Physical contact and carbon transfer between a lichen-forming *Trebuoxia* alga and a novel *Alphaproteobacterium*

Mieko Kono,1,* Hideyuki Tanabe,1 Yoshihito Ohmura,2 Yoko Satta1 and Yohey Terai1,*

Abstract

Recent progress in molecular techniques has begun to alter traditional recognition of lichens as symbiotic organisms comprised of a fungus and photosynthetic partners (green algae and/or cyanobacteria). Diverse organisms, especially various non-photosynthetic bacteria, are now indicated to be integral components of lichen symbiosis. Although lichen-associated bacteria are inferred to have functions that could support the symbiosis, little is known about their physical and nutritional interaction with fungi and algae. In the present study, we identified specific interaction between a lichen-forming alga and a novel bacterium. *Trebuoxia* alga was isolated from a lichen, *Usnea hakonensis*, and kept as a strain for 8 years. Although no visible bacterial colonies were observed in this culture, high-throughput sequencing of DNA isolated from the culture revealed that the strain is composed of a *Trebuoxia* alga and an *Alphaproteobacterium* species. *In situ* hybridization showed that bacterial cells were localized on the surface of the algal cells. Physiological assays revealed that the bacterium was able to use ribitol, glucose and mannitol, all of which are known to exist abundantly in lichens. It was resistant to three antibiotics. Bacteria closely related to this species were also identified in lichen specimens, indicating that *U. hakonensis* may commonly associate with this group of bacteria. These features of the novel bacterium suggest that it may be involved in carbon cycling of *U. hakonensis* as a member of lichen symbiosis and less likely to have become associated with the alga after isolation from a lichen.

INTRODUCTION

Lichens are traditionally described as symbiotic organisms composed of a fungal partner (mycobiont) and photosynthetic partners (photobionts). The symbiotic interaction provides a mycobiont with carbon nutrition, and photobionts with a suitable habitat that protects them from abiotic and biotic stresses [1]. In addition to the mycobiont and the photobiont, lichens are known to host various organisms on or within their symbiotic structures (thalli) and are sometimes considered to be self-contained ecosystems [1–3]. Recent progress in molecular techniques elucidated supportive roles of these ‘guests’ in functioning lichen symbioses. By using high-throughput sequencing techniques, Stribille et al. [4] identified a group of basidiomycete yeasts that ubiquitously associate with Parmeliaceae (Lecanoromycetes) lichens, indicating that more species than expected could be fundamentally involved in the establishment of lichen ecosystems.

Non-phototrophic bacteria associated with lichens were first reported in 1925 by Uphof [5], followed by studies that revealed the diversity of culturable lichen-associated bacteria and suggested their possible roles in lichen symbiosis [6, 7]. However, culture-dependent studies could not describe the overall composition of bacterial communities due to limitations in culturing techniques, and left the bulk of lichen-associated bacteria unknown [8]. Nowadays, culture-independent techniques, especially high-throughput sequencing techniques, have revealed the diversity and abundance of lichen-associated bacteria [2, 9–11]. Lichens are hypothesised to have mechanisms that select bacterial species beneficial for their symbioses [2, 9, 10, 12, 13]. Indeed, lichen-associated bacteria are inferred to have

Received 17 November 2016; Accepted 7 March 2017

Author affiliations: 1SOKENDAI (The Graduate University for Advanced Studies), Department of Evolutionary Studies of Biosystems, Shonan Village, Hayama, Kanagawa 240-0193, Japan; 2Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba, Ibaraki 305-0005, Japan.

*Correspondence: Mieko Kono, kono_mieko@soken.ac.jp; Yohey Terai, terai_yohei@soken.ac.jp

Keywords: *Usnea hakonensis*; carbon cycle; Sphingomonas.

Abbreviations: DAPI, 4’,6-diamidino-2-phenylindole; DNP, dinitrophenyl; FISH, fluorescence *in situ* hybridization; GCD, glucose dehydrogenase; ITS, internal transcribed spacer; KEGG, Kyoto Encyclopaedia of Genes and Genomes; PPP, pentose phosphate pathway; PQQ, pyrroloquinoline quinone; qPCR, quantitative PCR.

The DDBJ accession numbers for the genomic sequences of *Trebuoxia* sp. ‘TZW2008’ are BDIU01000001–BDIU01000077, for the genomic sequences of *Sphingomonas* sp. ‘TZW2008’ are BDJB01000001–BDJB01000024, and the accession numbers for the 16S rDNA sequences of *Usnea hakonensis* are: LC197944 (Uh_seq1); LC197945 (Uh_seq2).

One supplementary table is available with the online Supplementary Material.
functions such as nutrient supply and recycling of resources, which would enhance the persistence of lichens, which are often found in nutrient-poor environments [2, 10, 11, 14]. Moreover, recent metagenomic and proteomic studies suggested the contribution of bacteria to lichen survival under extreme and changing ecological conditions [10, 15].

The roles of bacteria in algal growth and survival are better understood in interactions between non-lichen-forming algae [16, 17]. Several studies reported that bacteria supply nutrients such as vitamin B12, iron, nitrogen, phosphorus and carbon to algae often, but not always, in exchange for photosynthetic products [18–21]. Such complementary functions are also predicted in interactions between lichen-associated non-phototrophic bacteria and the photobiont [6, 7]. Although addition of lichen extracts to media can improve the culturability of lichen-associated bacteria [22], little is known about their special requirements for nutrition and physical contact with mycobionts and photobionts.

Here, we present the physical contact and carbon transfer between a Trebouxia alga and a novel Alphaproteobacterium. A strain of Trebouxia algae isolated from a fruticose lichen, Usnea hakonensis, has been cultured for 8 years. The whole genome sequencing of this Trebouxia strain revealed the presence of a cryptic bacterium that covers the surface of algal cells. Our experiments showed that the bacterial growth is dependent on an algal photosynthetic product, ribitol, which Trebouxia algae are known to release from cells abundantly in lichens.

**METHODS**

Algal and bacterial strains and growth conditions

The algal strain used in this study was provided by Dr Yoshiaki Kon, Tokyo Metropolitan Hitotsubashi High School, Japan. It was isolated from U. hakonensis collected from Kanagawa Prefecture, Japan (35°26’N, 139°10’E) in 2008 by the method described previously [23]. A strain of the alga (Trebouxia sp. TZW2008) associated with the cryptic bacterium (Sphingomonas sp. TZW2008) has been maintained for 8 years. Since 2012, the algal strain has been kept on 2% agar plates of autotrophic C medium [containing 15 mg Ca(NO3)2·4H2O, 10 mg KNO3, 5 mg β-Na-glycerophosphate·5H2O, 4 mg MgSO4·7H2O, 0.01 μg vitamin B12, 0.01 μg biotin, 1 μg thiamin HCL, 0.3 ml PIV metals (100 mg Na2EDTA·2H2O, 19.6 mg FeCl3·6H2O, 3.6 mg MnCl2·4H2O, 1.04 mg ZnCl2, 0.4 mg CoCl2·6H2O, 0.25 mg Na2MoO4·2H2O, in 100 ml solution), 50 mg Tris, per 100 ml of medium (pH 7.5)] [24]. In addition, the culture was grown on 3% agar plates of heterotrophic medium MY [containing 2% (w/v) malt extract, 0.2% (w/v) yeast extract, pH 5.8] and checked for colonies of contaminant bacteria.

Genomic DNAs were extracted and concentrated using NucleoSpin Plant II (Macherey-Nagel, Düren, Germany) following the protocol using Buffer PL2. DNA libraries were constructed using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer’s instructions. Short DNA sequences (paired-end 125 bp) were determined from the libraries by Illumina HiSeq2500 platform (short reads). After removal of the adaptor sequences and low-quality reads, the reads were assembled into scaffolds using CLC genomic workbench (www.qiagenbioinformatics.com/) with a word size of 64.

Scaffold sorting by average coverage per site

The short reads were mapped to the assembled scaffolds and an average coverage per site was calculated for each scaffold using CLC genomic workbench. All scaffolds larger than 2 kb were sorted into two groups by the average coverage of ×2000 (Table 1). Partial sequences of scaffolds were submitted to BLASTN search [25] against the NCBI database (nr/nt: www.ncbi.nlm.nih.gov).

Phylogenetic analysis of the alga and bacterium

The internal transcribed spacer (ITS) rDNA sequence of Trebouxia sp. TZW2008 was amplified using AL1500bf [26] and LR3 [27] primers. Specific primers for the alga were designed to fill the gap left by the sequencing of the PCR products amplified with AL1500bf and LR3 (Treboko_n_ITS in Table 2). PCR in 25 µl reactions was run using Takara ExTaq DNA polymerase (Takara Bio, Shiga, Japan) with the following conditions: 30 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 58°C and extension for 1 min at 72°C, and final extension at 72°C for 5 min. The ITS rDNA sequence was aligned with the ITS rDNA sequences retrieved from Ohmura et al. [28] and the NCBI database. The 16S rDNA sequence of Sphingomonas sp. TZW2008 was found on a bacterial contig. The sequence was aligned with bacterial 16S rDNA sequences selected from Bates et al. [9] and those of Sphingomonas species additionally collected from the NCBI database. The phylogenetic trees were constructed in MEGA version 6 [29] using the p-distance algorithm and the neighbour-joining algorithm [30], with 1000 bootstrap replications.

Detection of Sphingomonas-like bacteria in field-collected lichens

We investigated field-collected lichens (U. hakonensis) to detect lichen-associated bacteria similar to Sphingomonas

<table>
<thead>
<tr>
<th>Table 1. Overview of the algal and bacterial genomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Trebouxia sp. TZW2008</strong></td>
</tr>
<tr>
<td>Predicted genome size (Mb)</td>
</tr>
<tr>
<td>No. of scaffolds (&gt;2 kb)</td>
</tr>
<tr>
<td>Average coverage</td>
</tr>
<tr>
<td>N50</td>
</tr>
</tbody>
</table>
sp. TZW2008. Lichens were sampled in 2013 at the same location as in 2008 (Kanagawa Prefecture, Japan; 35° 26' N, 139° 10' E). Total RNAs were extracted using RNeasy Plant mini kit (QIAGEN, Venlo, the Netherlands) and cDNAs were synthesized using SMARTer cDNA synthesis kit (Takara Bio, Shiga, Japan). We designed specific primers (Tr_bac in Table 2) to amplify the 16S rDNA sequence similar to that of Sphingomonas sp. TZW2008. We used cDNAs as PCR templates because cDNAs synthesized from total RNAs generally contain rDNA sequences. PCR in 25 µl reactions was run using Takara ExTaq DNA polymerase (Takara Bio, Shiga, Japan) with the following conditions: 35 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 66°C and extension for 30 s at 72°C, and final extension at 72°C for 5 min. A phylogenetic tree was constructed by the method described above using amplified sequences and sequences of Sphingomonas species used in the phylogenetic analysis of the bacteria.

Detection of a novel bacterium by fluorescence in situ hybridization

The bacterial 16S rDNA sequence was amplified by PCR using specific primers (Sphingo-like in Table 2) and genomic DNA of Sphingomonas sp. TZW2008 as a template. The PCR products were labelled with either Digoxigenin-11-dUTP (Roche, Basel, Switzerland) or DNP-11-dUTP (Invitrogen, Carlsbad, CA) were diluted to 1 : 200 and used as PCR templates because cDNAs synthesized from total RNAs generally contain rDNA sequences. PCR in 25 µl reactions was run using Takara ExTaq DNA polymerase (Takara Bio, Shiga, Japan) with the following conditions: 35 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 66°C and extension for 30 s at 72°C, and final extension at 72°C for 5 min. A phylogenetic tree was constructed by the method described above using amplified sequences and sequences of Sphingomonas species used in the phylogenetic analysis of the bacteria.

Detection of a novel bacterium by fluorescence in situ hybridization

The bacterial 16S rDNA sequence was amplified by PCR using specific primers (Sphingo-like in Table 2) and genomic DNA of Sphingomonas sp. TZW2008 as a template. The PCR products were labelled with either Digoxigenin-11-dUTP (Roche, Basel, Switzerland) or DNP-11-dUTP (Perkin Elmer, Waltham, MA) by secondary PCR. The labelled rDNA sequences were collected by ethanol precipitation and resuspended in hybridization solution (50% formamide and 10% dextran sulfate in 2×SSC) for use as probes. For fluorescence in situ hybridization (FISH), algal colonies cultured with the bacterium on an agar plate were cut out with agar substrate and placed on a poly-L-lysine-hydrobromide coated glass slide for 5 min until the colonies adhered to the slide surface. The samples were fixed with Carnoy solution (methanol:acetic acid= 3:1) for 30 min, dried for 30 min at room temperature and baked at 70°C for approximately 1 h. FISH was carried out in a previously described manner [31, 32] with slight modifications. The glass slide with the samples was denatured in 70% formamide in 2×SSC at 70°C for 2.5 min, and dehydrated in a series of ice-cold ethanol (70%, 85% and absolute) for 3 min each. The denatured probes were applied onto the glass slide and the slide was covered with a coverslip and sealed. Hybridization was performed in a moist chamber at 37°C for 3 h. The samples were washed twice in 2×SSC, three times in 0.1×SSC at 60°C for 5 min each, and blocked with 5% BSA in 4×SSC with 0.2% Tween-20 for 30 min at 37°C in the dark. Anti-Dinitrophenyl (DNP) rabbit antibody (Sigma, Kanagawa, Japan) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) were diluted to 1: 200 and used to detect DNP-labelled probes. Monoclonal anti-Digoxigenin mouse antibody (Sigma, Kanagawa, Japan) and Cy3-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted to 1:200 and used to detect DIG-labelled probes. The samples were treated with RNase (Sigma, Kanagawa, Japan; 100 µg ml⁻¹) during a series of antibody detections to reduce the non-specific background signals. Nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, Kanagawa, Japan) and TOPRO-3 (Molecular Probes, Eugene, OR) simultaneously, and the slides were mounted in Vectashield Antifade (Vector Laboratories, Burlingame, CA). Fluorescent images were observed using a Leica DM5000B epi-fluorescence microscope equipped with an Plan-Apochromat 63×/1.4 OIL objective lens and the filter set for DAPI, Green, and Cy3, captured by a Leica CW4000 system (Leica Microsystems, Hessen, Germany). Counterstained nuclei were coloured blue (DAPI) and FISH signals were coloured green (DNP-labelled probes) and magenta (DIG-labelled probes), respectively. For 3D image analysis, a confocal laser scanning microscope (LSM510META, Carl Zeiss Microscopy, Thuringia, Germany), equipped with a Plan-Apochromat 63×/1.4 OIL objective lens and argon (488 nm) and helium-neon (543/633 nm) lasers was used. Fluorescent images for each optical section were recorded in 200 nm z-intervals and collected sequentially in three separate RGB channels with Red (Cy3), Green (Alexa488) and Blue (Cy5; TOPRO-3), respectively. The image stacks were processed with microscope operating software (LSM5; Carl Zeiss Microscopy, Thuringia, Germany) and three-dimensional images were reconstructed using Amira 3.1.1 software (FEI, Hillsboro, OR).

Search for genes representing symbiotic interactions

To investigate interactions between Trebouxia sp. TZW2008 and Sphingomonas sp. TZW2008, genes reportedly involved in algae–bacteria symbiotic interactions were searched for. Sequences of the species closely related to Trebouxia sp. TZW2008 and Sphingomonas sp. TZW2008 were used as queries in the search. For green algae we chose Chlamydomonas reinhardtii, Volvox carteri f. nagariensis and Coccomyxa subellipsoidea. For Sphingomonas bacteria, we chose Sphingomonas taxi, Sphingomonas wittichii and Sphingomonas sp. WHSC-8. Gene sequences were retrieved from the Kyoto
Encyclopedia of Genes and Genomes (KEGG) and the NCBI database, which are listed in Table S1 (available in the online Supplementary Material). BLASTx searches [33] were locally performed against the algal cDNA and bacterial genome sequences. When the e-values of a result were larger than or equal to 1e-20, the gene was considered absent in the genome.

Effect of antibiotics on the alga and the bacterium

In an attempt to test the effect of antibiotics on algal and the bacterial growth, three antibiotics were selected. Ampicillin, kanamycin and chloramphenicol were added to the autotrophic C medium at final concentrations of 50, 250, 500 and 1000 µg ml⁻¹ for ampicillin, 50 µg ml⁻¹ for kanamycin and 32.5, 162.5, 325 and 650 µg ml⁻¹ for chloramphenicol. The strain of alga associated with the bacterium was inoculated onto media containing the antibiotics at each concentration. Three experimental replicates were conducted. The cultures were collected 1, 7 and 14 days after inoculation. At each sampling point, algal growth was measured by absorption at 680 nm normalized at 750 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). DNA was extracted from the samples and stored at −20 °C for use in quantitative PCR (qPCR) analysis.

To estimate the relative copy number of the bacterial genome to the algal genome, the copy number of algal actin gene and the bacterial 16S rRNA gene in the DNA extracted from the collected cultures were quantified by qPCR. Plasmid DNA was prepared as standard samples for quantification of each gene using specific primers for the algal actin gene (Trebo_act1 in Table 2) and the bacterial 16S rRNA gene (Tr_bac in Table 2). Partial sequences of the algal actin gene and the bacterial 16S rRNA gene were amplified and each PCR product was cloned into pMD20 vector (Takara Bio, Shiga, Japan) using Ligation Kit version 2 (Takara Bio, Shiga, Japan). qPCR reactions were performed with Thermal Cycler Dice TP800 (Takara Bio, Shiga, Japan) using the program of an initial denaturing step at 95 °C for 30 s, followed by 40 cycles of denaturing at 95 °C for 5 s and extension at 55 °C for 1 min. Two types of reaction mixture containing either the algal or bacterial primer set, were prepared with SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan). For every qPCR reaction, a series of diluted plasmid DNA (10 pg, 1 pg, 0.1 pg, 10 fg, and 1 fg, per µl) was prepared to construct a standard curve for the algal and bacterial genes. All qPCR reactions were run with three replicates. The copy number of the algal and bacterial genes in each sample DNA was quantified using the respective standard curves. The relative copy number of the bacterial genome to the algal genome in each sample DNA was estimated from these gene copy numbers.

Bacterial growth on medium supplemented with various carbohydrates

To identify the carbon source of the bacterium associated with the alga, 2 % ribitol, glucose, mannitol or sucrose solution (w/v) was spread over the surface of the heterotrophic MY media. The strain of alga associated with the bacterium was inoculated onto each medium with a platinum loop. The 16S rDNA sequences of bacterial colonies that grew on the ribitol-supplemented medium were amplified by PCR using Tr_bac primers (see the ‘Detection of Sphingomonas-like bacteria in field-collected lichens’ for the PCR condition. The condition was modified to 25 cycles with the annealing temperature 58 °C). Bacterial colonies were carefully sampled using micro glass tubes, avoiding any contamination by algal colonies.

Algal and bacterial growth on cycle and in constantautotrophic medium

Autotrophic C media inoculated with the strain of alga associated with the bacterium were cultured under a 12 h/12 h light/dark cycle and in constant darkness. Three experimental replicates were conducted for each condition, collected 1, 3, 6 and 12 days after inoculation. At each sampling point, 3 ml of liquid C medium was added to a medium and algal cells were scraped off the medium with a spreader. A 2 ml aliquot was collected for measurement of algal growth using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) and DNA was extracted for the qPCR analysis (described in the above sections). Algal and bacterial growth was estimated from the copy numbers of the algal actin and bacterial 16S rRNA genes, respectively.

Data deposition

The DDBJ accession numbers for the genomic sequences of Trebouxia sp. TZW2008 are BDJU01000001-BDJU01000677, for the genomic sequences of Sphingomonas sp. TZW2008 are BDJB01000001-BDJB01000024, and the 16S rDNA sequences of Usnea hakonensis are LC197944 (Uh_seq1) and LC197945 (Uh_seq2).

RESULTS

Culture of a green alga isolated from a lichen contained a novel bacterium

A green alga belonging to the genus Trebouxia (Trebouxia sp. TZW2008) was isolated from a U. hakonensis in 2008 by Dr Yoshiaki Kon and has been kept as a strain for 8 years. No bacterial contamination was observed during the culturing. Phylogenetic analysis using the sequences of ITS rDNA showed that Trebouxia sp. TZW2008 was closely related to Trebouxia corticola (Fig. 2a), which is a common symbiont of lichens [1].

Genomic DNA was isolated from cultured Trebouxia sp. TZW2008 and sequenced by Illumina Hiseq2500 platform. The 94 million reads (125 bp in length) were de novo assembled into 1997 scaffolds. Seven hundred and one scaffolds remained after the removal of scaffolds smaller than 2 kb.

We mapped the reads on the scaffolds and calculated the average number of reads mapped to each site of a scaffold and found that the scaffolds are distinguishable into two groups: a high-coverage group (average coverage per site ≥ 2×2000) and a low-coverage group (<×2000). When the scaffolds are
plotted with the length against the count of mapped reads, they are clearly separated into high- and low-coverage groups (Fig. 1). The BLASTN search against the NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov) showed that several large scaffolds in the high coverage group were homologous to the genomic sequence of the Gram-negative bacterium Sphingomonas taxi (CP009571.1), whereas large scaffolds in the low-coverage group were homologous to sequences of algal species belonging to Chlorophyta.

The BLASTN search of all the scaffolds in the high-coverage group revealed that these were homologous to algal chloroplastic, mitochondrial, rDNA sequences (shown as triangles in Fig. 1) and Sphingomonas sequences (squares). In consequence, 24 out of the 50 scaffolds were defined as bacterial sequences. The phylogenetic analysis of 16S rDNA sequences found on a bacterial scaffold also showed that the bacterium belonged to the genus Sphingomonas (Fig. 2b). This suggested the presence of a Sphingomonas species (hereafter called Sphingomonas sp. TZW2008) in the algal culture.

Genome size predicted from the sum of the scaffolds was 69 Mb for Trebouxia sp. TZW2008 and 3.5 Mb for Sphingomonas sp. TZW2008, which are comparable with closely related species: Trebouxia gelatinosa (57 Mb; www.ncbi.nlm.nih.gov/genome/?term=Trebouxia), Trebouxia decolorans (55 Mb) [34], S. taxi (4 Mb; www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+taxi), S. wittichii (6 Mb; www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+wittichii) and Sphingomonas sp. WHSC-8 (5 Mb; www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+WHSC-8). Average coverage for the predicted genome and N50 value are ×371 and 223 kb for the alga, and ×6245 and 273 kb for the bacterium (Table 1).

**Novel bacterium interacts with the lichen-forming alga through carbon transfer**

We cultured the algal strain on the heterotrophic MY medium for 1 month in order to promote growth of the cryptic bacterium associated with the alga. However, no visible bacterial colonies were observed (Fig. 3a). Therefore we performed FISH, targeting the 16S rDNA sequence of the bacterium. FISH not only confirmed its existence but also revealed the localization of bacterial cells on algal cells (Fig. 3b, c). Moreover, 3D analysis clearly visualized bacterial cells covering the surface of algal cells. A large number of bacterial cells (red and green signals) were detected even in deep positions between algal cells (Fig. 3d). These observations suggested that Sphingomonas sp. TZW2008 is unculturable without the alga. The quantification of the algal and bacterial growth on the autotrophic C medium under photosynthesis-activated and -inhibited conditions revealed that the alga and bacterium grew in a synchronized manner (Fig. 4). They grew dynamically only when algal photosynthesis was active (after day 6), compared to the photosynthesis-inhibited condition (t-test, alga, P=0.047; bacterium, P=0.025), indicating that both the alga and the bacterium are dependent on the algal photosynthetic product for growth.

To clarify the carbon source of Sphingomonas sp. TZW2008, the alga with the bacterium was cultured on heterotrophic MY medium supplemented with carbohydrates (ribose, glucose, mannitol or sucrose) previously identified in lichen thalli [35, 36]. As shown in Fig. 5(a–c), bacterial colonies distant from algal colonies were observed on the ribitol-, glucose- and mannitol-supplemented media. We amplified and sequenced the 16S rRNA gene from these colonies and verified that Sphingomonas sp. TZW2008 formed the colonies. Trebouxia photobionts are known to produce ribitol as a photosynthetic product [37]. Previous studies indicated that it is the main carbohydrate transferred from Trebouxia photobionts to mycobionts [35, 38] and exists in abundance in lichen thalli [36]. Within lichen thalli, ribitol is indicated to be metabolized by a fungal partner via
the pentose phosphate pathway (PPP) [38, 39]. Our gene search identified bacterial genes similar to those required in the proposed ribitol metabolism via the PPP, and inferred the ability of Sphingomonas sp. TZW200 to utilize ribitol (Table 3, Fig. 6).

Along with ribitol, glucose is a well-documented photosynthetic product that is transferred from a photobiont to a mycobiont in lichen thalli [40–42]. The gene search confirmed that the bacterial genome retains all the genes required in the glycolysis and Entner–Doudoroff pathway. Once taken up by the mycobiont, both ribitol and glucose are converted to mannitol that is abundantly contained in lichen thalli [36, 37]. Although no gene similar to the mannitol dehydrogenase gene (required in mannitol degradation) was found in the bacterial genome, we identified a gene similar to that of sorbitol dehydrogenase. This enzyme is reported to oxidize a number of sugar alcohols, including mannitol [43]. Therefore, the bacterium may use this enzyme to degrade mannitol rather than mannitol dehydrogenase. On the other hand, no bacterial colonies were observed on the sucrose-supplemented medium (Fig. 5d). Consistent with this, two genes encoding enzymes that metabolize sucrose were not identified in the bacterial genome. These results indicate the inability of Sphingomonas sp. TZW2008 to use sucrose as its carbon source.

The results of FISH and the culturing experiments indicate that, during its growth, the alga is tightly associated with bacterial cells as if wearing them around its cell wall, and provides ribitol to them. The gene search suggested that the bacterium does not have genes of enzymes that function in the digestion of cellulose, one of the major components of the algal cell wall. Therefore Sphingomonas sp. TZW2008 is likely to obtain extracellular ribitol released by Trebouxia sp. TZW2008.

**Bacterial roles in the interaction between the novel bacterium and the lichen-forming alga**

To examine whether the association of the bacterium and the alga is symbiotic or not, we searched for genes associated with algae–bacteria symbiotic interactions in the genome of the bacterium. The average coverage of the algal and the bacterial genomes sequenced in this study was \( \times 371 \) and \( \times 6245 \), respectively, and sequences were well assembled (Table 1). Therefore we consider that it is less likely to overlook genes by misassembly. Nitrogen fixation, phosphate solubilization and vitamin B\(_{12}\)

---

**Fig. 2.** Phylogenetic relationships of (a) the alga and (b) the bacterium based on ITS rDNA and 16S rDNA sequences, respectively. Scale bars represent 0.02 substitutions per site. The arrows show the position of Trebouxia sp. TZW2008 in (a) and Sphingomonas sp. TZW2008 in (b).
synthesis are well-studied bacterial roles in mutualistic interactions with algae. First, the ability of *Sphingomonas* sp. TZW2008 in nitrogen fixation was investigated. *Sphingomonas* species used as query sequences retain several enzymatic genes involved in nitrogen fixation. However, no similar sequence to those genes were found in the
bacterial genome. *Sphingomonas* sp. TZW2008 formed colonies distant from the alga on the MY medium supplemented with carbon sources. Therefore *Sphingomonas* sp. TZW2008 can obtain nitrogen from MY medium containing various nitrogen sources. According to our investigation, nitrate and nitrite reductase genes were found to be missing from the genome of *Sphingomonas* sp. TZW2008, indicating that neither nitrate nor nitrite could be the source of nitrogen for the bacterium. The strain of alga has been subcultured on the autotrophic C medium in our laboratory since 2012. This medium contains nitrate as the sole nitrogen source. Therefore, during subculture in our laboratory, *Sphingomonas* sp. TZW2008 is likely to have relied on the alga for its nitrogen source. Second, phosphate solubilization ability was examined. Although the genetic mechanism of mineral phosphate solubilization activity is not fully understood, biosynthesis of gluconic acid, carried out by the enzyme glucose dehydrogenase (GCD), is considered as a key step in many Gram-negative bacteria [44, 45]. In the genome of *Sphingomonas* sp. TZW2008 we identified a gene similar to that encoding pyrroloquinoline quinone (PQQ), the cofactor of GCD, but the gene encoding GCD was not found. In the genome of *S. taxi*, the GCD gene is encoded in the region where genes involved in phosphate regulation activity located. *Sphingomonas* sp. TZW2008 also retains all those regulatory genes but lacks the GCD gene. Finally, genes encoding enzymes involved in vitamin B<sub>12</sub> biosynthesis were also not identified. Therefore we predict that *Sphingomonas* sp. TZW2008 could not have supplied phosphate and vitamin B<sub>12</sub> to the alga.

Previous studies reported the release of amino acids by lichen-associated bacteria [11, 14, 46]. The constant supply of amino acids by a symbiotic partner can cause loss of genes involved in the biosynthesis of the supplied amino acids [47, 48]. Therefore, we searched the genomes of the alga for genes required in the biosynthesis of the 20 proteinogenic amino acids. We also searched the genomes of the bacterium in case there was amino acid supply from the alga to the bacterium. However, both alga and bacterium retained the genes in the biosynthetic pathways and may have been able to synthesize the amino acids per se.

**Tight association between the novel bacterium and lichen-forming alga**

To eliminate the bacterium from the algal cells, the strain of alga associated with the bacterium was grown on autotrophic C medium with antibiotics: ampicillin, kanamycin and chloramphenicol. As shown in Fig. 7(a), kanamycin at the final concentration of 50 µg ml<sup>-1</sup> almost halted algal growth.
7 days post-inoculation, whereas the other antibiotics had less effect even at higher concentrations (Fig. 7b). The low growth rate of the alga on the kanamycin-supplemented medium may have been due to the inhibitory effect of kanamycin on algal chloroplasts [49–51].

The effect of each antibiotic on the bacterial growth was observed by quantification of the 16S rRNA gene using qPCR (Fig. 7c). The relative copy number of the bacterial gene to the algal gene was used as an indicator of remaining bacterial cells. Although the alga showed variation in growth rate between the three antibiotics at the concentrations 50 µg ml\(^{-1}\) for ampicillin and kanamycin, and 32.5 µg ml\(^{-1}\) for chloramphenicol, the relative copy number reached the same value as that of the control after 14 days. Following antibiotic treatment, the culture was transferred to a fresh autotrophic medium without antibiotics. The survival of the bacterium was confirmed by amplification of the 16S rDNA sequence by PCR. The higher concentrations of ampicillin and chloramphenicol succeeded in reducing the relative copy number to almost one-tenth of that in the control after 14 days (Fig. 7d, \(t\)-test, \(P<0.05\)), but failed to eliminate bacterial cells completely. These results indicated that Sphingomonas sp. TZW2008 is resistant to antibiotics and could not be easily separated from the alga.

Bacterial 16S rDNA sequences highly similar to that of Sphingomonas sp. TZW2008 were amplified from lichen collected at the same location in a different year, and formed a monophyletic group with Sphingomonas sp. TZW2008 (Fig. 8). This suggested that Sphingomonas sp. TZW2008 is a lichen-associated bacterium tightly associated with a lichen-forming alga.

**DISCUSSION**

It is now widely recognized that diverse and abundant bacteria are associated with lichens [6]. In the last decade, the specificity of lichen-associated bacteria and their contribution to lichen symbiosis have been suggested. Bates et al. [9] reported that bacterial community composition in lichens is distinct from that of surrounding soils. The community composition of lichen-associated bacteria is suggested to be specific to mycobiont species [2, 9], whereas Hodkinson et al. [13] inferred that photobiont type (green algae or cyanobacteria) is the more significant factor that shapes the community. Large geographical scale, substrate type, lichen secondary metabolites, lichen growth type and age of thallus are also factors likely to affect the community composition [2, 9, 10, 12, 13, 52]. Although there is no clear answer to what shapes bacterial communities, they are assumed to reflect requirements in lichen symbioses [6]. Physiological assays on isolated strains of lichen-associated bacteria confirmed their functions in nitrogen fixation, phosphate solubilization, amino acid excretion, lytic activities and antagonistic activities [2, 10, 14, 53]. Metagenomic and proteomic data also inferred their diverse functions in vitamin and hormone biosynthesis, detoxification and
Table 3. Genes mapped onto the PPP in KEGG

<table>
<thead>
<tr>
<th>Annotation</th>
<th>EC number*</th>
<th>Scaffold no.</th>
<th>e-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>1.1.1.44</td>
<td>3</td>
<td>8.0E-168</td>
</tr>
<tr>
<td>Glucose 1-dehydrogenase</td>
<td>1.1.1.47</td>
<td>7</td>
<td>1.0E-31</td>
</tr>
<tr>
<td>Glucose-6-phosphate 1-dehydrogenase</td>
<td>1.1.1.49</td>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td>Quinoprotein glucose dehydrogenase</td>
<td>1.1.5.2</td>
<td>–†</td>
<td>–</td>
</tr>
<tr>
<td>Transketolase</td>
<td>2.2.1.1</td>
<td>3</td>
<td>0.0</td>
</tr>
<tr>
<td>Transaldolase</td>
<td>2.2.1.2</td>
<td>3</td>
<td>4.0E-168</td>
</tr>
<tr>
<td>2-Dehydro-3-deoxygluconokinase</td>
<td>2.7.1.45</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ribose-phosphate pyrophosphokinase</td>
<td>2.7.6.1</td>
<td>3</td>
<td>7.0E-176</td>
</tr>
<tr>
<td>Gluconolactonase</td>
<td>3.1.1.17</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6-Phosphogluconolactonase</td>
<td>3.1.1.31</td>
<td>7</td>
<td>8.0E-114</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatase II</td>
<td>3.1.3.11</td>
<td>13</td>
<td>3.0E-174</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>4.1.2.13</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-Dehydro-3-deoxyphosphogluconate aldolase</td>
<td>4.1.2.14</td>
<td>7</td>
<td>3.0E-96</td>
</tr>
<tr>
<td>Xylulose-5-phosphate</td>
<td>4.1.2.9</td>
<td>7</td>
<td>–</td>
</tr>
<tr>
<td>Phosphogluconate dehydratase</td>
<td>4.2.1.12</td>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td>Ribulose-phosphate 3-epimerase</td>
<td>5.1.3.1</td>
<td>1</td>
<td>2.0E-114</td>
</tr>
<tr>
<td>Ribose 5-phosphate isomerase A</td>
<td>5.3.1.6</td>
<td>3</td>
<td>3.0E-45</td>
</tr>
<tr>
<td>Ribose 5-phosphate isomerase B</td>
<td>5.3.1.6</td>
<td>9</td>
<td>2.0E-62</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>5.3.1.9</td>
<td>31</td>
<td>0.0</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>5.4.2.2</td>
<td>12</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Enzyme commission number.
†BLASTX results with e-value <1e-20 are considered significant.
‡Not found.

Fig. 6. The metabolism of ribitol via the PPP. The proposed pathway metabolizing ribitol into mannitol in lichens. The enzymes necessary for the reactions are shown in blue. Sequences homologous to the genes encoding the two enzymes were found in the bacterial genome. Dashed lines represent uninvestigated input and output pathways of the PPP.
resistance against biotic and abiotic stress [10]. These bacterial functions are assumed to sustain the integrity and robustness of lichens [2, 10, 12]. Therefore, lichen symbiosis is now considered to be a complex symbiotic assembly involving multiple species rather than a dual interaction between the mycobiont and the photobiont.

In this study, we sequenced the genome of a lichen-forming green alga, Trebouxia sp. TZW2008, as well as the genome of an Alphaproteobacterium, Sphingomonas sp. TZW2008. Our genome search and experiments provided strong evidence for the specific interaction between Trebouxia sp. TZW2008 and Sphingomonas sp. TZW2008. Sphingomonas sp. TZW2008 is strictly localized on algal cell walls and its growth is dependent on the photosynthetic product of the alga. The bacterium was able to use ribitol, which is known to be produced by and released from Trebouxia algae [35], as a carbon source. Closely related bacteria were identified in lichen samples collected in the field, and therefore we concluded that this bacterium is less likely to have become associated with algal cells after its isolation from the lichen, but rather associated with the lichen. Alphaproteobacteria have been reported as the dominant taxon in bacterial communities of several lichen species [2, 9–13, 46]. Since Alphaproteobacteria were hardly detected in culture-dependent studies, most of them are suspected to be unculturable and highly specific to lichen symbioses [12]. Previous studies identified several carbohydrates in

Fig. 7. Effect of antibiotics on bacterial growth. Growth of the alga and the bacterium on the autotrophic C medium with antibiotics. Effects of the three antibiotics on algal growth were tested at concentrations of (a) 50 µg ml⁻¹ for ampicillin (AMP) and kanamycin (KAN), and 32.5 µg ml⁻¹ for chloramphenicol (CHL); (b) five (×5), ten (×10) and 20 (×20) times higher than the initial concentrations (50 and 32.5 µg ml⁻¹) for ampicillin and chloramphenicol. CON indicates algal growth on media without antibiotics. The x- and the y-axes represent days post-inoculation and absorption at 680 nm, respectively. Relative copy numbers of the bacterial genome to that of the algal genome were estimated at: (c) 1, 7 and 14 days post-inoculation onto ampicillin- (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹)- and chloramphenicol (32.5 µg ml⁻¹)-supplemented media; (d) 14 days post-inoculation onto ampicillin (×5, ×10 and ×20 indicate concentrations 250, 500 and 1000 µg ml⁻¹)- and chloramphenicol (×5, ×10, ×20 indicate 162.5, 325 and 650 µg ml⁻¹)-supplemented media. The x-axes represent days post-inoculation (c) and the concentrations of the antibiotics (d), and the y-axes represent relative copy number ratio. The error bars represent the standard deviation of three biological replicates.
lichen thalli such as glucose, sucrose, trehalose, ribitol, arabitol, mannitol and also some sugar phosphates [35, 36, 42]. Among those, ribitol, glucose and mannitol are the major carbohydrates responsible for the carbon flow within lichens, are abundant in thalli [36, 42, 54]. The preference of Sphingomonas sp. TZW2008 for ribitol, glucose and mannitol in our experiments might represent the characteristic of Sphingomonas sp. TZW2008 as an endolithic bacterial species specialized in environments within lichen thalli. Lichen thalli are known to deposit various extracellular secondary metabolites, many of which are reported to have antibiotic effects [1, 55]. The fungal partner of Trebouxia sp. TZW2008, U. hakonensis, is known to produce a secondary metabolite called usnic acid [56], well known for its antibiotic effect [55]. Therefore, the resistance shown by Sphingomonas sp. TZW2008 towards the three types of antibiotic may possibly correlate with an endolithic environment that contains antibacterial secondary metabolites. The presence of closely related Sphingomonas bacteria in other U. hakonensis samples suggested that this group of bacteria might be involved in symbiotic association with U. hakonensis.

Our study clearly shows that the bacterium benefits from symbiotic interaction with the alga in terms of carbon source. On the other hand, the benefit for the alga, in return for the nutritional support given to the bacterium, remains unclear. In mutualistic interactions between algae and bacteria reported in previous studies, the latter often support algae in terms of nitrogen fixation, phosphate solubilization and vitamin B₁₂ supply [16, 17]. However, according to our gene searches we predict that Sphingomonas sp. TZW2008 may not have genes directly related to these functions and the benefit of the interaction seems unidirectional. It is worthy of note that Trebouxia sp. TZW2008 and Sphingomonas sp. TZW2008 are derived from a lichen where interactions with a mycobiont and/or other lichen-associated bacteria are expected. Taking this into account, the benefit might not be for the alga per se but for the lichen symbiosis as a unit. Considering its preference for ribitol, glucose and mannitol, Sphingomonas sp. TZW2008 may colonize thalli and play a supportive role in carbohydrate turnover in lichens. Previous studies showed that external carbohydrates, identical to photosynthetic products of the photobiont, inhibit carbon transfer from the photobiont to the mycobiont in lichen thalli [35, 42, 57]. Therefore if accumulation of excess ribitol within thalli interferes with carbon transfer between the photobiont and the mycobiont, one potential role of Sphingomonas sp. TZW2008 is to utilize excess ribitol within the thalli, which would support the transfer of ribitol from the alga to the fungus. Support for nutrient cycling can be a great benefit for lichens that grow widely in nutrient-poor habitats.

The close physical contact between Trebouxia sp. TZW2008 and Sphingomonas sp. TZW2008 shown in this study allows us to speculate that the latter could be vertically transferred with lichen propagules (symbiotic propagules of the mycobiont and the photobiont), like the bacterial starter community proposed by Aschenbrenner et al. [58]. Since inherited lichen-associated bacteria are suggested to influence the establishment of lichen symbiosis in a new environment [6], it might be advantageous for Trebouxia sp. TZW2008 to ‘wear’ this Sphingomonas sp. TZW2008 even outside lichen thalli to establish a new symbiotic ecosystem. However, further experiments on bacterial metabolic products and their effects on algal and fungal metabolism, as well as localization of the bacterium in lichen thalli, are required to elucidate the roles of Sphingomonas sp. TZW2008 in lichen

---

**Fig. 8.** Phylogenetic tree of 16S rDNA partial sequences similar to that of Sphingomonas sp. TZW2008 amplified from lichen samples (U. hakonensis). Scale bars represent 0.01 substitutions per site. The arrows show position of the sequences isolated from U. hakonensis.
symbiosis. Such experiments will help to further elucidate the sophisticated mechanism of symbiosis in lichens.

Funding information
This work was supported by an internal SOKENDAI grant to Y. T., and Ministry of Education, Culture, Sports, Science, and Technology of Japan grants to Y. S. (no. 15K14682).

Acknowledgements
We thank Dr Yoshiaki Kon (Tokyo Metropolitan Hitotsubashi High School, Japan) for providing the culture of Trebouxia sp. TZW2008 and helpful discussions on culture conditions.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


Edited by: G. Preston