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Detection and isolation of plant-associated bacteria scavenging atmospheric molecular hydrogen

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Summary

High-affinity hydrogen (H_2)-oxidizing bacteria possessing group 5 [NiFe]-hydrogenase genes are important contributors to atmospheric H_2 uptake in soil environments. Although previous studies reported the occurrence of a significant H_2 uptake activity in vegetation, there has been no report on the identification and diversity of the responsible microorganisms. Here, we show the existence of plant-associated bacteria with the ability to consume atmospheric H_2 that may be a potential energy source required for their persistence in plants. Detection of the gene *hhyL* – encoding the large subunit of group 5 [NiFe]-hydrogenase – in plant tissues showed that plant-associated high-affinity H_2-oxidizing bacteria are widely distributed in herbaceous plants. Among a collection of 145 endophytic isolates, seven *Streptomyces* strains were shown to possess *hhyL* gene and exhibit high- or intermediate-affinity H_2 uptake activity. Inoculation of *Arabidopsis thaliana* (thale cress) and *Oryza sativa* (rice) seedlings with selected isolates resulted in an internalization of the bacteria in plant tissues. H_2 uptake activity per bacterial cells was comparable between plant and soil, demonstrating that both environments are favourable for the H_2 uptake activity of streptomycetes. This study first demonstrated the occurrence of plant-associated high-affinity H_2-oxidizing bacteria and proposed their potential contribution as atmospheric H_2 sink.

Introduction

Hydrogen (H_2) is an important constituent of the atmosphere. With a typical mixing ratio of 0.530 parts per million by volume (ppmv), H_2 is the second most abundant reduced gas, after methane (CH_4; 1.7 ppmv). Emission sources of atmospheric H_2 include biomass burning, fossil fuel combustion, and photochemical oxidation of CH_4 and non-CH_4 hydrocarbons (Ehhalt and Rohrer, 2009). Atmospheric H_2 is consumed biologically at the soil surface and photochemically through reaction with hydroxyl radicals (OH·), which is produced by UV photolysis of ozone in the presence of water vapour. Because H_2 and CH_4 have a similar atmospheric removal rate by OH·, increased H_2 concentration would prolong the lifetime of the greenhouse gas CH_4 through the decrease in tropospheric OH· concentration. In addition, elevated H_2 emission is expected to result in an alteration of temperature and ozone loss in the stratosphere through the production of water vapour (Prather, 2003; Schultz *et al.*, 2003; Tromp *et al.*, 2003; Warwick *et al.*, 2004). Thus, mitigation of H_2 emission is of critical importance for atmospheric chemistry. The most part (~ 80%) of atmospheric H_2 is consumed by microorganisms in soil (Constant *et al.*, 2009). Dissection of microbiological processes associated with H_2 consumption is mandatory to fully understand the nature of global H_2 sink.

Recently, Constant and colleagues (2008) isolated *Streptomyces* sp. PCB7 – the first microorganism possessing the ability to consume atmospheric H_2. Further soil surveys unveiled that H_2-oxidizing Streptomycetes are ubiquitous and encode an atypical hydrogenase, so-called group 5 [NiFe]-hydrogenase (Constant *et al.*, 2011). *Streptomyces* isolates possessing group 5 [NiFe]-hydrogenase genes exhibited high-affinity H_2 uptake activity (apparent K_m [$_{(app)}K_m$], < 100 ppmv), which was clearly distinguished from previously reported H_2-oxidizing bacteria that are unable to scavenge atmospheric H_2 owing to their low affinity for this trace gas ($_{(app)}K_m$, > 1000 ppmv) (Constant *et al.*, 2010). A subsequent study of the hydrogenase-deleted mutants of a soil actinomycete *Mycobacterium smegmatis* clearly demonstrated that group 5 [NiFe]-hydrogenase is responsible for the oxidation of atmospheric H_2 (Greening *et al.*, 2014a).

Because of the greatest contribution of soil in global H_2 sink, most of the investigations dedicated to high-affinity H_2-oxidizing bacteria have focused on the soil microorganisms (Osborne *et al.*, 2010; Pumphrey *et al.*, 2011; Bontemps *et al.*, 2013; Greening *et al.*, 2014b; 2015). Detection of H_2 uptake activity in other compartments of

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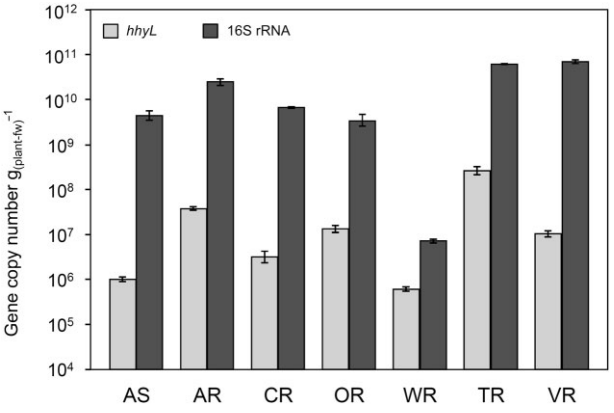

Fig. 1. Copy number of the *hhyL* and 16S rRNA genes in each tissue of natural plants. Values and error bars represent the mean and SDs of triplicate experiments. AS, *A. thaliana* shoot; AR, *A. thaliana* root; CR, *C. bursa-pastoris* root; OR, *O. sativa* root; WR, *A. fistulosum* root; TR, *T. repens* root; VR, *S. altissima* root.

terrestrial ecosystems suggests a broader distribution of this functional group in the environment. For instance, previous studies reported significant uptake activity of molecular tritium (the radioactive isotopologue of molecular H_2) in vegetation (McFarlane, 1978; Spencer and Dunstall, 1986; Ichimasa *et al*., 1989; 1999). The biological nature of the reaction was confirmed using sterilized control experiments, suggesting the occurrence of plant-associated H_2-oxidizing bacteria (Spencer and Dunstall, 1986).

Despite the previous suggestion, there has been no attempt to assess the H_2 uptake ability of epiphytic and endophytic bacteria. Epiphytes or endophytes are defined as those microorganisms that live on plant surface or inside plant tissues without infection respectively (Reinhold-Hurek and Hurek, 1998). Bacteria are abundant inside plant tissues, with biomass ranging between 10^2 and 10^7 cells per gram of plant fresh weight (Compant *et al*., 2010). Plant colonization processes are usually initiated with rhizosphere and rhizoplane colonization by soil-borne bacteria that can penetrate roots and spread in various plant organs through xylem vessels (Compant *et al*., 2010). Endophytic bacteria have attracted much interest in their plant growth promotion and phytopathogen suppression abilities via releasing phytohormone mimics or antimicrobial compounds, supplying nitrogen or soluble mineral nutrients and degrading pathogenicity factors (Hardoim *et al*., 2008; Ryan *et al*., 2008; Francis *et al*., 2010). Despite their abundance in plants, other ecological roles such as contribution to the biogeochemical cycle of atmospheric trace gases remain largely unexplored.

In this study, we seek to verify whether plant-associated high-affinity H_2-oxidizing bacteria contribute to the uptake of atmospheric H_2. We first investigated the presence of *hhyL* gene in various plant species. Cultivable high-affinity H_2-oxidizing bacteria were isolated from plants. Sterile seedlings were finally inoculated with selected isolates possessing high-affinity H_2 uptake activity to verify their ability to penetrate and disseminate in plant tissues and scavenge atmospheric H_2 in plant.

Results and discussion

Plant-associated bacteria possessing hhyL *gene are widespread in various plants*

Distribution, diversity and abundance of high-affinity H_2-oxidizing bacteria in plant tissues were first assessed by the polymerase chain reaction (PCR) detection of the *hhyL* gene. The gene encodes for the large subunit of group 5 [NiFe]-hydrogenase, which is the enzyme responsible for high-affinity H_2-oxidation. The *hhyL* gene has been used as a functional biomarker to evaluate the distribution, taxonomic diversity and abundance of high-affinity H_2-oxidizing bacteria in soil environments (Constant *et al*., 2011; Pumphrey *et al*., 2011; Bontemps *et al*., 2013; Greening *et al*., 2014a). In the present study, genomic DNA has been extracted from the root tissues of a variety of herbaceous plants to cover a wide range of families of the plant kingdom, including *Arabidopsis thaliana*, *Capsella bursa-pastoris*, *Oryza sativa*, *Allium fistulosum*, *Trifolium repens* and *Solidago altissima* – comprising the families *Brassicaceae*, *Poaceae*, *Amaryllidaceae*, *Fabaceae* and *Asteraceae*. DNA extracted from the stem tissues of *A. thaliana* was also used to determine the occurrence and compare the abundance of high-affinity H_2-oxidizing bacteria in stem with those in root. The *hhyL* gene was successfully detected in all tested plant species, indicating a wide distribution of presumptive high-affinity H_2-oxidizing bacteria in plants (Fig. 1).

The deduced amino acid sequences of 42 clones obtained from the *hhyL* PCR products were incorporated into a phylogenetic analysis (Fig. 2). A previous study demonstrated that the *hhyL* homologues were distributed across the phyla *Actinobacteria*, *Proteobacteria*, *Acidobacteria* and *Chloroflexi* (Constant *et al*., 2011). These genes were classified into four clusters: clusters I, II and IV mainly composed of actinobacterial isolates, and cluster III that comprises isolates belonging to the other phyla (Constant *et al*., 2011). Incongruence between *hhyL* and 16S rRNA gene phylogenies implies a lateral inheritance of group 5 [NiFe]-hydrogenases and thus impairs the utilization of *hhyL* gene sequence as a phylogenetic marker (Constant *et al*., 2011). In this study, most of the clones derived from plant tissues were affiliated with cluster I (36 clones, 85.7%) (Fig. 2). Other clones belonged to clusters II (four clones, 9.5%) and IV (two clones, 4.8%). No clone belonged to cluster III. Diversity of *hhyL* gene sequences obtained in this study appeared

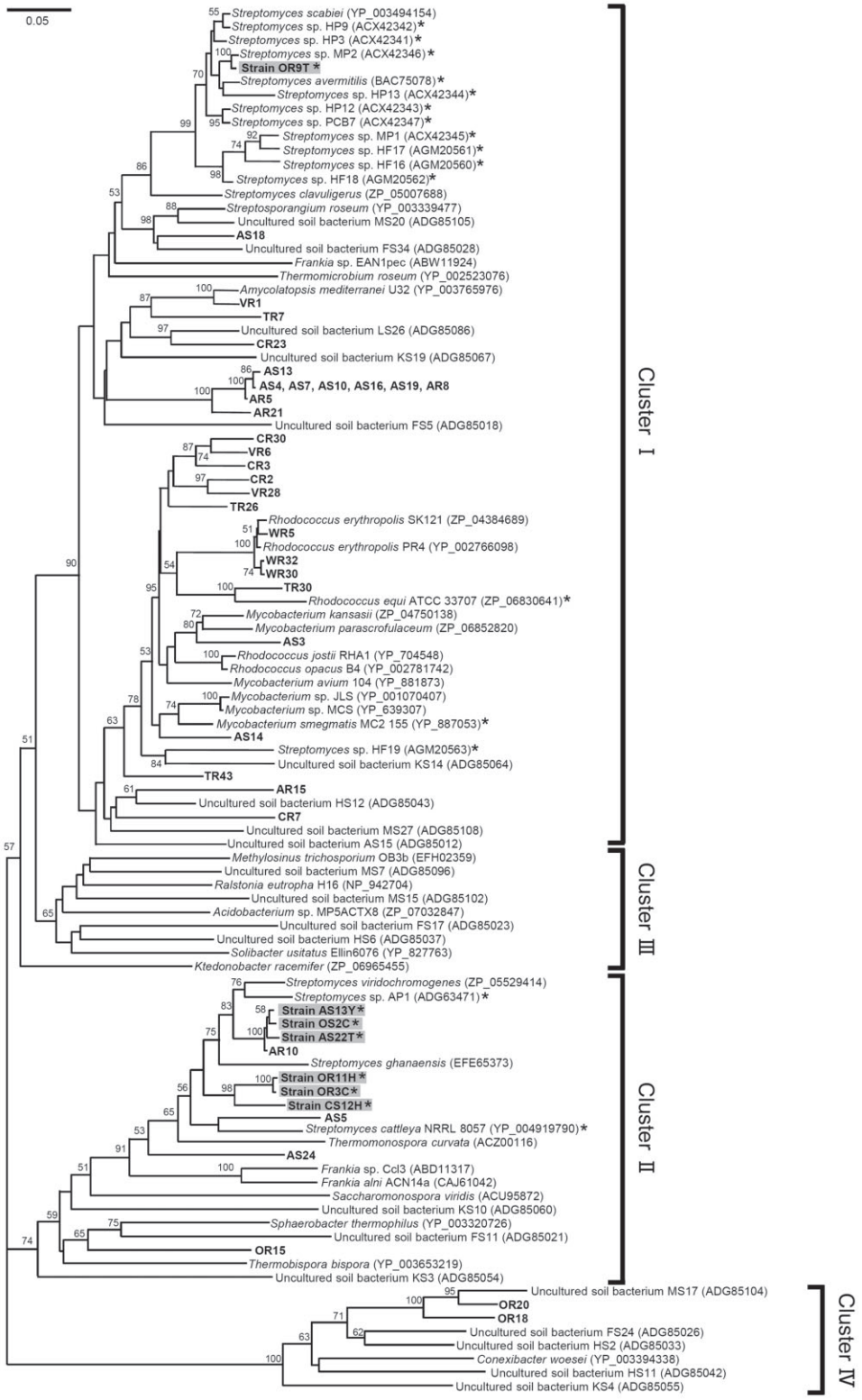

Fig. 2. Neighbour-joining tree based on deduced amino acid sequences from partial *hhyL* gene sequences retrieved from seven isolates (bold + shaded clusters), clone libraries from plant tissues (bold) and public databases. The prefixes of *hhyL* clone sequences indicate the plant origin, as follows: AR, *A. thaliana* root; AS, *A. thaliana* shoot; CR, *C. bursa-pastoris* root; OR, *O. sativa* root; WR, *A. fistulosum* root; TR, *T. repens* root; VR, *S. altissima* root. High-affinity H_2-oxidizing bacteria for which the H_2 oxidation activity has been characterized were indicated by asterisks. Four *hhyL* gene clusters are represented. Only bootstrap values ≥ 50% (1000 resamplings) are presented. The tree is based on 361 aligned amino acids. The scale bar represents 5% sequence divergence.

Table 1. H_2 uptake activity of seven strains isolated from surface-sterilized plants.

Strains	(app)K_m (ppmv)[a]	Threshold (ppmv)[b]	Origin	Closest relative species (Accession no.)[c]	Identity (%)
CS12H	275	0.021	*C. bursa-pastoris* shoot	*Streptomyces lannensis* strain TA4-8 (NR025212)	100.0
AS13Y	36	< 0.020	*A. thaliana* shoot	*Streptomyces thermocarboxydus* strain AT37 (NR026072)	99.9
AS22T	42	< 0.020	*A. thaliana* shoot	*Streptomyces thermocarboxydus* strain AT37 (NR026072)	99.5
OS2C	16	< 0.020	*O. sativa* shoot	*Streptomyces thermocarboxydus* strain AT37 (NR026072)	99.9
OR3C	95	0.249	*O. sativa* root	*Streptomyces koyangensis* strain VK-A60 (NR025662)	98.3
OR9T	78	< 0.020	*O. sativa* root	*Streptomyces scabiei* strain EF-52 (FJ007405)	99.6
OR11H	103	0.282	*O. sativa* root	*Streptomyces thermospinosisporus* strain AT10 (NR025147)	98.3

a. Strains CS12H, AS13Y, AS22T and OS2C were grown on R2A agar, whereas strains OR3C, OR9T and OR11H were grown on mannitol soya flour agar.
b. H_2 threshold concentration was determined by measuring H_2 concentration time series in the static headspace of microcosms comprising sterile dry (CS12H, AS13Y and AS22T) or wet soil (OS2C, OR3C, OR9T and OR11H) inoculated with the isolates.
c. The closest relatives were identified based on 16S rRNA gene phylogenetic analysis with sequences retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/).

relatively limited compared with that of soil bacteria clone sequences. For instance, 12 of the 19 clones from *A. thaliana*, and all four clones from *A. fistulosum* were most closely related to the *hhyL* gene sequences of *Amycolatopsis mediterranei* U32 and *Rhodococcus erythropolis* PR4, with 84–86% and > 99% amino acid sequence identity respectively. The limited diversity is partly due to the interference of plant DNA. For instance, the *hhyL* gene library from the rice plant-associated bacteria contained a large number of clones assigned as rice chromosomal DNA, resulting in recovery of merely three *hhyL* positive clones. These unspecific amplifications of plant DNA was known as a main cause of interference for culture-independent analysis of plant-associated microorganisms (Saito *et al*., 2007).

The abundance of plant-associated high-affinity H_2-oxidizing bacteria was also investigated using the *hhyL* quantitative PCR (qPCR) assay. According to the assay, plant samples contained 10^5–10^8 *hhyL* gene copies per $g_{(fw)}^{-1}$, which were lower than 16S rRNA gene copy number (10^6–10^{10} copies per $g_{(fw)}^{-1}$) detected in same plant samples (Fig. 1). It is noteworthy that this is of the same order of magnitude as the abundance of *hhyL* gene detected in soil (Constant *et al*., 2011). When comparing the abundance of *hhyL* gene in different tissues of *A. thaliana*, we found that root tissues were more than one order of magnitude higher than stem (Fig. 1), suggesting that only a fraction of the H_2-oxidizing bacteria population may have the ability to spread systemically from root to stem. Although quantification of *hhyL* gene might not be a suitable proxy for H_2 uptake activity owing to the occurrence of cryptic hydrogenase operons and the inherent bias of PCR technique, detection of *hhyL* gene sequences in plant tissues unveils the occurrence of plant-associated high-affinity H_2-oxidizing bacteria. This is further supported by the collection of bacterial isolates described in the next section.

Isolation of endophytic bacteria consuming H_2 *at ambient levels*

To confirm the presence of plant-associated high-affinity H_2-oxidizing bacteria, we screened cultivable endophytic bacteria possessing high-affinity H_2 uptake activity. The endophytic bacteria were isolated from surface-sterilized *C. bursa-pastoris*, *A. thaliana* and *O. sativa* root/shoot samples. To date, only a few actinobacterial strains, including *Streptomyces* isolates (Constant *et al*., 2008; 2010; Meredith *et al*., 2013), *Mycobacterium smegmatis* (Greening *et al*., 2014a) and one acidobacterial strain *Pyrinomonas methylaliphatogenes* (Greening *et al*., 2015), have been reported to show high-affinity H_2 uptake activity. Thus, the isolation was performed by using four actinobacteria-selective cultivation media combined with PCR screening for *hhyL* gene. In total, 145 endophytic bacteria were isolated. About half of them were presumptive actinobacteria, distinguished by their morphology (data not shown). Among 145 isolates, seven strains displayed a positive PCR signal of *hhyL* gene, and thus were selected for further analyses. Sequencing of PCR-amplified 16S rRNA gene sequences revealed that all selected isolates were actinobacteria belonging to the genus *Streptomyces*, with > 98.3% sequence identity with known species (Table 1 and Fig. S1). Although considered as soil-dwelling microorganisms, actinobacteria are ubiquitous in the environment, as demonstrated by their detection in airborne particles, freshwater and marine ecosystems (Glöckner *et al*., 2000; Ward and Bora, 2006; Després *et al*., 2007). Over the last decade, more than 300 endophytic actinobacteria (including *Streptomyces* as well as *Frankia*, *Microbispora*, *Micromonospora* and *Nocardia* spp.) have been isolated from a range of plants, and investigated as agents to produce useful natural compounds and improve agricultural crop production (Franco *et al*., 2007; Schrey and Tarkka, 2008; Qin *et al*., 2011).

The *hhyL* gene sequences of the seven isolates were incorporated into the phylogenetic analysis (Fig. 2). The *hhyL* gene sequences of the isolates encompassed cluster I and cluster II, and were closely affiliated with those of *Streptomyces ghanaensis* (CS12H, OR3C and OR11H), *Streptomyces* sp. AP1 (AS13Y, AS22T and OS2C) and *Streptomyces avermitilis* (OR9T). No *hhyL* gene sequence corresponded to the sequences retrieved from the clone libraries constructed in this study likely due to cultivation bias and the low sequencing effort in clone libraries. Indeed, no endophytic isolates showed affiliation with *hhyL* gene sequences of *Amycolatopsis* spp., *Rhodococcus* spp. or *Mycobacterium* spp., whereas all clones derived from *C. bursa-pastoris*, and 15 out of the 19 clones derived from *A. thaliana* were affiliated to these representatives.

The H_2 uptake activity of the seven isolates was evaluated for testing whether these endophytic bacteria could scavenge atmospheric H_2. Efficient scavenger of atmospheric H_2 should show a high affinity for H_2 ($_{(app)}K_m$, < 100) and a minimal H_2 threshold concentration supporting oxidation activity should be below 0.530 ppmv, which is the concentration of tropospheric H_2 (Conrad, 1996). Previous characterizations of *Streptomyces* soil isolates indicated that spores rather than mycelia are responsible for the H_2 uptake activity (Constant *et al.*, 2008; 2010; Meredith *et al.*, 2013). To obtain a large proportion of spore-forming cells, the isolates were grown on $NiCl_2$-amended R2A agar, mannitol soya flour agar or in sterile soils for more than 2 weeks. The H_2 uptake activity of the isolates followed typical Michaelis–Menten kinetics, with $_{(app)}K_m$ values between 16 and 275 ppmv (Table 1). Although three strains (CS12H, OR3C and OR11H) displayed intermediate affinity for H_2 ($_{(app)}K_m$, 100–1000 ppmv), all strains reduced H_2 concentration from 2 ppmv to less than the ambient level, with threshold H_2 concentration lower than 0.3 ppmv. These results clearly show that all isolates have the ability to consume H_2 at ambient level. To date, only members of *Proteobacteria* (including *Rhizobium* spp.) have been reported as plant-associated H_2-oxidizing bacteria (Emerich *et al.*, 1979; Maimaiti *et al.*, 2007). However, they are unable to consume atmospheric H_2 owing to their low affinity for H_2 (Constant *et al.*, 2010), unlike the plant-associated high-affinity H_2-oxidizing *Actinobacteria* found in this study.

Colonization of plants by high-affinity H_2*-oxidizing bacteria*

The ability of *Streptomyces* isolates to penetrate, colonize and survive within plant tissues was confirmed by recovery of viable cells from surface-sterilized host plants cultivated for 4–5 weeks following inoculation with selected isolates, strain AS22T or strain OS2C (Table S1). To further confirm the endophytic colonizing abilities, microscopic observation by fluorescence *in situ* hybridization (FISH) of selected isolates was employed. After 4 weeks of inoculation, spores and mycelia of selected strains inside both shoot and root tissues of host plants were visualized (Fig. 3). In accordance with the abundance of *hhyL* gene in *A. thaliana* (Fig. 1), the strains were seen more frequently in root tissues than in shoots. Especially, microcolonies were often observed inside epidermis and exodermis of root tissues. Some bacterial cells were found in close proximity to the plant cell walls, indicating localization in extracellular space of the plant tissues, which is in accordance with a previous study of endophytic *Streptomyces* (Coombs and Franco, 2003a). Taken together, it was found that the isolates could penetrate and thrive inside plants, implying the ability of high-affinity H_2-oxidizing bacteria to adapt to colonize plants as well as soil. According to previous reports (Constant *et al.*, 2008; 2010), sporulation is a key property for assuming the H_2 uptake activity in streptomycetes. Although the study was not designed to assess the life cycle of *Streptomyces* in plants, spores were observed in both above- and underground plant organs, suggesting the occurrence of H_2 oxidation in plants.

H_2 *uptake activity of plant-associated bacteria in plant–soil microcosm*

Measurement of H_2 consumption in soil microcosms containing seedlings was performed to investigate the ability of plant-associated bacteria to scavenge atmospheric H_2. Surface-sterilized seeds of *A. thaliana* and *O. sativa* were placed into sterile soil bottles, mimicking upland and wetland soil conditions. *Arabidopsis thaliana* and *O. sativa* seedlings were then inoculated with 10^9 spores of either strain AS22T or strain OS2C, respectively, allowing colonization of the bacteria both in soil and in plant tissues. After 4–5 weeks of inoculation, bacterial biomass reached 10^8 to 10^9 colony-forming units (cfu) $g_{(dw)}^{-1}$ in soil and plant tissues (Table 2). All microcosms displayed H_2 uptake activity, demonstrating that H_2-oxidizing bacteria were active either in soil or in plant tissues, or both (Fig. S2). A fractionation experiment was then performed to determine the relative proportion of the activity in soil and plant fractions. The total H_2 uptake activity (corresponding to 100% activity) was first measured in the microcosm containing both soil and plant. Plants were then removed from the microcosms, surface-washed and transferred into an empty microcosm. H_2 uptake activity was measured in microcosm containing residual soil (soil fraction of the activity) and the microcosm containing only plant (plant fraction of the activity). In the plant fraction, plant-associated bacteria were dominated by epiphytic cells (> 92%) rather than endophytic cells (Fig. S3 and

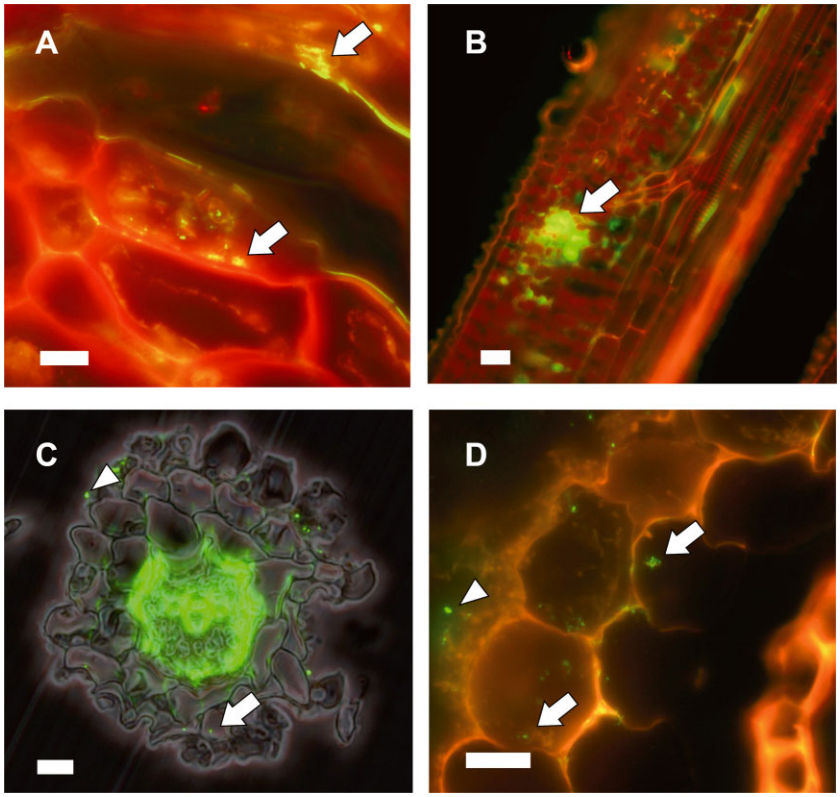

Fig. 3. Endophytic colonization of the *Streptomyces* isolates inside *A. thaliana* (A, C) and *O. sativa* (B, D). The image of FITC fluorescence detection by the actinobacteria-specific probes (shown in green) was merged with a micrograph of phase-contrast (C) or plant autofluorescence (shown in red) (A, B, D). The arrows indicate the parts of bacterial cells colonizing xylem or parenchyma (A, B) of stem tissues, and cortex (C) or epidermis and exodermis (D) of root tissues. Some bacteria were outside the root tissues (C, D: arrowhead). The green fluorescence signal around endodermis (C) was plant autofluorescence. Scale bars, 10 μm.

Table S1). This fractionation of the activity showed that the plant fraction can oxidize trace concentrations of H_2 (Fig. S2B and D). Even shoot sections exhibited significant H_2 uptake activity (Fig. S2E). H_2 oxidation rates measured in plant fractions ranged from 1079 to 3472 pmol $g_{(dw)}^{-1}$ h^{-1} (Fig. 4A). These rates are comparable with the activity of atmospheric tritium uptake observed in other plants. Ichimasa and colleagues (1989; 1999) quantified the oxidation rate of tritium in various woody and herbaceous plant leaves and roots. The tritium oxidation rates in the surface-washed roots were high, ranging from 123 to 9285 pmol $g_{(fw)}^{-1}$ h^{-1} (Ichimasa *et al.*, 1999). Although the activity in plant leaves was generally low (around 10 pmol $g_{(fw)}^{-1}$ h^{-1}), high oxidation rates were also observed in the leaves of the woody plant *Vaccinium smallii* (70 pmol $g_{(fw)}^{-1}$ h^{-1}) and in the leaves of the herbaceous plant *Phalaris arundinacea* (282 pmol $g_{(fw)}^{-1}$ h^{-1}) (Ichimasa *et al.*, 1989; 1999). Importantly, both previous and present studies showed that atmospheric H_2 is not scavenged in plants grown aseptically (Boyer *et al.*, 2009) (Fig. S2F). These results clearly demonstrate that plant-associated bacteria oxidized H_2 in plant tissues. However, the determination of H_2 uptake activity in plant-associated bacteria and soil bacteria revealed that the plant-associated activity accounted for $0.05 \pm 0.02\%$ and $6.6 \pm 5.9\%$ of the total activity in the *A. thaliana* and *O. sativa* microcosms respectively (Fig. 4B). This implied that soil fractions were responsible for the large proportion of the total activity. As expected, measured activity was proportional to the number of viable cells determined by agar plate enumeration. Indeed, specific H_2 oxidation rates per bacterial cell measured in plant and soil fractions were not significantly different both in *A. thaliana* and in *O. sativa* microcosms ($P > 0.05$, t-test) (Table 2).

Table 2. Fractionation of the H_2 uptake activity and bacterial biomass (expressed as colony forming units; cfu) in plant and soil fractions of *A. thaliana* and *O. sativa* microcosms.

Microcosms	Fractions	H_2 oxidation (pmol $g_{(dw)}^{-1}$ h^{-1})	cfu $g(dw)^{-1}$	specific H_2 oxidation rate (amol cfu^{-1} h^{-1})
A. thaliana + strain AS22T	Soil	7994 (1110)	$1.5\ (0.34) \times 10^9$	5.8 (2.12)
A. thaliana + strain AS22T	Plant	1836 (659.2)	$2.3\ (0.81) \times 10^8$	8.1 (0.05)
O. sativa + strain OS2C	Soil	387 (354.4)	$6.7\ (0.95) \times 10^8$	0.6 (0.46)
O. sativa + strain OS2C	Plant	3340 (120.0)	$3.0\ (0.87) \times 10^9$	1.2 (0.34)

Standard deviations are presented in parentheses.

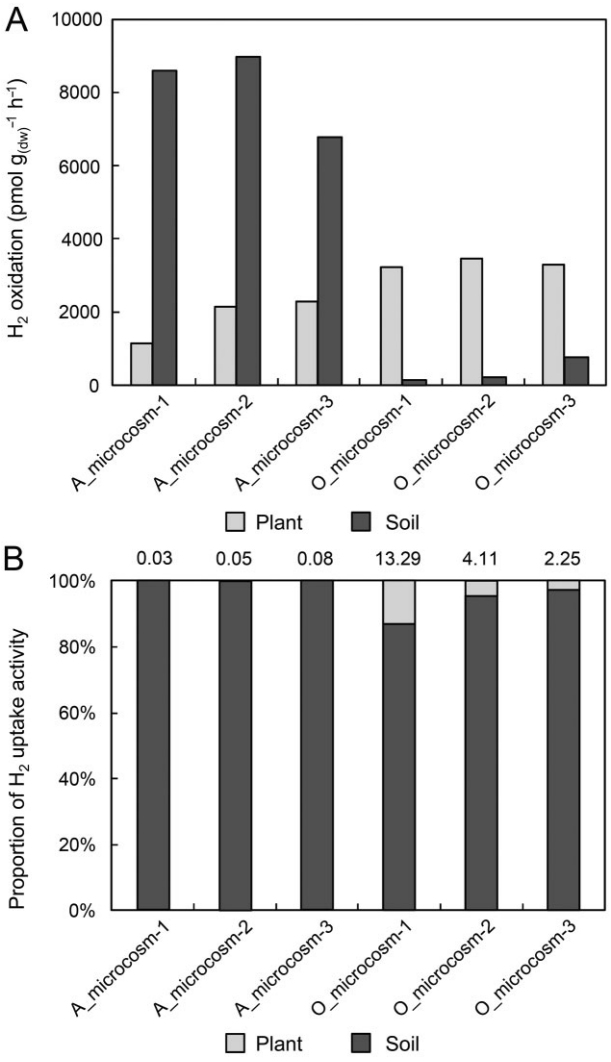

Fig. 4. Fractionation of the H_2 oxidation activity measured in microcosms. H_2 oxidation rate measured in soil and plant fractions (A), and proportion of activity catalysed by soil and plant fractions (B) in triplicate *A. thaliana* and *O. sativa* microcosms. The numbers above the bars in (B) indicate the percentage of H_2 uptake activity catalysed by the plant fraction. A_microcosm and O_microcosm refer to *A. thaliana* and *O. sativa* microcosms respectively.

Our studies imply that soil microorganisms are much more responsible for the oxidation of atmospheric H_2 than plant-associated organisms because of their different cell abundance. However, considering that (i) as most of high-affinity H_2-oxidizing bacteria associated with plants were not cultivable in our experiments, we were not able to estimate their contributions in plants to H_2 consumption; (II) a much more variety of plants than we examined may harbour high-affinity H_2-oxidizing bacteria and their total surface area and inside spaces should be considerably greater than the seedlings we used, the contribution of plant-associated bacteria has to be even more noteworthy and thus should be elaborated to better understand the global cycling of atmospheric H_2.

Our findings will stimulate further questions to assess physiological and ecological significance of atmospheric H_2 oxidation in plant–microbe interactions. (i) Does high-affinity H_2 oxidation in streptomycetes facilitate their localization within or near the plant? Considering the ubiquity of high-affinity H_2-oxidizing streptomycetes in soil, H_2 diffusion from plant-associated nitrogen-fixing bacteria as well as atmospheric H_2 may support their endophytic or epiphytic localization. In fact, the colonization of *Streptomyces* species in the surface of root nodules of peas was found in the field (Tokala *et al*., 2002). We also observed the presence of *Streptomyces* species in plant tissues, which supports the hypothesis. (II) Does high-affinity H_2 oxidation provide an advantage for the persistence of streptomycetes in plant tissues? It seems to be true. Recently, it has been proposed that atmospheric H_2 may serve as the maintenance energy during starvation and sporulation of high-affinity H_2-oxidizing *Actinobacteria* (Greening *et al*., 2014a,b; 2015; Liot and Constant, 2015). Indeed, we observed *Streptomyces* spores in plant tissues in this study, implying that high-affinity H_2 oxidation in streptomycetes may be involved in generating energy required for their long-term survival in plants. (III) Does high-affinity H_2 oxidation in streptomycetes positively affect the growth of host plants? H_2 treatment of soil was known to promote plant growth of both legume and non-leguminous crops (Dong *et al*., 2003). Interestingly, H_2-oxidizing lineages of *Actinobacteria* including streptomycetes reproducibly increased in abundance in soils treated with low H_2 concentration, although the overall bacterial community was not significantly altered (Osborne *et al*., 2010). Taken together with the fact that several *Streptomyces* species promote plant growth (Franco *et al*., 2007; Schrey and Tarkka, 2008), these reports suggest that high-affinity H_2-oxidizing streptomycetes may positively affect growth of host plants. Although it is not conclusive to address these questions in this study, further investigations of plant-associated high-affinity H_2-oxidizing bacteria will advance our knowledge of both biogeochemical cycle of H_2 and plant–microbe interactions associated with H_2 uptake.

Conclusions

The *hhyL* gene clone library analyses indicated that high-affinity H_2-oxidizig bacteria were ubiquitous in herbaceous plants. We were successful in isolating seven endophytic bacteria that have the ability to consume H_2 at ambient level. Microcosm experiments revealed that the isolates could colonize and scavenge atmospheric H_2 in plant tissues. As far as we know, this is the first report focusing on plant-associated bacteria possessing high-affinity H_2 uptake activity. From a global perspective, herbaceous

plant biomass represent 64 Pg (1 Pg = 10^{15} g) (Woodwell *et al*., 1978). Assuming that high-affinity H_2-oxidizing bacteria are present and active in this plant biomass, plant-associated bacteria as well as soil bacteria may exert a significant contribution to the global budget of atmospheric H_2.

Experimental procedures

Plant samples

All plant samples were grown in nature except *A. thaliana* (thale cress), which was cultivated in greenhouses. In total, six plant species were utilized. *Arabidopsis thaliana*, *C. bursa-pastoris* (shepherd's purse), *T. repens* (white clover) and *S. altissima* (tall goldenrod), were harvested in Ibaraki Prefecture, Japan, whereas *O. sativa* (rice) and *A. fistulosum* (welsh onion) were collected in Miyagi Prefecture. These plants were used as the source material for clone library analysis and the isolation of high-affinity H_2-oxidizing bacteria. Samples of *in vitro* cultivated *A. thaliana* ecotype Columbia (March 2012), *C. bursa-pastoris* (April 2012) and *S. altissima* (November 2012) were collected during flowering period. *Trifolium repens* (November 2012) and *A. fistulosum* (March 2012) were collected during vegetative period. Samples of *O. sativa* (Japonica cultivar, Hitomebore) grown in wetland rice field were collected at ripening stage (October 2010). Seeds of *A. thaliana* ecotype Columbia and *O. sativa* (Japonica cultivar, Nipponbare) were used for microcosm experiment.

Isolation of endophytic bacteria

Each plant sample was washed with tap water to remove attached clay. Then, the tissues were sterilized by immersing in 70% ethanol and 1.0% hypochlorite solution as described previously (Sun *et al*., 2008). Plant tissues were rolled onto R2A agar (Fluka) and incubated at 30°C for 1 week. No growth was observed using this procedure, thus confirming the reliability of the sterilization procedure. Surface-sterilized *C. bursa-pastoris*, *A. thaliana* and *O. sativa* tissues were cut into ~ 1 cm long pieces and crushed aseptically by sterile mortar and pestle with bacterial cell extraction (BCE) buffer, pH 7.5 (Ikeda *et al*., 2009). Each homogenate was spread onto R2A agar, tap water-yeast extract (TWYE) agar ((Crawford *et al*., 1993); containing (g l^{-1}) yeast extract (0.25), K_2HPO_4 (0.5), and agar (18)), modified HV agar ((Hayakawa and Nonomura, 1987); containing (g l^{-1}) humic acid (1), KCl (1.71), Na_2HPO_4 (0.5), $MgSO_4{\cdot}7H_2O$ (0.05), $CaCO_3$ (0.02), $FeSO_4{\cdot}7H_2O$ (0.01), 1 ml l^{-1} vitamin solution (Hanada *et al*., 1997), and agar (18)), yeast extract-casein hydrolysate (YECD) agar ((Coombs and Franco, 2003b); containing (g l^{-1}) yeast extract (0.3), glucose (0.3), K_2HPO_4 (2), and agar (18)), and modified starch casein agar ((Li *et al*., 2008); containing (g l^{-1}) starch (10), casein (0.3), KNO_3 (2), K_2HPO_4 (2), NaCl (2), $MgSO_4{\cdot}7H_2O$ (0.05), $CaCO_3$ (0.02), $FeSO_4{\cdot}7H_2O$ (0.01), and agar (18)). These media were utilized for the enrichment and isolation of actinobacteria (Mackay, 1977; Coombs and Franco, 2003b; Misk and Franco, 2011). All media were adjusted to pH 7.2 and supplemented with 50 μg ml^{-1} cycloheximide to inhibit fungal growth. Plates were incubated at 30°C for up to 12 days and colonies were isolated and purified.

Plant microcosm re-inoculation experiment

The overall workflow for this microcosm experiment is shown in Fig. S4. For plant cultivation in sterile soil, 30 g soil mixture (commercially available black soil:vermiculite; 2:1, w/w) was prepared for *A. thaliana*, whereas 35 g black soil was utilized for *O. sativa*. Soil water content was adjusted to 70% in *A. thaliana* and 85% in *O. sativa* microcosms using sterile water. Soil preparation was transferred into a 720 ml glass bottle (Gibco) and heat sterilized (20 min, 121°C) in two cycles separated by a 24 h time interval. Seeds of *A. thaliana* and seeds of *O. sativa* without husk were sterilized with 70% ethanol and 1.0% hypochlorite solution with 0.02% Triton-X. Seeds were then incubated on one-half-strength Murashige and Skoog medium containing 1.0% sucrose and 0.5% agar (pH 5.8) to confirm aseptic germination at 25°C (*A. thaliana*) or 28°C (*O. sativa*) (Murashige and Skoog, 1962). After 1 week, seedlings were individually transferred into sterile soil bottles and inoculated with 0.5 ml spore suspensions of the strain AS22T (*A. thaliana*) or the strain OS2C (*O. sativa*) ($1.1–1.3 \times 10^9$ cfu per bottle). *Arabidopsis thaliana* and *O. sativa* were grown at room temperature (ca. 25°C) and at 28°C respectively, with a 16 h-light/8 h-dark cycle. Sterile water was added in soil every week. No extra H_2 was added into the bottle's headspace. To ensure aerobic condition in the bottle's headspace while avoiding contamination by airborne microorganisms, bottles were closed with sterile sponge stopper throughout the whole incubation period. H_2 uptake activity measurements were performed 34 days (*A. thaliana*) or 28 days (*O. sativa*) after microbial inoculation. The number of cells in the inoculants and each microcosm bottles (harvested plants and soils) was estimated using agar plate enumeration technique (Constant *et al*., 2010). All the experiments were performed using three biologically independent replicates.

H_2 uptake activity measurement

H_2 uptake measurement were performed using biomass grown in sterile soils, on R2A agar or on mannitol soya flour agar (Hobbs *et al*., 1989) containing (g l^{-1}) soya flour (20), mannitol (20) and agar (18), pH 7.2 amended with 10 μM $NiCl_2{\cdot}6H_2O$. Agar plates were incubated at 30°C for 2 weeks, whereas soil bottles were incubated at 25–28°C for 4–5 weeks. Before H_2 uptake activity was measured, agar plates covered by spore mats were transferred into a previously described static flux chamber (Constant *et al*., 2008), whereas soil microcosms were sealed with screw caps equipped with septum. H_2 uptake activity measurements were performed in triplicate using a reduced gas analyzer (ta3000 Gas Analyzer, Trace Analytical, Ametek), as described by Constant and colleagues (2010). H_2 oxidation kinetic parameters ($K_{m(app)}$) and thresholds, which is a minimal H_2 concentration below which no uptake occurs, were obtained according to the method described by Constant and colleagues (2008; 2010). No significant H_2

Table 3. Primers and characteristics of the standard curves used for qPCR of *hhyL* gene in plant.

Primer name	Primer sequence (5′ to 3′)[a]	Intercept point	Efficiency (%)	R^2	Non-template control
NiFe-380f NiFe5-R	KSGTSGACTWCTGCGARRMSA ATGAGRCAGAACATCTCYCGGGT	70.1	51.1	0.997	Not detected

a. Amplicon length was 202 bp in *S. avermitilis hhyL* gene.

uptake activity was observed in bottles involving aseptically grown plants or sterile soil established as negative controls (Fig. S2F).

FISH for bacteria inside plants

The shoot and root samples of *A. thaliana* or *O. sativa* were used for microscopic analysis, after 4 weeks of inoculations with the isolates, strain AS22T (*A. thaliana*) or strain OS2C (*O. sativa*). Samples were washed with sterile water, cut into ~ 1 cm long pieces and immediately fixed in 5% formaldehyde: 45% ethanol: 5% acetic acid:water solution (1:9:1:9) for 2 days. Then they were sequentially rinsed, dehydrated and embedded in Technovit 7100 resin (Heraeus Kulzer) according to the manufacturer's instructions. The plant samples were then sliced with a RM2165 microtome (Leica) into sections of 5–8 μm. Fluorescence hybridizations of slices with the combination of actinobacteria-specific probe HGC236 labelled with FITC and helper probe HGC270H were done according to Glöckner and colleagues (2000). By increasing the hybridization time to 15 h, both spore and mycelium of *Streptomyces* isolates could be detected as fluorescence (Fig. S5). More than 10 fields in each part of plant tissues were examined using an Axio Observer Z1 epifluorescence microscope (Zeiss). Endophytic colonization of the *Streptomyces* isolates was visualized with an Endow GFP filter set (excitation 450–490 nm, emission 500–550 nm). The structure of the plant tissue was visualized by phase-contrast or fluorescence with a 4'-6-diamidino-2-phenylindole (DAPI) filter set (excitation 365 nm, emission 420–470 nm) using the autofluorescence of the tissue itself. The image generated from the green-light detection of the microbial cell was digitally coloured green, whereas the image generated from the blue-light detection of the plant tissue was digitally coloured red. The images were then overlaid by using AXIO VISION 4.8 software (Zeiss). The FITC-labelled cells did not show any fluorescence under blue-light detection. The slices of aseptically grown plants were additionally used as negative controls, confirming the specificity of the observed signal.

DNA extraction, PCR amplification of 16S rRNA and hhyL *genes*

Frozen samples of surface-sterilized plant tissues were pre-homogenized prior to DNA extraction by using FastPrep instrument (MP Biomedicals) at a speed of 5.5 m s^{-1} for 20 s with freezing in liquid nitrogen. Total DNA was extracted from surface-sterilized plant tissues or biomass collected from agar plates using FastDNA Spin Kit for Soil (MP Biomedicals) and subsequently purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. All PCR reactions were performed in 50 μl reaction volumes containing 1 U TaKaRa Ex Taq HS DNA polymerase (TaKaRa), 5 μl PCR buffer (TaKaRa), 5 μl dNTP mixture (TaKaRa), 0.5 μM primer sets and 1 μl sample DNA. *hhyL* genes were amplified using NiFe-244f and NiFe-1640r primer pair (Constant *et al*., 2010) with T100 Thermal Cycler (Bio-Rad) as follows: 95°C for 2 min, followed by 35 cycles of 96°C for 30 s, 60°C for 30 s, 72°C for 90 s and a final extension period of 5 min at 72°C. PCR amplification of 16S rRNA gene sequences (*Escherichia coli* positions 28–1491) of each isolate was performed as previously described (Tamaki *et al*., 2009).

qPCR of hhyL *and 16S rRNA genes*

SYBR Green-based qPCR was performed for quantification of the copy number of *hhyL* gene in plant using NiFe-380f and NiFe5-R primer pairs (Table 3). These primers were newly designed to avoid unspecific amplification of plant DNA, as well as to generate shorter PCR products appropriate for qPCR (202 bp in *S. avermitilis hhyL* gene) by using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) in National Center for Biotechnology Information (NCBI) (Ye *et al*., 2012). Dilution series of the PCR-amplified *hhyL* genes from strain OS2C were made to generate a standard curve covering 96 to 9.6×10^8 copies of template per assay. The assay was performed using the Mx3000P qPCR system (Stratagene) with either non-diluted or 1:200 diluted plant DNA extract as the template (efficiency = 51.1%, $R^2 = 0.997$). The reaction mixture (20 μl) contained 10 μl of SYBR Select Master Mix (Applied Biosystems), 0.5 μM each primer and 1 μl template DNA. The thermal cycling was performed in triplicate for each DNA template as follows: 50°C for 2 min and initial denaturation at 95°C for 6 min followed by 50 cycles of 94°C for 35s, 55°C for 25s and 72°C for 45 s. For the determination of melting curve, the temperature was increased 1°C every 30 s from 55°C to 90°C. Specific amplification of the *hhyL* gene was confirmed via agarose gel and melting curve analysis. The qPCR of 16S rRNA gene was also performed in the same way as described above by using Eub341f and Eub534r primer sets (efficiency = 85.1%, $R^2 = 0.999$) (Muyzer *et al*., 1993).

Phylogenetic analysis

PCR products were directly sequenced in both directions or sequenced after cloning into pT7Blue-2 T-vector (Novagen) using the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). All sequences were compared with the GenBank database using a standard nucleotide–nucleotide BLAST search (Altschul *et al*., 1990) and were aligned with their relatives by using GENETYX software (Software Development Co). The *hhyL* gene sequences were translated *in silico* by using both NCBI Open Reading Frame (ORF) Finder and

ExPasy translation tool (Gasteiger *et al*., 2003), and were also aligned. A phylogentic tree was constructed with the neighbour-joining method (Saitou and Nei, 1987). The robustness of tree topology was assessed by bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

Nucleotide sequence accession numbers

All sequences determined in this study were deposited in the GenBank/EMBL/DDBJ with accession numbers AB894405 to AB894411, LC076443 for 16S rRNA genes and AB894412 to AB894418, AB894496 to AB894537 for *hhyL* gene sequences.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequence (1366 bp) retrieved from the seven isolates (bold), the closest relative species, the reported 14 *Streptomyces* isolates in which high-affinity H_2 uptake activity was demonstrated (shaded clusters) and related *Streptomyces* type strains. Bootstrap values (1000 re-samplings) are indicated only ≥ 50%. *Streptomyces albus* subsp. *albus* DSM 40313^T (AJ621602) was used as an outgroup. The scale bar represents 1% sequence divergence.

Fig. S2. H_2 uptake activity of strain AS22T (A, B) and strain OS2C (C, D) in the microcosms of *A. thaliana* and *O. sativa*, represented in Fig. 4. as A_microcosm-1 and O_microcosm-1 respectively. Typical trends of the H_2 headspace concentrations of bottles containing plants plus soils, or soil fraction (A, C), or plant fraction (B, D) were shown. H_2 uptake activity of rice shoot section (E) was also shown. The absence of the activity was shown in bottles involving aseptically grown plants or sterile soil (F).

Fig. S3. Pie charts representing the efficiency of surface-wash treatment and the proportions of the epiphytic or endophytic cells in surface-washed *A. thaliana* (A) and *O. sativa* (B). The percentage based on colony-forming units (cfu) in total volume of rinsing solution (10 ml) or total cfu per plant individual.

Fig. S4. Schematic representation of the experimental flow of microcosm experiment. Photographs of rice seeds without husk (A), spore mat of strain OS2C on R2A agar medium (B), microcosm bottle of *A. thaliana* (C) and *O. sativa* (D) cultivated for 5–6 weeks, bottle for H_2 uptake activity measurement of isolate in soil (E) and plant (F) prepared by transferring plant to another bottle. Scale bars in A represent 1 cm, and scale bars in B–F represent 3 cm.

Fig. S5. Detection of spore and mycelium of the strain OS2C by prolonging hybridization time in FISH to 15 h with actinobacteria-specific probe. Biomass was harvested from R2A agar. The arrows point to spores. Scale bars, 10 μm.

Table S1. Efficiency of the rinsing procedure to remove cells attaching to plant surface. Bacterial biomass (expressed as colony forming units; cfu) in rinsing solution, surface-washed, or surface-sterilized plant tissues was examined after 4 weeks of inoculation. Both epiphytic and endophytic cells exist in surface-washed plants, whereas only endophytic cells can survive in surface-sterilized plants. The cfu enumerations were performed using three biologically independent replicates. Standard deviations are presented in parentheses.