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Stress resistance and C1 metabolism involved in plant colonization of a methanotroph *Methylosinus* sp. B4S

Hiroyuki Iguchi · Izuru Sato · Hiroya Yurimoto · Yasuyoshi Sakai

Abstract Methanotrophs are widespread and have been isolated from various environments including the phyllosphere. In this study, we characterized the plant colonization by *Methylosinus* sp. B4S, an α-proteobacterial methanotroph isolated from plant leaf. The *gfp*-tagged *Methylosinus* sp. B4S cells were observed to colonize *Arabidopsis* leaf surfaces by forming aggregates. We cloned and sequenced the general stress response genes, *phyR*, *nepR* and *ecfG*, from *Methylosinus* sp. B4S. In vitro analysis showed that the *phyR* expression level was increased after heat shock challenge, and *phyR* was shown to be involved in resistance to heat shock and UV light. In the phyllospheric condition, the gene expression level of *phyR* as well as *mmoX* and *mxaF* was found to be relatively high, compared with methane-grown liquid cultures. The *phyR*-deletion strain as well as the wild-type strain inoculated on *Arabidopsis* leaves proliferated at the initial phase and then gradually decreased during plant colonization. These results have shed light firstly on the importance of general stress resistance and C1 metabolism in methanotroph living in the phyllosphere.

Keywords PhyR · General stress response · Phyllosphere · Methane monooxygenase · Methanol

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Introduction

Methanotrophs are a unique group of bacteria that utilize methane as their sole source of carbon and energy and are important players in carbon and nitrogen cycle (Hanson and Hanson 1996). Methane oxidation by methanotrophs also contributes to the mitigation of global warming. Methanotrophs have been found in a wide variety of environments in which methane–air interfaces develop, including wetlands, peat bogs, lakes, groundwater, rice paddies, landfills, volcanoes and marine sediments (Semrau et al. 2010).

Our previous study showed that methanotrophs inhabit the phyllosphere of various plant species (Iguchi et al. 2012). Plant-associated environments are known to be important methane sources and sinks. Some plants generate methane from the methyl ester groups of pectin (Keppler et al. 2006). Wetland plants that develop aerenchyma tissue transport methane generated by methanogens from ground into the atmosphere (Aulakh et al. 2000). Plant roots supply organic compounds and oxygen, thus stimulating microbial activities including methane oxidation in the rhizosphere (Hanson and Hanson 1996). Further, the phyllosphere is clearly distinct from soil and aqueous environments for microorganisms, in terms of nutrient availability, stress and physical structure. However, little is known about colonization of methanotroph, carbon metabolism and adaptation to stresses in the phyllosphere.

The phyllosphere is thought to be a hostile environment since the inhabitants are exposed to various stresses such as temperature change, UV radiation, desiccation, osmotic pressure, reactive oxygen species (ROS) and starvation (Lindow and Brandl 2003; Whipps et al. 2008). It was recently reported that PhyR, which is involved in the general stress response, was necessary for plant colonization by several *α-Proteobacteria* (Gourion et al. 2006, 2008, 2009; Kaczmarczyk et al. 2011). The *phyR* gene encodes a response regulator, and PhyR induces resistance to stresses such as heat shock, UV radiation, desiccation and osmotic pressure (Gourion et al. 2006, 2008, 2009; Kaczmarczyk et al. 2011). *phyR* orthologues are predicted to be present in essentially all *α-Proteobacteria* (Gourion et al. 2006), which are known to be the predominant population in the phyllosphere (Delmotte et al. 2009; Redford et al. 2010; Vorholt 2012). Therefore, the general stress system regulated by PhyR might be a strategy to enhance fitness in plant-associated environments. According to the proposed regulatory model, in the absence of stress, PhyR is inactivated and the ECF σ factor EcfG is sequestered by its anti-σ factor NepR. In response to stress, PhyR is phosphorylated by a histidine kinase PhyK and interacts with NepR, thus releasing EcfG and allowing EcfG to associate with RNA polymerase to transcribe stress genes (Francez-Charlot et al. 2009; Lourenco et al. 2011). These *phyR*, *nepR*, *ecfG* and *phyK* genes are often clustered in the genome (Staron and Mascher 2010).

Most of methanotrophs are obligate C1-utilizers (Semrau et al. 2011). In C1 metabolism, methane is oxidized by methane monooxygenase (MMO) to methanol, which is subsequently oxidized to formaldehyde by methanol dehydrogenase. Formaldehyde is assimilated into biomass or further oxidized to CO_2, providing reducing equivalents for biosynthesis. MMO exists in two distinct forms, pMMO and sMMO, and methanotrophs possess both or either form of MMO (Semrau et al. 2010). pMMO and sMMO are expressed under the conditions of high and low copper concentrations, respectively, and the expression is regulated at the transcriptional level (Lieberman and Rosenzweig 2004; Nielsen et al. 1997).

We have isolated *α*- and *γ*-proteobacterial methanotrophs belonging to the genus *Methylosinus Methylocystis* and *Methylmonas* from plants (Iguchi et al. 2012). In this study, we used a methanotroph *Methylosinus* sp. B4S isolated from reed leaf to characterize plant colonization of methanotroph. We observed colonization of GFP-tagged *Methylosinus* sp. B4S on *Arabidopsis* leaves and examined the involvement of PhyR and C1 metabolism in plant colonization. These results reveal life of methanotroph in the phyllosphere.

Materials and methods

Bacterial strains and media

The strains and plasmids used in the present study are listed in Table 1. Methanotrophic strains were usually cultivated in nitrate mineral salt (NMS) medium (ATCC 1306 medium, plus 3 nM $CuCl_2$) with 20 % atmospheric methane at 28 °C, as described previously (Iguchi et al. 2012). When required, ampicillin (Ap), kanamycin (Km) or gentamycin (Gm) was added to a final concentration of 50, 20 or 10 μg/ml, respectively.

Utilizable carbon and nitrogen sources

The ability of strain B4S to utilize various carbon sources was tested in liquid NMS medium. Methanol, formamide and ethanol were added at 0.05 % (v/v). The following compounds were tested at a concentration of 0.1 % (w/v): methylamine, formate, glucose, sucrose, galactose, lactose, fructose, citrate, succinate, pyruvate and acetate. Utilization of various nitrogen sources was tested in liquid NMS medium with methane, in which potassium nitrate was replaced by one of the following compounds at 0.05 % (w/v): ammonium chloride, sodium nitrite, urea, glycine, alanine, lysine, arginine, glutamate, glutamine, aspartate, asparagine, tryptophan, methionine, threonine, cysteine or histidine.

Construction of a *gfp*-expressing strain

The *gfp* gene under the control of the *mxaF* gene promoter was introduced into the chromosome using a transposon delivery system. The primers used in this study are listed in Table S1. Here, PCR was performed using KOD FX DNA polymerase (Toyobo, Osaka, Japan), which generates the blunt-ended amplicon. At first, to obtain the *mxaF* promoter sequence in strain B4S, the upstream region of *mxaF* was cloned by inverse PCR using primers mxaF1023-INV and mxaF1541-INV and sequenced. Then, the *mxaF* gene promoter region (300 bp) was amplified by PCR from the genomic DNA of strain B4S with the primers PmxaF-Fw and PmxaF-Re and cloned into the *Eco*RV site of pBluescript II SK+, resulting in pSG1201. The *gfp* gene that was amplified by PCR from pDCG-1 with the primers gfp-Fw and gfp-Re was cloned into the *Eco*RV site of pSG1201, resulting in pSG1202. The correct orientation of the insert was confirmed by sequencing. The *Not*I fragment of pSG1202 was cloned into the *Not*I site of pUTmini-Tn5Km, resulting in pSG1203.

The procedure for conjugating plasmids from *E. coli* S17-1 *λpir* into methanotrophs was based on the

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
Methylosinus sp.		
B4S	Wild type	Iguchi et al. (2012)
B4S-gfp	B4S P*mxaF*-*gfp* Km^R	This study
B4S-phyR	B4S *ΔphyR*::Gm^R	This study
B4S-gfp-phyR	B4S-gfp *ΔphyR*::Gm^R	This study
E. coli S17-1 *λpir*	*recA thi pro hsdRM*$^+$ RP4-2-Tc::Mu-Km::Tn7 *λpir*	Biomedal Inc.
Plasmids		
pUC19	Cloning vector, Ap^R	Takara Bio Inc.
pBluescript II SK+	Cloning vector, Ap^R	Stratagene Inc.
pBR322	Source of Ap^R cassette, Ap^R	Takara Bio Inc.
p34S-Gm	Source of Gm^R cassette, Ap^R Gm^R	Dennis and Zylstra (1998)
pDCG-1	Source of *gfp*, Ap^R Cm^R	Kobayashi (2007)
pUTmini-Tn5 km	Transposon vector, Ap^R Km^R mini-Tn5 RP4-*mob*	Biomedal Inc.
pK18mob	Mobilizable cloning vector, Km^R RP4-*mob*	Schafer et al. (1994)
pK18mob-bla	pK18mob carrying Ap^R replacing Km^R, Ap^R	This study
pSG1101	pUC19 carrying upstream and downstream of *phyR*, Ap^R	This study
pSG1102	pSG1101 carrying *ΔphyR*::Gm^R, Ap^R Gm^R	This study
pSG1103	pK18mob-bla carrying *ΔphyR*::Gm^R, Ap^R Gm^R	This study
pSG1201	pBluescript II SK + carrying the *mxaF* promoter, Ap^R	This study
pSG1202	pSG1201 carrying the *mxaF* promoter fused with *gfp*, Ap^R	This study
pSG1203	pUTmini-Tn5 km carrying the *mxaF* promoter fused with *gfp*, Ap^R Km^R	This study

methods (Csaki et al. 2003). Donor cells (*E. coli* harboring pSG1203) and recipient cells (*Methylosinus* sp. B4S strains) were grown to the stationary phase in LB medium and NMS medium, respectively. The cultures were harvested by centrifugation and mixed in a polypropylene tube. Cells were spotted on a NMS agar plate containing 0.01 % Bacto tryptone, and the plate was incubated at 28 °C under methane/air gas mixture for 1 day. Then, the cells were resuspended in NMS medium and spread on NMS agar containing 0.01 % Bacto tryptone and Km. After the cells were grown under a methane/air gas mixture, Km-resistant and Ap-sensitive colonies were selected.

Cloning of *phyR* gene

The partial fragment of *phyR* was amplified by PCR from the genomic DNA of strain B4S with the primers phyR424-Fw and phyR668-Re, which were designed on the basis of the conserved region of *phyR* (Fig. S2). Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3130 genetic analyzer (Applied Biosystems, Foster city, CA).

Based on the partial *phyR* sequence obtained, the *phyR* locus was cloned by inverse PCR and sequenced. The genomic DNA of strain B4S was digested with *Eco*RI or *Hin*dIII, and the resultant fragments were self-ligated with

T4 DNA ligase. One fragment, the 5′ region of *phyR*, was amplified by PCR from the *Hin*dIII ligation product with the primers phyR-INVu2 and phyR-INVd1. Another fragment, 3′ region of *phyR*, was amplified from the *Eco*RI ligation product with the primers phyR-INVu1 and phyR-INVd1.

Construction of a *phyR*-deletion mutant

The *phyR*-deletion mutant was generated by homologous recombination. The upstream (1,210 bp) and downstream (770 bp) fragments of *phyR* were amplified by PCR from the genomic DNA. The PCR primers used were phyR381-Fw and phyR1590-Re for the upstream fragment, and phyR2001-Fw and phyR2770-Re for the downstream fragment. The upstream fragment was cloned into pUC19 using the *Bam*HI and *Kpn*I sites, and then the downstream fragment was cloned using the *Kpn*I and *Eco*RI sites, resulting in pSG1101. The gentamycin resistance gene generated from p34S-Gm by *Kpn*I digestion was cloned into the *Kpn*I site of pSG1101, resulting in pSG1102. The Km resistance gene in pK18mob was replaced by the ampicillin resistance gene that was amplified by PCR from pBR322 with the primers bla-Fw and bla-Re, using *Bgl*II and *Nco*I sites, resulting in pK18mob-bla. The *Bam*HI-*Sph*I-digested fragment of pSG1102 was ligated to pK18mob-bla using

the same sites, resulting in pSG1103. This construct is expected to result in a 410-bp deletion and insertion of the Gm resistance gene in the *phyR* gene of strain B4S. pSG1102 was introduced into the methanotroph strains by conjugation, as described above. Gm-resistant and Ap-sensitive colonies (i.e., double-crossover strain) were selected. Deletion of *phyR* was confirmed by diagnostic PCR and Southern blotting.

Phenotypic assays

Strains B4S and B4S-phyR were grown in NMS medium with methane to exponential growth phase. The cells were harvested by centrifugation, washed with NMS medium and adjusted OD_{600} to 0.5 with NMS medium. This cell suspension was used for the following assays. (1) *Heat shock resistance assay*. Cell suspensions (100 µl each) were placed in 1.5-ml tubes and incubated at 46 °C for 5, 10, 15 or 20 min. Triplicate samples were prepared for each incubation time. After incubation, the tubes were immediately cooled on ice. Untreated cell suspension served as the control. The suspensions were diluted and spread on NMS agar plates. The colonies were counted after 14 days of incubation with methane. (2) *UV resistance assay*. Cell suspensions (100 µl each) were placed on the lids of 1.5-ml tubes and exposed to 254 nm UV light for 3 min. The suspensions were diluted and spread on NMS agar plates, and colonies were counted after 14 days of incubation. Triplicate samples were conducted. (3) *Disk diffusion assay*. Each 1 ml cell suspension was mixed with 50 ml of NMS soft agar (0.75 % agar), and 4 ml portions of this mixture was poured on NMS agar (2 % agar). A disk (8 mm diameter) was placed at the center of each plate, and aliquots (5 µl) of 1 M H_2O_2 or 2 % methylglyoxal were deposited on the filter disks. The diameter of the inhibition circle was measured after 9 days of incubation. Four replicate samples were conducted. (4) *Osmotic stress tolerance assay*. Aliquots of serial dilutions of the cell suspension were spotted on NMS agar containing 0.3 % NaCl or 7 % sucrose. Plates were incubated with methane until colonies appeared. (5) *Desiccation resistance assay*. Aliquots of serial dilutions of the cell suspension were spotted on mixed cellulose ester filters (0.45 µm pore size). Filters were placed on NMS agar either immediately (control) or after drying under sterile airflow on a clean bench for 30 min. Plates were incubated with methane until colonies appeared.

Plant cultivation

Arabidopsis thaliana seeds (Col.) were sown on rock wool cubes immersed in Hoagland medium prepared with tap water. The medium contained macronutrients (mM): 2.5 $Ca(NO_3)_2$, 2.5 KNO_3, 0.5 KH_2PO_4 and 1.0 $MgSO_4$, and micronutrients (µM): 42 KCl, 23 H_3BO_3, 4.6 $MnCl_2$, 0.4 $ZnSO_4$, 0.1 H_2MoO_4, 0.16 $CuSO_4$ and 64 Fe-EDTA. The plants were grown under open air in a plant growth chamber (Biotron: Nippon Medical & Chemical Instruments, Osaka, Japan) at 25 °C (14 h day/10 h night) with 65 % humidity. Three-week-old plants (6–8 leaf stage) were used for experiments.

Inoculation of methanotroph on plant leaves

Methanotroph cells grown in liquid NMS medium with methane were harvested by centrifugation and washed with water, and the suspension was adjusted to an OD_{600} of 0.5 (for transcriptional analysis), 0.05 (for microscopic observation) or 0.01 (for growth analysis) with water. For the former two experiments, the cell suspension was sprayed on *Arabidopsis* plants. For growth analysis, 5 µl of the cell suspension (ca. 10^6 cells) was spotted on the upper side of *Arabidopsis* leaf.

Confocal laser scanning microscopy

Methanotroph cells were observed using a Zeiss LSM510 META laser scanning confocal microscope equipped with a Plan-FLUAR 100x, NA 1.45 oil objective on an inverted microscope Axiovert 200 M (Carl Zeiss, Oberkochen, Germany). GFP was excited with a 488-nm argon laser with a 505–550-nm filter for emission.

Reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was extracted and purified using a TRIzol reagent (Invitrogen, Carlsbad, CA). Methanotroph cells or ca. 20 leaves (from 4 to 5 individual plants) were frozen in liquid nitrogen and then were disrupted with zirconia beads in TRIzol reagent. After purification according to the manufacturer's instructions, contaminating DNA was removed by DNase I treatment. The RNA was purified with acidic phenol, precipitated with ethanol and dissolved in water.

RT reactions were done using 1 µg of total RNA, SuperScript III Reverse Transcriptase (Invitrogen) and random primers (Promega, Madison, CA). qPCR analysis was carried out using a LightCycler system (Roche Diagnostics, Tokyo, Japan), SYBR Premix ExTaq (Takara Bio, Shiga, Japan) and 1 µl of cDNA according to the manufacturer's instructions. PCR primers were designed for 16S rRNA, *pmoA*, *mmoX*, *mxaF* and *phyR* of strain B4S (Table S1). Specificity of these primers was confirmed by lack of amplification from cDNA of untreated leaves. The copy number of each gene was calculated with a standard curve that was created with plasmid DNA harboring each gene. Because RNA extracted from leaves is contaminated with

RNA from naturally occurring bacteria and *Arabidopsis*, transcriptional levels between samples were normalized using the 16S rRNA gene transcript as the endogenous control.

Growth measurement by qPCR

Four leaves were harvested and frozen at −80 °C. Genomic DNA was quantitatively extracted from leaves by incubating at 65 °C in TEN buffer (50 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, pH8) containing 1 % SDS and 200 µg/ml proteinase K, followed by purification with phenol–chloroform. qPCR was carried out using a LightCycler system, SYBR Premix ExTaq and the genomic DNA as template. PCR primers targeting for *gfp* were used (Table S1).

Nucleotide sequence accession numbers

The sequences obtained in this study have been submitted to GenBank and assigned the following accession numbers: *mmoX* gene, AB683148; *phyR* gene locus, AB741957; and *mxaF* gene locus, AB741958.

Results

Characterization and plant colonization of *Methylosinus* sp. B4S

We isolated a methanotrophic bacterium, *Methylosinus* sp. B4S, from reed leaf in the previous study (Iguchi et al. 2012). Strain B4S possesses the *pmoA* and *mmoX* genes, which encode particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO), respectively. Strain B4S grew only on C1-compounds, i.e., methane, methanol and formate, among the tested carbon sources including organic acids and sugars, and thus is an obligate methanotroph. Utilizable nitrogen sources were nitrate, ammonium, urea, alanine, glutamine, aspartate, asparagine and cysteine. At least methanol as a carbon source and nitrate and ammonium as nitrogen sources were reported to be present on plant leaves (Nemecekmarshall et al. 1995; Parangan-Smith and Lindow 2013).

We investigated the colonization of *Methylosinus* sp. B4S on *Arabidopsis* leaves using green fluorescent protein (GFP)-tagged strains. A construct carrying the *gfp* gene fused to the *mxaF* gene promoter was introduced into the chromosome by mini-Tn5 transposon system. The generated GFP strain showed the similar growth rate as the wild-type strain under standard culture conditions (Fig. S1).

Strain B4S-gfp cells that were inoculated by spraying onto *Arabidopsis* plants were monitored with a confocal laser scanning microscopy. Figure 1a shows the leaf image of two vertical sections at 1 day post-inoculation (dpi). We detected methanotroph cells exhibiting GFP fluorescence on *Arabidopsis* leaves. The inoculated cells were located on the surface of leaf epidermal cells and often were aggregated in the grooves between the leaf epidermal cells (Fig. 1a right panel), which was similar to the localization pattern of *Methylobacterium* cells sprayed on leaves of clover or strawberry (Omer et al. 2004; Verginer et al. 2010). Since a part of strain B4S cells showed motility in liquid culture, this localization may be due to water drift on leaves. GFP fluorescence of the methanotroph was still observed at 7 dpi, although their fluorescence intensity declined during plant habitation (Fig. 1b).

Cloning of *phyR* gene locus

Next, we cloned and analyzed *phyR* to characterize general stress resistance in *Methylosinus* sp. B4S. A partial fragment of *phyR* was obtained from *Methylosinus* sp. B4S by PCR with primers designed within the conserved sequence regions of *phyR* (Fig. S2 and Table S1). Then, the surrounding region was cloned by inverse PCR, as described in "Materials and Methods." PhyR of *Methylosinus* sp. B4S shared 54 % amino acid identity with PhyR of *Methylobacterium extorquens* AM1 (Gourion et al. 2006). Figure 2 shows the organization of the *phyR* gene locus of *Methylosinus* sp. B4S. The *phyR*, *nepR* and *sigT* genes were found in an organization typical of *α-Proteobacteria* where *phyR* is adjacent to and divergently oriented from *nepR-sigT* (Staron and Mascher 2010). A gene encoding putative histidine kinase was not present in the sequenced region. *orf1* encodes a hypothetical protein containing the stress-induced bacterial acidophilic repeat motif and has not been assigned to the PhyR regulatory system. This motif is characterized as the amino acid sequence KGG and is found in proteins that are expressed under stress conditions. Gene organization of the locus from *ecfG* to *orf1* was conserved among the sequenced *α*-proteobacterial methanotrophs except *Methylocella* lacking *orf1* (Chen et al. 2010; Dam et al. 2012; del Cerro et al. 2012; Stein et al. 2010; Stein et al. 2011).

The ECF σ factor EcfG recognizes an ECF-type promoter sequence (consensus GGAAC-$N_{16,17}$-CGTT) to activate the transcription of genes (Alvarez-Martinez et al. 2007; Gourion et al. 2009). In *Methylosinus* sp. B4S, an ECF-type promoter sequence was found for *nepR* (GGAAC-N_{16}-CGTT-N_{148}-ATG) and *orf1* (GGAAC-N_{14}-CCTT-N_{48}-ATG), but not for *phyR*. Other methanotroph strains in the genus *Methylosinus* and *Methylocystis* also do not have an ECF-type promoter sequence upstream of *phyR*; however, *Methylocella* has the sequence (Chen et al. 2010; Dam et al. 2012; del Cerro et al. 2012; Stein et al.

Fig. 1 Colonization of *Methylosinus* sp. B4S-gfp on *Arabidopsis* leaves. Confocal laser scanning microscopic images of *Arabidopsis* leaves at 1 day (**a**) and 7 days (**b**) post-inoculation with *Methylosinus* sp. B4S-gfp. Pictures include orthogonal sections of a stack image. *Panel a* represents two *vertical* sections in one viewpoint, showing colonization on the leaf surfaces (*left*) and in the leaf grooves (*right*). *Scale bar*: 10 μm
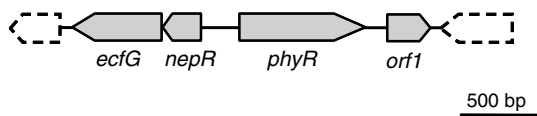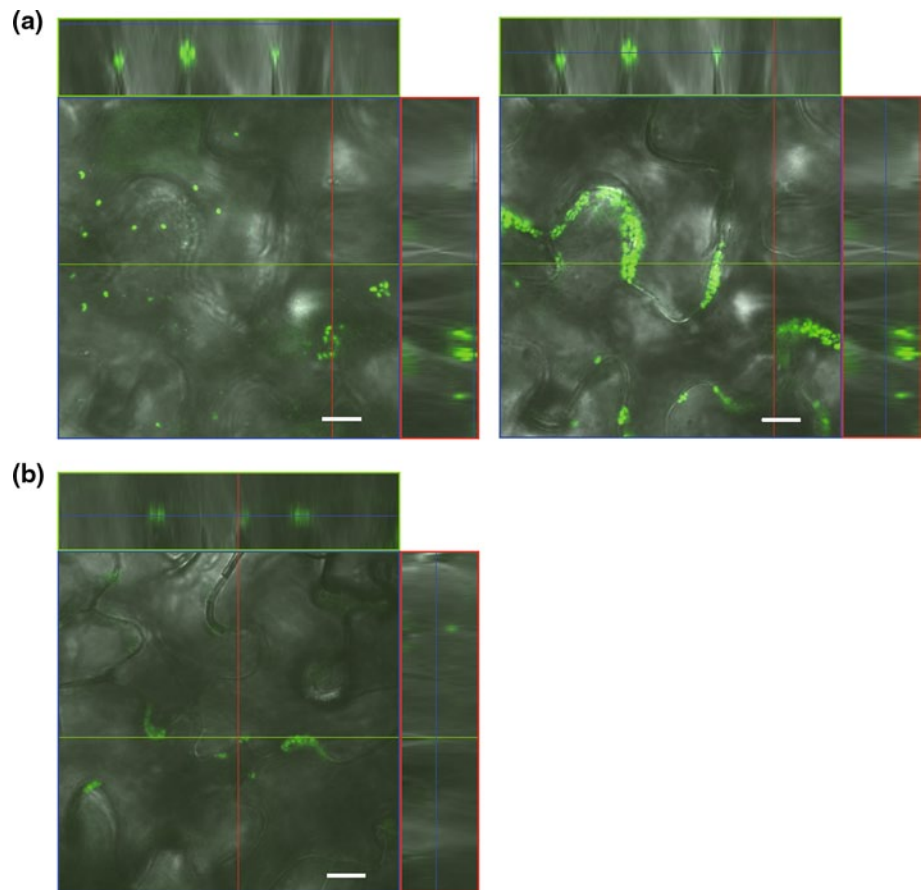
Fig. 2 Organization of the *phyR* gene locus in *Methylosinus* sp. B4S. Truncated genes found in the sequenced border region are shown with *dotted lines*

2010, 2011). This trait of methanotrophs, the absence of an ECF-type promoter sequence for *phyR*, is unlike those of previously studied bacteria (Bastiat et al. 2010; Gourion et al. 2008, 2009; Kaczmarczyk et al. 2011; Lourenco et al. 2011). These observations stimulated an interest in the expression manner of *phyR* in *Methylosinus* sp. B4S.

PhyR is involved in resistance to heat shock and UV radiation

To examine the stress resistance regulated by PhyR in *Methylosinus* sp. B4S, we constructed a *phyR*-deletion mutant via homologous recombination. The mutant strain B4S-phyR (*ΔphyR*) showed the almost same growth rate as strain B4S (wild type) in methane-grown cultures (Fig. S1),

indicating that PhyR is not required for methanotrophic metabolism under standard culture conditions.

Resistance to heat shock, UV radiation, oxidative stress (H_2O_2 and methylglyoxal), osmotic stress (NaCl and sucrose) and desiccation was evaluated for wild-type and *ΔphyR* strains. The *ΔphyR* strain was more sensitive to heat shock and UV radiation than wild type (Fig. 3a, b). The survival rate after a challenge at 46 °C for 20 min was 20-fold lower for the *ΔphyR* strain than for the wild-type strain. UV radiation for 3 min reduced the viability of the *ΔphyR* strain but not that of the wild-type strain. In contrast, no differences were detected in the sensitivity to H_2O_2, methylglyoxal, NaCl, sucrose or desiccation between the wild-type and *ΔphyR* strains (Fig. 3c–f).

We then analyzed the expression of *phyR* in response to stress in flask cultures by RT-qPCR. When the *Methylosinus* sp. B4S culture was shifted from 28 to 40 °C, transcription of *phyR* was elevated by 1.5-fold (Fig. 3g). Although the induction was small, it was reproducible at 40 °C and was also observed in response to heat shock at 37 °C (1.7-fold induction, Fig. 3g). These results, together with mutant analysis, indicate that in *Methylosinus* sp. B4S, PhyR contributes to resistance to heat shock and UV stress and responds to stress at the transcriptional level.
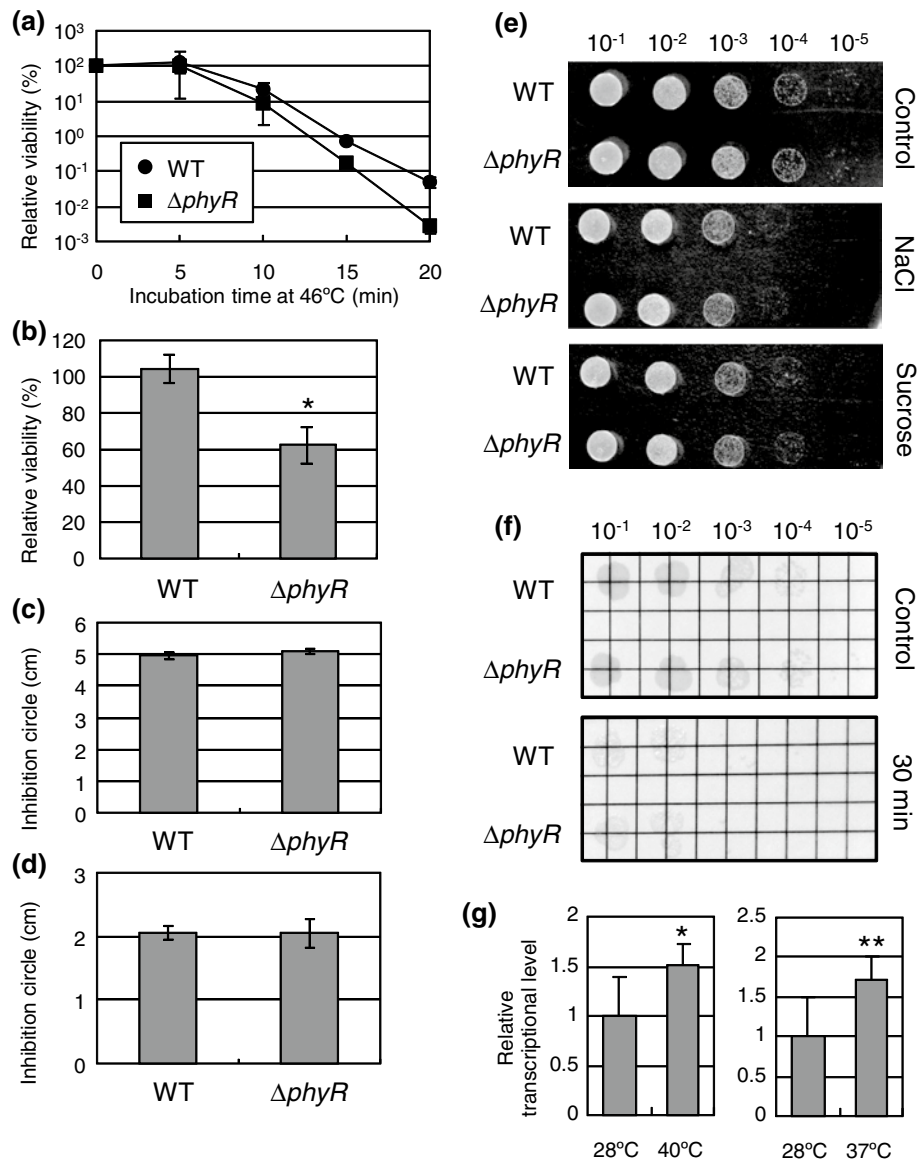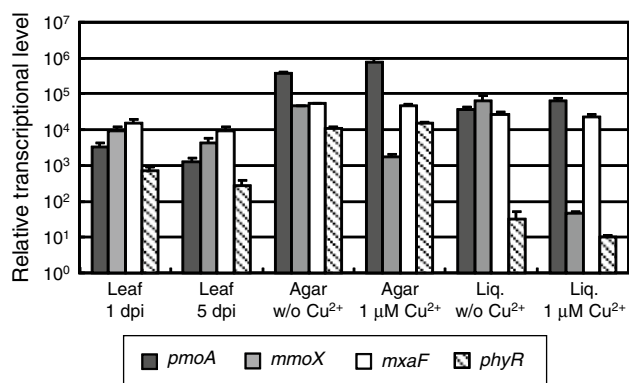
Fig. 3 Stress resistance and response in *Methylosinus* sp. B4S (WT) and B4S-phyR (*ΔphyR*) strains. Thermal (**a**) and UV light (**b**) resistance assessed by viability after cells were incubated at 46 °C for indicated time or exposure to UV light (254 nm) for 3 min. The percentage of viability corresponds to the CFU ratio of treated to untreated cells. Resistance to H_2O_2 (**c**) and methylglyoxal (**d**) determined by disk diffusion assays. Data represent the diameter of the inhibition halo created on agar plates. **e** Ability to grow in media containing 0.3 % NaCl or 7 % sucrose. A dilution series of cells was spotted on NMS agar. Shown is the plate after cultivation for 7 days (control), 9 days (NaCl plate) and 11 days (sucrose plate). **f** Cell viability after desiccation challenge. A dilution series of cells was spotted on filter paper, and the filters were laid on NMS agar plates immediately or after 30-min drying under sterile air flow. Shown is the filter after cultivation for 10 days (control) and 14 days (30 min dry). **g** Transcriptional level of *phyR* in the absence (28 °C) and presence (40 or 37 °C, 1 h) of heat stress was analyzed by RT-qPCR for liquid cultures of strain B4S. Data are the means of three replicates (**a**, **b**) and four replicates (**c**, **d**, **g**), and one representative from three replicates (**e**, **f**). *Error bars* represent standard deviations. *Asterisks* indicate significant increases between two samples (*t* test; *P < 0.05; **P < 0.1)

Gene expression of *phyR* and C1 metabolism genes in the phyllosphere

Expression of methane monooxygenase (*pmoA* and *mmoX*) and methanol dehydrogenase genes (*mxaF*) as well as *phyR* on *Arabidopsis* leaves was examined. Inoculation was done by spraying the cell suspension on growing *Arabidopsis* plants, and the plants were cultivated under a 14-h day/10-h night cycle. RNA was extracted in the daytime from leaves, and gene expression was analyzed by RT-qPCR. To set the culture condition, growing agar and liquid cultures were experienced 1-day cultivation in the plant growth chamber before RNA extraction. Transcriptions were calculated as copy number with standard samples, and the transcriptional
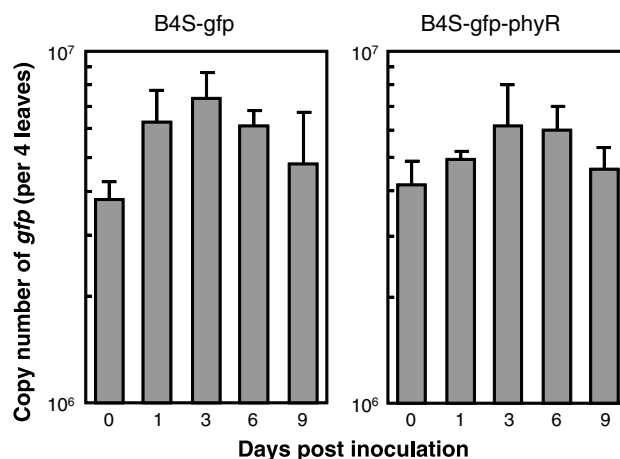
Fig. 4 Gene expression on *Arabidopsis* leaves. Gene expression of *Methylosinus* sp. B4S was analyzed by RT-qPCR on *Arabidopsis* leaves, NMS agar plates and NMS liquid cultures. Before RNA extraction, the agar and liquid cultures were incubated for 1 day in the plant growth chamber. Shown are the relative transcriptional levels in which the copy numbers of each gene calculated with standard samples were normalized with 16S rRNA transcript. Data are the means of three replicate samples. *Error bars* represent standard deviations

Fig. 5 Growth profile on *Arabidopsis* leaves. The number of plant-colonized cells of *Methylosinus* sp. B4S strains was determined by qPCR. Data are the means of four replicates. *Error bars* represent standard deviations

level of *pmoA*, *mmoX*, *mxaF* and *phyR* was normalized with 16S rRNA transcript in each sample. In general, the 16S rRNA transcriptional level changes depending on the growth phase and culture condition and was tenfold higher in liquid culture than on agar plates in this study. On the other hand, copper concentration in the media did not affect the 16S rRNA transcriptional level. Hence, we evaluated the transcriptional levels of *phyR* and C1 metabolism genes relative to the 16S rRNA transcript within each culture (leaf, agar and liquid culture).

It was found that *mmoX* was more highly expressed than *pmoA* on *Arabidopsis* leaves (Fig. 4). Expression of *pmoA* and *mmoX* was reported to be regulated by copper ion in some methanotrophs (Semrau et al. 2010), and such copper-dependent regulation was confirmed in vitro in *Methylosinus* sp. B4S. As shown in Fig. 4, both on agar plates and in liquid culture, *mmoX* (sMMO-encoding gene) was strongly repressed in the presence of copper compared to in the absence of copper addition while the repression level of *pmoA* (pMMO-encoding gene) was low without copper addition. Based on these expression profiles, *Arabidopsis* leaf surfaces were suggested to represent low copper conditions. In this study, *Arabidopsis* plants were cultivated in Hoagland medium (Cu^{2+}, 0.16 μM) and tap water with daily replenishment; thus, sufficient copper for plant growth was available. Furthermore, relatively high expression of *mxaF* was found on leaves (Fig. 4), suggesting that the methanotrophic cells may respond to methanol present on leaf surfaces (Hanson and Hanson 1996).

High expression level of *phyR*, relative to other three C1 metabolism genes, was found on leaves and agar plates

than in liquid culture (Fig. 4). We suspect that solid culture (i.e., leaf and agar plate) elevated *phyR* gene expression. During plant habitation, the transcriptional level of *phyR* was decreased similarly to C1 metabolism genes (Fig. 4), which may represent the decline in whole cellular activity, as observed with the GFP fluorescence (Fig. 1).

Growth of methanotroph in the phyllosphere

Growth on leaves between *phyR*-intact and deletion strains was compared by qPCR. Inoculation with cells on *Arabidopsis* leaves, DNA extraction and calculation were subjected to the quantitative methods. The inoculated cells of strain B4S-gfp divided once until 3 dpi (Fig. 5). After that, the cells gradually decreased but survived for at least 9 days, which supports the results of gene expression and GFP fluorescence on *Arabidopsis* leaves (Figs. 1, 4). The *phyR*-deletion strain (B4S-gfp-phyR) also showed similar pattern of growth (Fig. 5) and GFP fluorescence profile (data not shown). These results indicated that *phyR*-regulated stress resistance is not necessary for plant colonization under the growth chamber condition where methanotrophs were not exposed to heat and UV stresses.

Discussion

The phyllosphere is a distinct environment from soils and aqueous environments that have been the major targets of studies of methanotrophs; thus, the life of methanotrophs in the phyllosphere would be of interest. In this study, we examined the expression of *phyR* and C1 metabolism genes on plant leaves, firstly with a methanotroph *Methylosinus*

sp. B4S. We also assessed the ability of methanotrophs to adapt to rapid environmental changes and stresses occurring in the phyllosphere by functional analysis of PhyR.

Methylosinus sp. B4S was demonstrated to colonize *Arabidopsis* leaves (Figs. 1, 5), although *Arabidopsis* plants were reported not to produce methane (Nisbet et al. 2009). The cell proliferation occurred in the initial 3 days after inoculation, presumably by utilizing methanol emitted from leaves and/or nutrients accumulated during preculture (Fig. 5). Moreover, though the cell proliferation did not continue, methanotroph was able to survive on leaves (Figs. 1, 5). The *phyR*-deletion strain colonized and survived on *Arabidopsis* leaves similarly to the wild-type strain (Fig. 5). We think that cultivation of *Arabidopsis* plants in the growth chamber did not afford the *phyR*-regulated stresses, heat shock and UV radiation, to *Methylosinus* sp. B4S. These stresses are serious for phyllospheric microorganisms in natural environments where plant surfaces are directly irradiated by sunlight and the surface temperature is rapidly affected by the day/night cycle.

In our previous study, a microbial sensor determined the methanol concentration present on *Arabidopsis* leaf surfaces to be between 4 and 64 mM (Kawaguchi et al. 2011). *Methylosinus* sp. B4S cannot grow with methanol concentrations above 25 mM (Iguchi et al. 2012), and hence, cells may suffer and accumulate a damage derived from methanol and its metabolite formaldehyde during plant habitation. Nevertheless, this methanotroph seems to be able to survive and colonize on plant leaves in nature. This might be explained by the fact that methanotrophs often cohabitate on plant leaves with other methanol utilizers (Iguchi et al. 2012), which can reduce the local methanol concentration in the phyllosphere.

Methane and methanol are the main carbon sources for methanotrophs in natural environments and are assumed to be available in the phyllosphere. To characterize the C1 metabolism of methanotrophs in the phyllosphere, gene expression was examined on *Arabidopsis* plants. Methanotrophs oxidize methane with pMMO and sMMO, depending on copper availability in the environment. Some studies of in situ methanotroph activity indicated that pMMO was largely responsible for methane oxidation in soils, since the transcript of *pmoA* but not *mmoX* was detected (Chen et al. 2007; Han and Semrau 2004). These results also suggested that soil contained sufficient amount of copper to induce *pmoA* gene expression. Our present results indicated that both *pmoA* and *mmoX* were expressed in the phyllosphere (Fig. 4).

We confirmed that the copper-dependent regulation of MMO genes occurs in *Methylosinus* sp. B4S on agar and in liquid cultures (Fig. 4) although the repression level of *pmoA* transcript was very small, probably due to an influence of trace amount of copper ion contaminated in the medium. When inoculated on *Arabidopsis* leaves, *Methylosinus* sp. B4S exhibited a higher level of *mmoX* transcription than that of *pmoA* (Fig. 4), which represents the expression profile in copper-limited conditions. These results suggest that copper availability for methanotrophs is low on leaf surfaces, and hence, sMMO can respond to methane oxidation in the phyllosphere. Plants regulate copper uptake using a copper transporter and employ copper chaperones to avoid the toxic effects of copper (Klaumann et al. 2011). The copper concentration is estimated to be 2–50 µg/g dry weight of plant material and 4 µg/g dry weight of *Arabidopsis* leaves (Burkhead et al. 2009; Klaumann et al. 2011). However, these data cannot fully explain the amount of copper that microorganisms sense and utilize. To this end, the quantitative technique of in situ gene expression of microorganisms is practical. Quantitative RT-PCR analysis was used in this study to define this physiological aspect of methanotrophs on leaf surfaces.

The present study characterized the in situ activity of methanotroph in the phyllosphere. It was found that PhyR of the methanotroph *Methylosinus* sp. B4S regulates resistance to heat and UV stress, and C1 metabolism genes were expressed and regulated in the phyllosphere. These cellular functions are expected to play an important role in habitation of methanotrophs in natural environment.

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