR-DGGE examined according to procedures described previously (10).

To examine whether the interaction between *S. leopoliensis* CCAP1405/1 and C-Pink1 occurs in other heterotrophic bacteria, several bacterial strains isolated from a soil and river biofilms (Table 1) were tested for their ability to support *S. leopoliensis* CCAP1405/1 on agar media. To collect the soil bacteria, a diluted soil suspension made from Honjo A alluvial soil (Matsue, Shimane, Japan) (4) was mixed with a cell suspension of axenic *S. leopoliensis* CCAP1405/1 grown in CT liquid medium and spread onto 1/16PYG+Sp agar medium. After 15 days of incubation under the above mentioned conditions, seven bacterial colonies that allowed *S. leopoliensis* CCAP1405/1 to grow in and/or around them were isolated. In addition, four bacterial strains isolated from Akagawa river biofilms (Izumo, Shimane, Japan) in a previous study (8) were used. They were isolated independently of this study by a dilution plating method using 1/16PYG+Sp agar medium.

### Ability of co-inoculated heterotrophic bacteria to support growth of *S. leopoliensis* CCAP1405/1 on agar media

The ability of the heterotrophic bacterial strains to support *S. leopoliensis* CCAP1405/1 on 1.5% agar was examined using 1/16PYG+Sp, 1/10PTYG, CT, and modified BG11 (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 7.15 g, NaNO<sub>3</sub> 1.5 g, K<sub>2</sub>HPO<sub>4</sub> 0.04 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.075 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.036 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, EDTA 0.001 g, Na<sub>2</sub>CO<sub>3</sub> 0.02 g, trace metal mix A5+Co 1.0 mL L<sup>-1</sup> [pH 7.8], trace metal mix A5+Co: H<sub>3</sub>BO<sub>3</sub> 2.86 g, MnCl<sub>2</sub>·4H<sub>2</sub>O 1.81 g, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.222 g, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.39 g, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.079 g, Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O 0.00494 g L<sup>-1</sup>). (23). *S. leopoliensis* CCAP1405/1 was precultured in CT liquid medium under the conditions mentioned above, diluted with sterilized distilled water, and spread on the agar plates (90 mm id) at 10<sup>5-6</sup> cells plate<sup>-1</sup> as determined based on the OD<sub>600</sub> of the culture. The bacterial strains were precultured on 1/10 PTYG agar medium at 25°C for several days, and each of eight independent colonies (*n*=8) was removed with a sterilized toothpick, and then spotted at 1-cm intervals onto the agar media, which were already inoculated with *S. leopoliensis* CCAP1405/1. Two strains were inoculated onto a plate with *S. leopoliensis* CCAP1405/1. During incubation, the growth of *S.*

**Table 1.** Bacterial strains used in this study

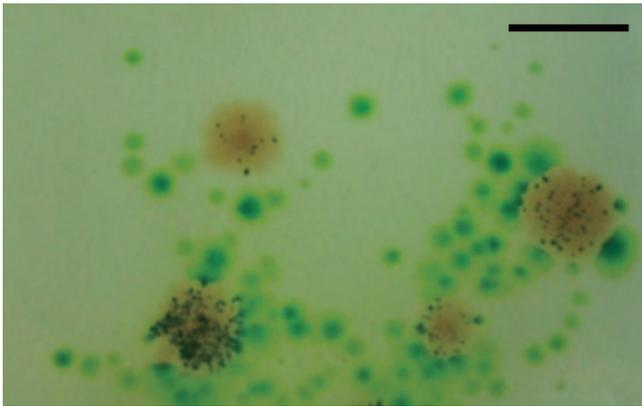
Strain	Genus/species <sup>a)</sup>	Similarity (%)	Accession number	Class	Source <sup>b)</sup>	Catalase activity <sup>c)</sup>	Motility <sup>d)</sup>
CCAP1405/1	<i>Synechococcus leopoliensis</i>	100	AP008231	Cyanobacteria	CCAP	–	–
C-Pink1	<i>Porphyrobacter</i> sp.	100	AB578868	Alphaproteobacteria	CCAP1405/1	–	–
SH-4-1	<i>Bacillus</i> sp.	98	AB578869	Bacilli	soil	–	–
SH-4-2	<i>Sinorhizobium</i> sp.	99	AB578870	Alphaproteobacteria	soil	+	+
SH-4-3-1	<i>Paenibacillus</i> sp.	97	AB578871	Bacilli	soil	++	+
SH-4-3-2	<i>Microbacterium</i> sp.	99	AB578872	Actinobacteria	soil	–	–
SH-5-1	<i>Phycoccus</i> sp.	98	AB578873	Actinobacteria	soil	++	–
SH-5-2	<i>Bacillus</i> sp.	99	AB578874	Bacilli	soil	++	–
SH-7-1	<i>Arthrobacter</i> sp.	96	AB578875	Actinobacteria	soil	+	+
7-11	<i>Pedobacter</i> sp.	99	AB578876	Bacteroidetes	river biofilm	–	–
T-5	<i>Aquamonas</i> sp.	98	AB578877	Betaproteobacteria	river biofilm	–	+
3-7	<i>Stenotrophomonas</i> sp.	99	AB578878	Gammaproteobacteria	river biofilm	++	–
3-8	<i>Pseudomonas</i> sp.	99	AB578879	Gammaproteobacteria	river biofilm	++	+

<sup>a)</sup> Most homologous genus of 16S rRNA gene in DDBJ.

<sup>b)</sup> CCAP, Culture Collection of Algae and Protozoa; CCAP1405/1, isolated from a culture of *S. leopoliensis* CCAP1405/1; soil, isolated from Honjo A alluvial soil; river biofilm, isolated from Akagawa river biofilm.

<sup>c)</sup> Relative activity of bubble formation with H<sub>2</sub>O<sub>2</sub>. ++, + and – represent vigorous, moderate and no bubbling, respectively.

<sup>d)</sup> Microscopic observation.



**Fig. 1.** Growth of *Synechococcus leopoliensis* CCAP1405/1 (green colonies) around/in the colonies of the heterotrophic bacterial strain C-Pink1 (pink colonies) on 1/16PYG+Sp agar (1.5%) medium after incubation for one month. Scale bar, 10 mm.

*leopoliensis* CCAP1405/1 was observed with the naked eye based on the presence or absence of a green color.

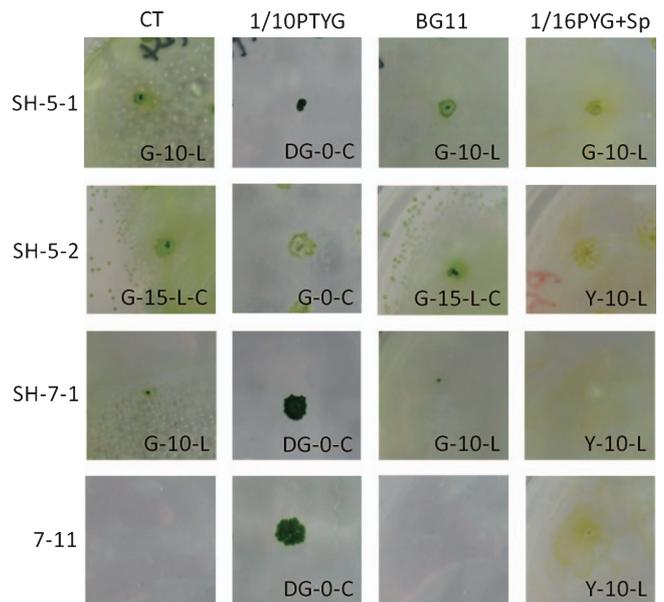
#### *Effects of conditions and initial cell density of S. leopoliensis CCAP1405/1 on the growth on solid media*

The ability of *S. leopoliensis* CCAP1405/1 to grow on solid media was examined using several solidifying agents and a range of concentrations as follows: agar (Wako, Osaka, Japan) from 0.5 to 1.5%, agarose HT (Amresco, Ohio, USA) from 0.5 to 1.5%, agar noble (Difco, Detroit, USA) from 0.5 to 1.5%, gelrite (31) (Wako) at 1.5%, and gelatin (Wako) at 5.0%. The components of the media other than the solidifying agent were the same as for CT and 1/10 PTYG. Each agent was added before autoclaving. *S. leopoliensis* CCAP1405/1 was precultured in CT liquid medium as mentioned above, and spread at  $10^{5-6}$  cells plate<sup>-1</sup>. After incubation for 2 months under the conditions described above, the growth of the strain was observed with the naked eye. The effects of the solidifying agent were also examined in the presence of another heterotrophic bacterial strain. Strain T-5 was precultured on 1/10 PTYG agar medium at 25°C for several days, and each of three independent colonies ( $n=3$ ) was removed with a sterilized wooden toothpick, and spotted at 3-cm intervals onto the solid media which were already inoculated with *S. leopoliensis* CCAP1405/1.

In the presence of other heterotrophic bacterial strains, the effects of the initial cell density of *S. leopoliensis* CCAP1405/1 and agar concentration on the growth of the cyanobacteria on CT agar medium were examined by using a 96-well microplate (5 mm id × 10 mm depth) with 100 μL of the agar medium. The cell densities ranged from  $4.7 \times 10^2$  to  $4.8 \times 10^5$  cells well<sup>-1</sup>, and the agar concentrations from 0.25 to 2.0%. After inoculation of *S. leopoliensis* CCAP1405/1, the plates were left for about two hours at ambient temperature until disappearance of the liquid layer, and two independent colonies ( $n=2$ ) of the selected bacterial strains, SH-4-3-2, T-5 and 3-8, were inoculated with a sterilized toothpick on the corresponding wells. The appearance of green colonies was observed for 2 months during the incubation.

#### *Effects of addition of sodium thiosulfate, vitamins, and catalase on the growth of S. leopoliensis CCAP1405/1 on agar media*

The efficacy of adding sodium thiosulfate to the media was evaluated by using a 96-well microplate (7 mm id × 10 mm depth) with 260 μL of the media. Two times the concentration of CT liquid medium was separately autoclaved from a 3% agar solution containing sodium thiosulfate at 0.04, 0.4, 4, 40, and 400 mM, and equal volumes of the solutions were mixed to make 1.5% CT agar media containing sodium thiosulfate at 0.02, 0.2, 2, 20, and 200 mM. The control wells without sodium thiosulfate were



**Fig. 2.** Growth of *Synechococcus leopoliensis* CCAP1405/1 around/in the colonies of the heterotrophic bacteria, strains SH-5-1, SH-5-2, SH-7-1 and 7-11 on CT, 1/10PTYG, BG11 and 1/16PYG+Sp agar (1.5%) media after incubation for one month. Letters in the photos indicate color of *S. leopoliensis* CCAP1405/1 (DG, dark green; G, green; Y, yellowish green), growth area as a distance (mm) from a heterotrophic bacterial colony, and the form of culture (L, lawn; C, colony; L-C, colony with lawn). Scale bar, 10 mm.

prepared by the same procedures. *S. leopoliensis* CCAP1405/1 was inoculated onto the agar media at  $3 \times 10^4$  cells well<sup>-1</sup> and seven wells were inoculated for each condition ( $n=7$ ). The growth of *S. leopoliensis* CCAP1405/1 on the wells was examined according to the procedures described above.

The effects of adding vitamin B<sub>12</sub>, biotin, thiamine HCl, and catalase on the growth of *S. leopoliensis* CCAP1405/1 were examined with the microplate system described above. Aliquots of filter-sterilized stock solutions of vitamin B<sub>12</sub> at 1 μg mL<sup>-1</sup>, biotin at 1 μg mL<sup>-1</sup>, thiamine HCl at 100 μg mL<sup>-1</sup>, and catalase (bovine liver, Wako) at 12,000 U mL<sup>-1</sup> were added to the autoclaved CT agar medium to prepare concentrations 10, 100, and 1000 times higher than the concentrations of the CT medium. For catalase, the final concentrations were set at 2.3, 23 and 231 U mL<sup>-1</sup> based on a previous report (19).

#### *Catalase activity of bacterial strains*

The catalase activity of the bacterial strains was assayed by Morris' method (19). After addition of a drop of 3 and 30% H<sub>2</sub>O<sub>2</sub> solution to the colonies grown on the 1/10 PTYG agar plate, the production of O<sub>2</sub> on the colonies was observed at room temperature.

#### *Inhibitive concentration of H<sub>2</sub>O<sub>2</sub> for the growth of S. leopoliensis CCAP1405/1 and effect of catalase*

The inhibitive concentration of H<sub>2</sub>O<sub>2</sub> for the growth of *S. leopoliensis* CCAP1405/1 was determined by using a 96-well microplate (7 mm id × 10 mm depth) with 260 μL of CT agar media at high initial cell density ( $4.8 \times 10^5$  cells/well), at which *S. leopoliensis* CCAP1405/1 grows on the media by itself. An aliquot of 30% H<sub>2</sub>O<sub>2</sub> solution was added to the autoclaved CT agar medium before solidification. The final concentrations ranged from 32 mM to 320 nM with a factor ratio of 10. In addition, the effect of adding catalase to the H<sub>2</sub>O<sub>2</sub>-containing CT agar medium was examined. An aliquot of the filter-sterilized stock solutions of catalase was added at 231 U mL<sup>-1</sup> before solidification. After the inocu-

**Table 2.** Ability to support growth of *Synechococcus leopoliensis* CCAP1405/1 on agar media and morphological characteristics of *S. leopoliensis* CCAP1405/1

Strain	Media <sup>a)</sup>							
	CT		1/10PTYG		BG11		1/16PYG+Sp	
	Growth <sup>b)</sup>	Morphology <sup>c)</sup>	Growth	Morphology	Growth	Morphology	Growth	Morphology
CCAP1405/1	0		0		0		0	
C-Pink1	0/8		0/8		5/8*	DG-0-L	8/8*	G-5-C/L
SH-4-1	8/8*	G-10-L	2/8	G-0-C	6/8*	G-10-C	5/8*	Y-0-L
SH-4-2	8/8*	G-10-L	8/8*	G-15-L-C	8/8*	G-10-L-C	5/8*	Y-5-L
SH-4-3-1	8/8*	G-10-C/L	8/8*	DG-5-C	8/8*	G-10-L	8/8*	Y-0-L
SH-4-3-2	8/8*	G-10-C/L	4/8*	DG-0-C	8/8*	G-10-L-C	8/8*	Y-5-C/L
SH-5-1	8/8*	G-10-L	6/8*	DG-0-C	8/8*	G-10-C/L	8/8*	G-10-C/L
SH-5-2	8/8*	G-15-L-C	8/8*	G-0-C/L	8/8*	G-15-L-C	8/8*	Y-10-C/L
SH-7-1	8/8*	G-10-L	5/8*	DG-0-C/L	8/8*	G-10-L	8/8*	Y-10-L
7-11	0/8		8/8*	DG-0-C	0/8		8/8*	Y-10-L
T-5	8/8*	G-10-L	8/8*	DG-0-C/L	8/8*	G-10-L-C	6/8*	Y-0-L
3-7	0/8		8/8*	DG-0-C	4/8*	DG-0-C	8/8*	Y-10-L
3-8	8/8*	G-10-C	0/8		8/8*	DG-0-L	0/8	

<sup>a</sup> Components of each medium are described in the text.

<sup>b</sup> Number of cultures that allowed *S. leopoliensis* CCAP1405/1 to grow on agar media among eight inoculated cultures. Asterisks indicate the growth of *S. leopoliensis* CCAP1405/1 at a significantly different frequency ( $P < 0.05$ ) analyzed by Pearson's chi-square test.

<sup>c</sup> Expressed by color (DG, dark green; G, green; Y, yellowish green), growth area as a distance (mm) from a heterotrophic bacterial colony, and form of culture (L, lawn for all inoculants; C, colony for all inoculants; C/L, colony or lawn; L-C, colony with lawn for all inoculants).

**Table 3.** Growth of *Synechococcus leopoliensis* CCAP1405/1 on solid media at a low initial cell density with and without heterotrophic bacterial strain T-5

Solidifying agent	Concentration (%)	CT		1/10PTYG	
		N <sup>a)</sup>	T-5 <sup>a)</sup>	N	T-5
Agar	0.5–1.5	0/3 <sup>b)</sup>	3/3	0/3	3/3
Agarose HT	0.5	0/3	3/3	0/3	2/3
Noble agar	1.0–1.5	0/3	3/3	0/3	0/3
	0.5–1.0	0/3	3/3	0/3	0/3
Gelrite	1.5	0/3	0/3	0/3	0/3
	1.5	0/3	3/3	0/3	3/3
Gelatin	5.0	0/3	0/3	0/3	0/3

<sup>a</sup> T-5 and N indicate incubation with and without strain T-5, respectively.

<sup>b</sup> Number of cultures that allowed *S. leopoliensis* CCAP1405/1 to grow on agar media among three inoculated cultures.

lation of *S. leopoliensis* CCAP1405/1 onto the agar media, growth was observed according to the procedures described above. Eight wells were prepared for each condition ( $n=8$ ).

### Statistics

In the examinations of the effects of co-inoculated heterotrophic bacteria and additives on *S. leopoliensis* CCAP1405/1 growth on agar media, significant differences in growth frequency among the eight cultures were determined using a Pearson's chi-square test.

## Results

### Ability of co-inoculated heterotrophic bacteria to support growth of *S. leopoliensis* CCAP1405/1 on agar media

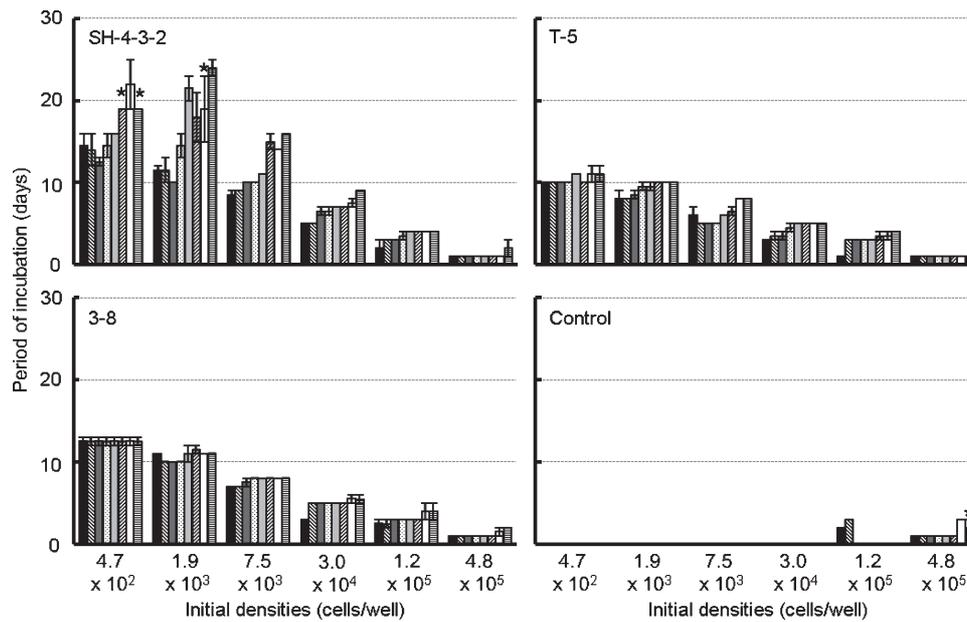
All the heterotrophic bacteria used in this study, which belong to several classes (Table 1), supported the growth of *S. leopoliensis* CCAP1405/1 on either all or some of the agar (1.5%) media. The morphological characteristics of *S. leopoliensis* CCAP1405/1 are summarized in Table 2, and representative images are shown in Fig. 2. In some cases,

the growth of *S. leopoliensis* CCAP1405/1 was observed only around 2–6 colonies of the inoculated eight colonies on the same plate, but the growth frequency was significant ( $P < 0.05$ ) in almost all cases as summarized in Table 2.

The heterotrophic bacteria grew faster on the 1/16 PYG+Sp and 1/10 PTYG than CT and BG11 agar media. The growth of *S. leopoliensis* CCAP1405/1 was also generally faster on 1/16 PYG+Sp and 1/10 PTYG than CT and BG11 agar. There was a tendency for the *S. leopoliensis* CCAP1405/1 culture to turn yellowish green within one month on 1/16 PYG+Sp, to remain green for over three months on CT and BG11, and to become dark green on 1/10 PTYG. In most cases, the growth of *S. leopoliensis* CCAP1405/1 was observed a distance of 5–15 mm from the inoculated heterotrophic bacteria on CT, BG11 and 1/16 PYG+Sp, while it was within the colonies of the inoculated bacteria on 1/10 PTYG. The growth of *S. leopoliensis* CCAP1405/1 was in the form of colonies, a lawn, or colonies with a lawn depending on the heterotrophic bacterial strains and composition of the agar media (Table 2 and Fig. 2), and in some cases, a colony or lawn was formed around/in each colony of the same strain on the same medium. There was a tendency to form a lawn on 1/16 PYG+Sp (Table 2).

### Effects of conditions and initial cell density of *S. leopoliensis* CCAP1405/1 on the growth on solid media

The growth of *S. leopoliensis* CCAP1405/1 on the solid media is summarized in Table 3. On both the CT and 1/10 PTYG media, *S. leopoliensis* CCAP1405/1 did not grow on any solid media made from agar (0.5–1.5%), agarose HT (0.5–1.5%), noble agar (0.5–1.5%), gelrite (1.5%), and gelatin (5.0%) within 2 months. In the presence of a bacterial strain, T-5, however, it grew on CT agar (0.5–1.5%), agarose HT (0.5–1.5%), noble agar (0.5–1.0%), and gelrite (1.5%) media, and on 1/10 PTYG agar (0.5–1.5%) and gelrite (1.5%) media. On 1/10 PTYG agarose HT at 0.5%, growth



**Fig. 3.** Effects of initial density of *Synechococcus leopoliensis* CCAP1405/1 and agar concentration on the growth on CT agar media in the presence of heterotrophic bacteria presented as period of incubation at which colonies were observed with the naked eye. Agar concentrations examined were 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00%, shown at each initial density from left to right. Bars indicate the range of data ( $n=2$ ). Asterisks indicate that no colony was observed in one of the duplicates for 2 months.

was observed around two of the three colonies of strain T-5.

The period of incubation at which the growth of *S. leopoliensis* CCAP1405/1 on the agar media was observed with the naked eye is shown in Fig. 3. In the absence of the other heterotrophic bacteria, *S. leopoliensis* CCAP1405/1 made colonies only in the high density culture of  $4.8 \times 10^5$  cells well<sup>-1</sup> at agar concentrations from 0.25 to 2.0%. When the initial density was  $1.2 \times 10^5$  cells well<sup>-1</sup>, it made colonies only at the lower agar concentrations of 0.25 and 0.5%. The presence of the other heterotrophic bacterial strains, SH-4-3-2, T-5, and 3-8, allowed *S. leopoliensis* CCAP1405/1 to grow in a range of initial densities from  $4.7 \times 10^2$  to  $4.8 \times 10^5$  cells well<sup>-1</sup> and agar concentrations from 0.25 to 2.0%. In all strains, the period of incubation increased when the initial density decreased at all agar concentrations. Effects of the agar concentration on the incubation period were not observed for strains T-5 and 3-8 at any initial density, while in strain SH-4-3-2, the period increased at the higher agar concentrations especially with the lower initial densities, and no colony was made in one of the two wells in several cases.

#### *Effects of addition of sodium thiosulfate, vitamins, and catalase on the growth of S. leopoliensis CCAP1405/1 on agar media*

As summarized in Table 4, all of the additives used in this study except catalase were significantly ( $P<0.05$ ) effective in the range of concentrations for making *S. leopoliensis* CCAP1405/1 on CT agar (1.5%) medium at a low initial cell density at which it can not grow by itself. *S. leopoliensis* CCAP1405/1 grew on the agar medium with sodium thiosulfate at 2–20 mM, vitamin B<sub>12</sub> at 1–100  $\mu\text{g L}^{-1}$ , biotin at 10–100  $\mu\text{g L}^{-1}$ , and thiamine HCl at 1–10 mg L<sup>-1</sup> within 1 month of incubation. In the case of biotin at 1  $\mu\text{g L}^{-1}$  and thiamine HCl at 100  $\mu\text{g L}^{-1}$ , it grew in five and six of seven wells, respectively. In the other conditions, no growth was

**Table 4.** Effect of additives on growth of *Synechococcus leopoliensis* CCAP1405/1 on CT agar medium at a low initial cell density<sup>a)</sup>

Additive	Concentration	Growth <sup>b)</sup>
Sodium thiosulfate	0.02–0.2 mM	0/7
	2–20 mM	7/7*
	200 mM	0/7
Vitamin B <sub>12</sub>	1–100 $\mu\text{g L}^{-1}$	7/7*
Biotin	1 $\mu\text{g L}^{-1}$	5/7*
	10–100 $\mu\text{g L}^{-1}$	7/7*
Thiamine HCl	100 $\mu\text{g L}^{-1}$	6/7*
	1–10 mg L <sup>-1</sup>	7/7*
Catalase	2.3–231 U mL <sup>-1</sup>	0/7

<sup>a)</sup> CT standard medium contains 0.1  $\mu\text{g L}^{-1}$  of vitamin B<sub>12</sub> and biotin, 10  $\mu\text{g L}^{-1}$  of thiamine HCl, and no sodium thiosulfate and catalase.

<sup>b)</sup> Number of *S. leopoliensis* CCAP1405/1 cultures that grew on agar media among seven inoculated cultures. Asterisks indicate the growth of *S. leopoliensis* CCAP1405/1 at a significantly different frequency ( $P<0.05$ ) analyzed by Pearson's chi-square test.

observed until 2 months of incubation.

#### *Inhibitive concentration of H<sub>2</sub>O<sub>2</sub> for the growth of S. leopoliensis CCAP1405/1 and effect of catalase*

The growth of *S. leopoliensis* CCAP1405/1 at a high initial cell density on CT agar medium supplemented with H<sub>2</sub>O<sub>2</sub> and catalase is summarized in Table 5. The growth of *S. leopoliensis* CCAP1405/1 was completely inhibited by H<sub>2</sub>O<sub>2</sub> at 320  $\mu\text{M}$ , while the same growth was observed as the control at less than 3.2  $\mu\text{M}$ . At 32  $\mu\text{M}$ , *S. leopoliensis* CCAP1405/1 grew for 5 days, but the green color disappeared thereafter. When catalase was added, *S. leopoliensis* CCAP1405/1 grew on the agar medium supplemented with H<sub>2</sub>O<sub>2</sub> at from 320 nM to 3.2 mM.

## Discussion

All bacterial strains used in this study allowed *S. leopoliensis* CCAP1405/1 to grow on agar media around/in their colonies. The bacterial strains belong to various taxonomic groups, and there was no phylogenetic relationship among the bacterial strains with this ability. A similar phenomenon was reported by Morris *et al.* (19), in which taxonomically different bacterial strains belonging to *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes*, helped *Prochlorococcus* sp. strains to grow on Pro99 noble agar medium. The ability seems to be wide spread in bacteria, but it is unclear if the mechanism is the same or not because different features were observed in the morphology of *S. leopoliensis* CCAP1405/1 and the effects of components of the media (Fig. 2 and Table 2). A supplementary experiment was conducted to examine if the bacterial strains survived on the agar media where *S. leopoliensis* CCAP1405/1 failed to grow. Strains C-Pink1 and 7-11 could not be recovered from the inorganic medium, 1/16PYG+Sp and 1/10PTYG, respectively (data not shown). Therefore, it is likely that they did not allow *S. leopoliensis* CCAP1405/1 to grow on CT medium because they failed to thrive themselves. On the other hand, the viability of strain 3-7 on CT agar medium, where *S. leopoliensis* CCAP1405/1 failed to grow, was confirmed (data not shown), suggesting an absence of factors aiding the growth of *S. leopoliensis* CCAP1405/1.

The growth of *S. leopoliensis* CCAP1405/1 around/in the heterotrophic bacterial colonies was usually in the form of not a lawn but an independent colony, even though it was evenly inoculated on the agar media at  $10^{5-6}$  cells plate<sup>-1</sup> and certain substances excreted from the bacterial colonies seemed to diffuse evenly into the agar media (Fig. 2 and Table 2). This observation suggests that each cell of *S. leopoliensis* CCAP1405/1 had different threshold levels for the substance and/or growth, and that not all inoculated cells could propagate under the conditions. In addition, the composition of the media might affect the threshold level of *S. leopoliensis* CCAP1405/1. The behavior of *S. leopoliensis* CCAP1405/1 suggested the presence of a mutator locus as in the formation of papillae by a mutator strain of *Escherichia coli* (21). Then, we examined whether the independent colonies of *S. leopoliensis* CCAP1405/1 around the heterotrophic bacterial colonies were mutants or not, by transferring them onto CT agar. Because the culture of *S. leopoliensis* CCAP1405/1 did not grow on CT agar as their wild type, it was supposed that the independent colonies of *S. leopoliensis* CCAP1405/1 were not mutant strains. In addition, all of the colonies of a strain of heterotrophic bacteria in a plate did not show the same ability to aid *S. leopoliensis* CCAP1405/1, that is, there were variables in the ability among colonies under the same conditions. This variability sometimes made the results confusing, and required standardization of the cultivation conditions of the bacterial strains.

*S. leopoliensis* CCAP1405/1 did not grow at a low initial cell density by itself on the solidified media examined in this study even though it grew in the liquid media containing the same components. The growth inhibition was observed in all of the solidifying agents tested (Table 3), therefore, it

seemed that the common impurities in the agents did not directly inhibit the growth of *S. leopoliensis* CCAP1405/1. Co-inoculation of the heterotrophic bacterial strain T-5 allowed *S. leopoliensis* CCAP1405/1 to grow on the solid media around/in the colonies of strain T-5, but the effectiveness differed depending on the solidifying agents and their concentrations, and on the media (Table 3). These results suggest that the mechanisms of both growth inhibition and recovery of growth ability are different among the solidifying agents as well as among the media. *S. leopoliensis* CCAP1405/1 might be inhibited by drying stress on the solid media or by the inhibitory products made when media were autoclaved with the solidifying agent, as Allen and Gorham (1) reported.

When *S. leopoliensis* CCAP1405/1 was inoculated at a high initial density of  $4.8 \times 10^5$  cells well<sup>-1</sup>, it could grow by itself at agar concentrations from 0.50 to 2.0%. As quorum sensing systems are suggested in cyanobacteria for enhancing photosynthetic activity (29) and heterocyst differentiation (25), the same system might be involved in the growth of *S. leopoliensis* CCAP1405/1 on the agar plate at a high cell density. It has been reported that *Anabaena* sp. PCC7120 (25) and *Gloeothece* sp. PCC6909 (29) use acyl homoserine lactone (AHL) as an autoinducer for quorum sensing, and the same compounds are reported in several Gram-negative bacterial strains for their quorum sensing (17, 41). Therefore, *S. leopoliensis* CCAP1405/1 and some Gram-negative strains might excrete AHL for the growth of *S. leopoliensis* CCAP1405/1 at a high cell density. In our preliminary experiments, however, the addition of supernatant from a late-stage *S. leopoliensis* CCAP1405/1 culture in liquid CT medium and a representative Gram-negative strain T-5 culture in liquid 1/10PTYG medium failed to support growth of *S. leopoliensis* CCAP1405/1 on CT agar medium (data not shown). Thus, it was supposed that quorum sensing was not involved in the growth of *S. leopoliensis* CCAP1405/1 on agar media, but further studies are necessary to confirm the assumption.

Co-inoculation of T-5 or 3-8 allowed *S. leopoliensis*

**Table 5.** Effects of H<sub>2</sub>O<sub>2</sub> and catalase on growth of *Synechococcus leopoliensis* CCAP1405/1 on CT agar medium at a high initial cell density<sup>a)</sup>

H <sub>2</sub> O <sub>2</sub> addition	Growth	
	– Catalase	+ Catalase <sup>b)</sup>
32 mM	0/8 <sup>c)</sup>	0/8
3.2 mM	0/8	8/8
320 μM	0/8	8/8
32 μM	8/8*	8/8
3.2 μM	8/8	8/8
320 nM	8/8	8/8
– H <sub>2</sub> O <sub>2</sub>	8/8	8/8

<sup>a)</sup> The initial density of *S. leopoliensis* CCAP1405/1 was  $4.8 \times 10^5$  cells/well which allows the strain to grow on the CT agar (1.5%) medium by itself.

<sup>b)</sup> Catalase was added at 231 U mL<sup>-1</sup>.

<sup>c)</sup> Number of *S. leopoliensis* CCAP1405/1 cultures that grew on agar media among eight inoculated cultures. Asterisk indicates that *S. leopoliensis* CCAP1405/1 grew for 5 days, but its green color disappeared thereafter.

CCAP1405/1 to grow almost equally at agar concentrations from 0.50 to 2.0% with initial cell density of  $4.7 \times 10^2$  to  $4.8 \times 10^5$  cells well<sup>-1</sup>, whereas the effects of SH-4-3-2 became weak at agar concentrations higher than 1.0% and with initial density lower than  $3.0 \times 10^4$  cells well<sup>-1</sup> (Fig. 3), suggesting different mechanisms among the strains. Strain 3-8 is catalase-positive while strains SH-4-3-2 and T-5 are catalase-negative, therefore, there might be no relation between the different properties of the strains and the presence or absence of catalase activity.

The addition of sodium thiosulfate at 2–20 mM allowed *S. leopoliensis* CCAP1405/1 to grow on CT agar medium. The effectiveness of sodium thiosulfate was also reported by Thiel *et al.* (36) for the growth of *Anabaena* sp. PCC7120, *Pseudanabaena* sp. PCC7409, *Nostoc* sp. PCC8009, *A. variabilis* ATCC29413, *Synechococcus* sp. PCC7942 and *Synechocystis* sp. PCC6803 on BG11 agar medium. Because sodium thiosulfate is a reducing agent known to neutralize H<sub>2</sub>O<sub>2</sub> (28), H<sub>2</sub>O<sub>2</sub> might be produced during autoclaving as Marler and Baalen (16) reported, and might have inhibited the growth. It was also reported that the addition of catalase to Pro99 noble agar (0.42%) semisolid medium at 50–200 U mL<sup>-1</sup> allowed *Prochlorococcus* sp. MIT 9215 to grow robustly on the medium to form lawns (19). In this study, however, the addition of catalase (2.3–231 U mL<sup>-1</sup>) had no effect on the growth of *S. leopoliensis* CCAP1405/1 on CT agar (Table 4). The growth of *S. leopoliensis* CCAP1405/1 was observed on the H<sub>2</sub>O<sub>2</sub>-containing agar medium supplemented with catalase at 231 U mL<sup>-1</sup> (Table 5). The results indicate that the catalase decomposed H<sub>2</sub>O<sub>2</sub> to less than 3.2 μM, at which *S. leopoliensis* CCAP1405/1 can grow. Therefore, no growth of *S. leopoliensis* CCAP1405/1 on agar (not an H<sub>2</sub>O<sub>2</sub>-containing medium) with the catalase at 231 U mL<sup>-1</sup> suggests that H<sub>2</sub>O<sub>2</sub> is not involved in the growth inhibition of *S. leopoliensis* CCAP1405/1.

The addition of high concentrations of one of any vitamins examined allowed *S. leopoliensis* CCAP1405/1 to grow on CT agar medium. As the effect of each vitamin seems to be different, the reasons for the effects are unclear. Croft *et al.* (6) reported that *Halomonas* sp. contaminating the culture of a dinoflagellate, *Amphidinium operculatum*, was able to synthesize vitamin B<sub>12</sub>, which supported the growth of the dinoflagellate. Because sufficient amounts of vitamins are contained in CT medium for growth in the liquid state, a reduction in the availability of the vitamins by solidification is a possible reason for the inhibition, and the heterotrophic bacteria might provide the deficient essential nutrients for *S. leopoliensis* CCAP1405/1.

Growth of the heterotrophic bacterial strains was observed on inorganic CT and BG11 agar media when they were co-cultured with *S. leopoliensis* CCAP1405/1. The microbial behavior indicates mutual interactions between *S. leopoliensis* CCAP1405/1 and the heterotrophic bacteria. The heterotrophs could utilize photosynthetic products from *S. leopoliensis* CCAP1405/1 for their growth, while they helped *S. leopoliensis* CCAP1405/1 to grow on the agar media. It was reported that some bacterial strains can proliferate in inorganic media with algae (6, 37, 39), and a symbiotic association between algae and bacteria was suggested. It is likely that the bacteria utilize photosynthetic

products as carbon sources provided by the algal strain.

Morris *et al.* (19) reported the ability of heterotrophic bacteria to support the growth of cyanobacterial *Prochlorococcus* sp. strains on a solid medium and the involvement of heterotrophic bacterial catalase. This study provides the second example of the interaction of *S. leopoliensis* CCAP1405/1 with heterotrophic bacteria on solid media, and different mechanisms other than catalase although it was not specified in this study. Additionally, this study showed the growth at a high initial cell density of *S. leopoliensis* CCAP1405/1 and by addition of the vitamins as well as sodium thiosulfate. These results could provide useful information to clarify the mechanisms in the future.

Mutual interactions between cyanobacteria and heterotrophic bacteria as revealed in this study may exist in natural habitats where the cyanobacteria and bacteria cannot survive by themselves. In fact, the presence of *Synechococcus* sp. in biofilms with other bacteria has been reported in estuarine environments (11, 20). In harsh environments in which water and nutrients are deficient, H<sub>2</sub>O<sub>2</sub> is produced, and cell densities are low, certain bacteria, in return for their proliferation, allow cyanobacteria to grow and receive photosynthetic products.

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