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Short Communication

Isolation and culture of lichen bacteriobionts

Lichens are increasingly regarded as a complicated microcosm formed by a large number of lichen-associated fungi and non-photoautotrophic bacteria, in addition to the photobionts and mycobionts (Grube *et al.* 2012). Some non-photoautotrophic bacteria harboured in lichen microbiomes are considered by some as integral players in the lichen symbiosis and have been referred to as bacteriobionts (Grube & Berg 2009).

The first studies on lichen-associated bacteria using culture-independent molecular methods were conducted by Cardinale *et al.* (2006). Subsequent research with molecular and microscopic methods clearly showed that Alphaproteobacteria is the predominant bacterial group in growing parts of lichens (Cardinale *et al.* 2008, 2012; Grube *et al.* 2009; Bates *et al.* 2011; Mushegian *et al.* 2011; Schneider *et al.* 2011; Hodkinson *et al.* 2012). In some cases, host species specificity of the lichen-associated bacteria has been shown (Grube *et al.* 2009; Bates *et al.* 2011; Bjelland *et al.* 2011). These studies provide evidence that bacteriobionts are integral components in lichen symbioses. *Rhizobiales* (Alphaproteobacteria) was shown to be the predominant order among lichen bacteriobionts (Hodkinson *et al.* 2012) and includes many nitrogen-fixing taxa forming plant-associated root nodules (Sy *et al.* 2001; Ngom *et al.* 2004). A recently discovered and uncultured lineage of this order, LAR1, has been found in many lichens (Hodkinson & Lutzoni 2009; Bates *et al.* 2011; Hodkinson 2011; Hodkinson *et al.* 2012). Furthermore, the bacterial nitrogen-fixing genes were also detected in chlorolichens using PCR (Grube *et al.* 2009). These studies suggest that bacteriobionts are potential nitrogen-fixers in the lichen symbioses.

Despite living in nutrient-poor conditions, most lichen mycobionts produce high levels of secondary compounds. For example, the usnic acid content in *Alectoria ochroleuca* (Hoffm.) A. Massal. is 8% of the dry weight of the lichen thallus, while lecanoric acid may constitute up to 23·5% of the thallus dry weight of *Parmotrema tinctorum* (Despr. ex Nyl.) Hale (Wei 1998). More than 700 lichen compounds have been reported, almost all of which are insoluble in water (Huneck & Yoshimura 1996; Dembitsky & Tolstikov 2005). Most lichen compounds are aromatics (containing one or more benzene rings), while terpenoids are also very common in lichens (Huneck & Yoshimura 1996; Dembitsky & Tolstikov 2005).

Considering the high content of secondary compounds in lichen thalli and the enormous diversity of carbon sources utilized by bacteria, we first investigated the culturable bacteria from the chlorolichens *Umbilicaria esculenta* (Miyoshi) Minks and *Parmelia omphalodes* (L.) Ach., and the cyanolichen *Lobaria retigera* (Bory) Trevis., on media with acetone extracts of corresponding lichens as the nutrient source. However. some bacteria can use acetone as a carbon source (Platen & Schink 1989). Therefore, we selected dimethyl sulfoxide (DMSO) to dissolve the acetone extracts, and DMSO media were used to clarify whether or not culturable bacteriobionts utilize DMSO as a substrate. All the lichen compounds extracted from these species are water-insoluble, but soluble in acetone and DMSO.

Specimens of *U. esculenta* and *P. omphalodes* were collected from Mt. Tulaopodingzi, Wangqing, Jilin, China. The specimens of *L. retigera* were collected from Mt. Jiaozixueshan, Luquan, Yunnan, China. Samples were placed in plastic bags, stored at 4 °C and processed within 7 days of collection. A specimen of each lichen species was used to isolate and culture bacteria, while the remaining specimens were used to prepare acetone extracts.

Air-dried lichen thalli (2 g) were cut into pieces (1 × 1 mm) and shaken in 100 ml acetone (24 h, 150 rpm) at room temperature. The acetone extracts were filtered using Whatman No. 1 filter paper and then concentrated *in vacuo* at 40 °C using a rotary evaporator. The dry extract was stored at 4 °C.

The isolation was performed as follows. For each lichen species five segments were cut from healthy growing parts of the thallus after washing in sterile water for 10 min. Each segment (about 5 mg) was treated to 10 × 1 min. washes in 0·8% NaCl and crushed in 0·5 ml of sterile deionized water using a pestle and mortar. 0·1 ml of the resulting suspension was plated on the water medium plus DMSO and lichen extract (water medium: 0·4 g KH_2PO_4, 0·2 g $MgSO_4.7H_2O$, 0·1 g NaCl, 0·02 g $CaCl_2.2H_2O$, 0·01 g $FeCl_3.6H_2O$, 0·002 g $Na_2MoO_4.H_2O$, 17 g agar, 1000 ml deionized water, pH 7·0–7·2). The DMSO solution of lichen acetone extract (5ml, 20mg ml^{-1}) was filter sterilized and added to the medium after it was autoclaved and cooled. Plates were incubated at 20 °C and inspected every day until no new colonies appeared. For each lichen species, all the colonies with distinctive phenotypes were purified by the streak inoculation method to seed a single colony on a new plate filled with the same medium.

Strains of bacteria from a single colony were put into a sterile centrifuge tube (1·5 ml), washed three times using sterile deionized water, centrifuged and then mixed with

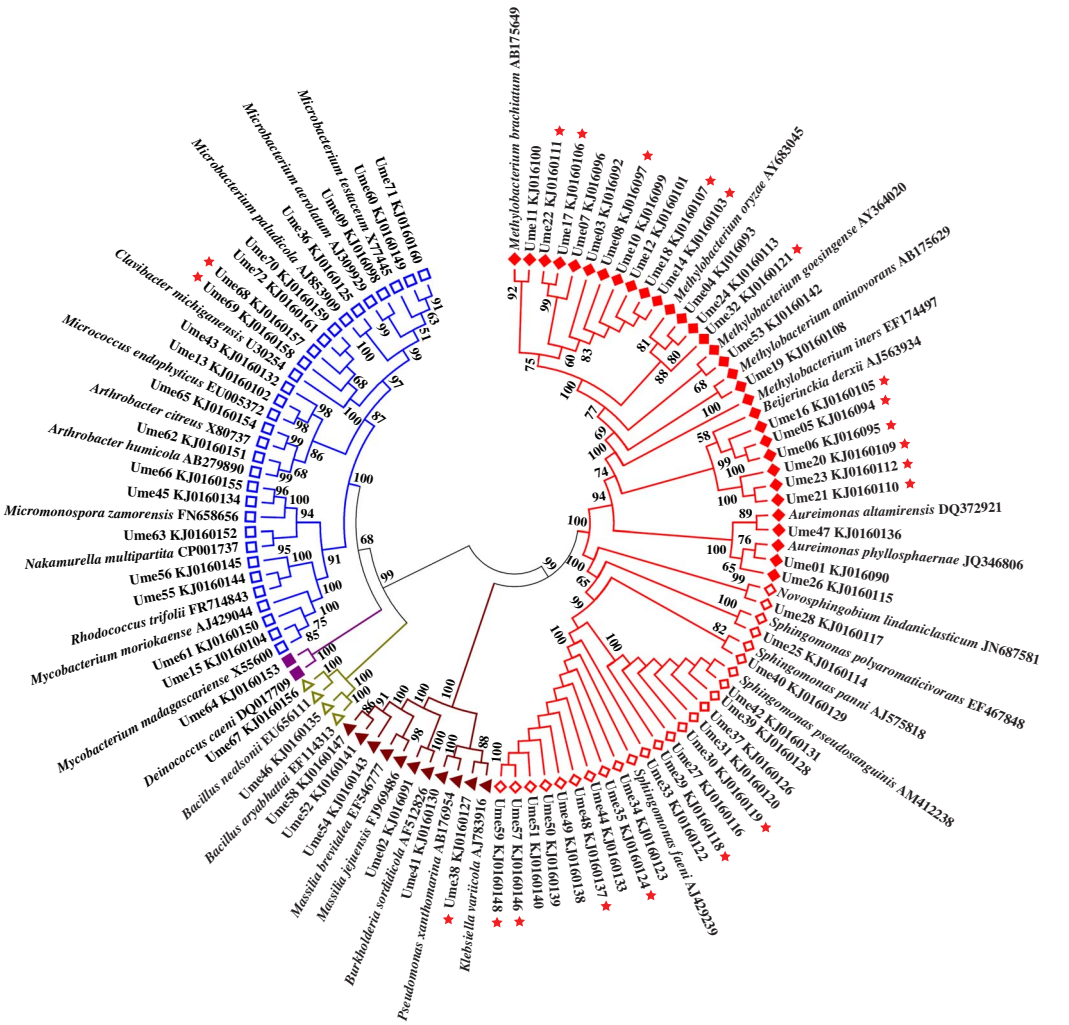

FIG. 1. Unrooted phylogenetic tree for bacterial isolates from *Umbilicaria esculenta*. ◆ = *Rhizobiales*; ◇ = *Sphingomonadales*; ▲ = Gammaproteobacteria; △ = Bacilli; ■ = Deinococci; □ = Actinobacteria. ★ = nifH gene positive. In colour online.

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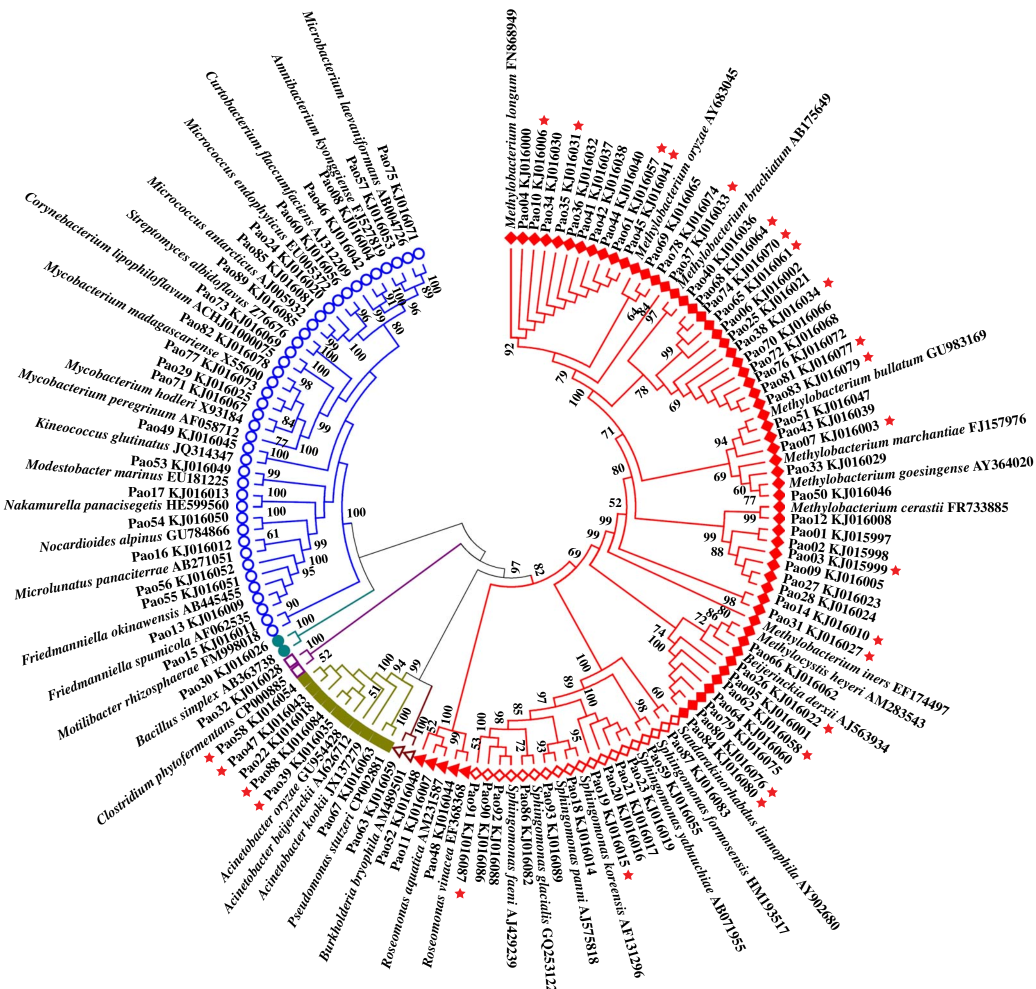

FIG. 2. Unrooted phylogenetic tree for bacterial isolates from *Parmelia omphalodes*. ◆ = *Rhizobiales*, ◇ = *Sphingomonadales*, ▲ = *Rhodospirillales*, △ = Betaproteobacteria, ■ = Gammaproteobacteria, □ = Clostridia, ● = Bacilli, and ○ = Actinobacteria. ★ = nifH gene positive. In colour online.

1·2 ml sterile deionized water after discarding the supernatant. 100 µl of bacterial suspension was plated on each of the isolation media, namely water medium plus DMSO and lichen extract, water medium and water medium plus DMSO(5 ml DMSO (5·5 g) added to the water media), and cultures incubated at 20 °C.

Total DNA from the bacteria was extracted using the DNA Extraction Mini Kit (SANGON). The primers 27F and 1492R (Dees & Ghiorse 2001) were used for PCR amplification of the 16S rDNA. The PCR temperature profile was as follows: initial denaturation at 95 °C for 3 min followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 2 min extension at 72 °C and completed with a final 10 min extension at 72 °C. PCR products were purified with the Gel Extraction Mini Kit (SABC). Sequencing reactions were carried out by Shanghai SANGON Corporation. All the 16S rDNA sequences obtained are available in GenBank (KJ015997–KJ016230) and the phylogenetic analysis was performed as described by Wang *et al.* (2013). Primers F1 (5'-TAYGGNAARGGNGGNAT YGGNAARTC-3') and nifH-r (5'-ADNGCCATCA TYCTNCC-3') were used to amplify a partial stretch of the nifH gene.

Bacterial colonies appeared on the isolation plates after three days with no new colonies appearing after approximately two weeks. A total of 234 single bacterial colonies (72 from

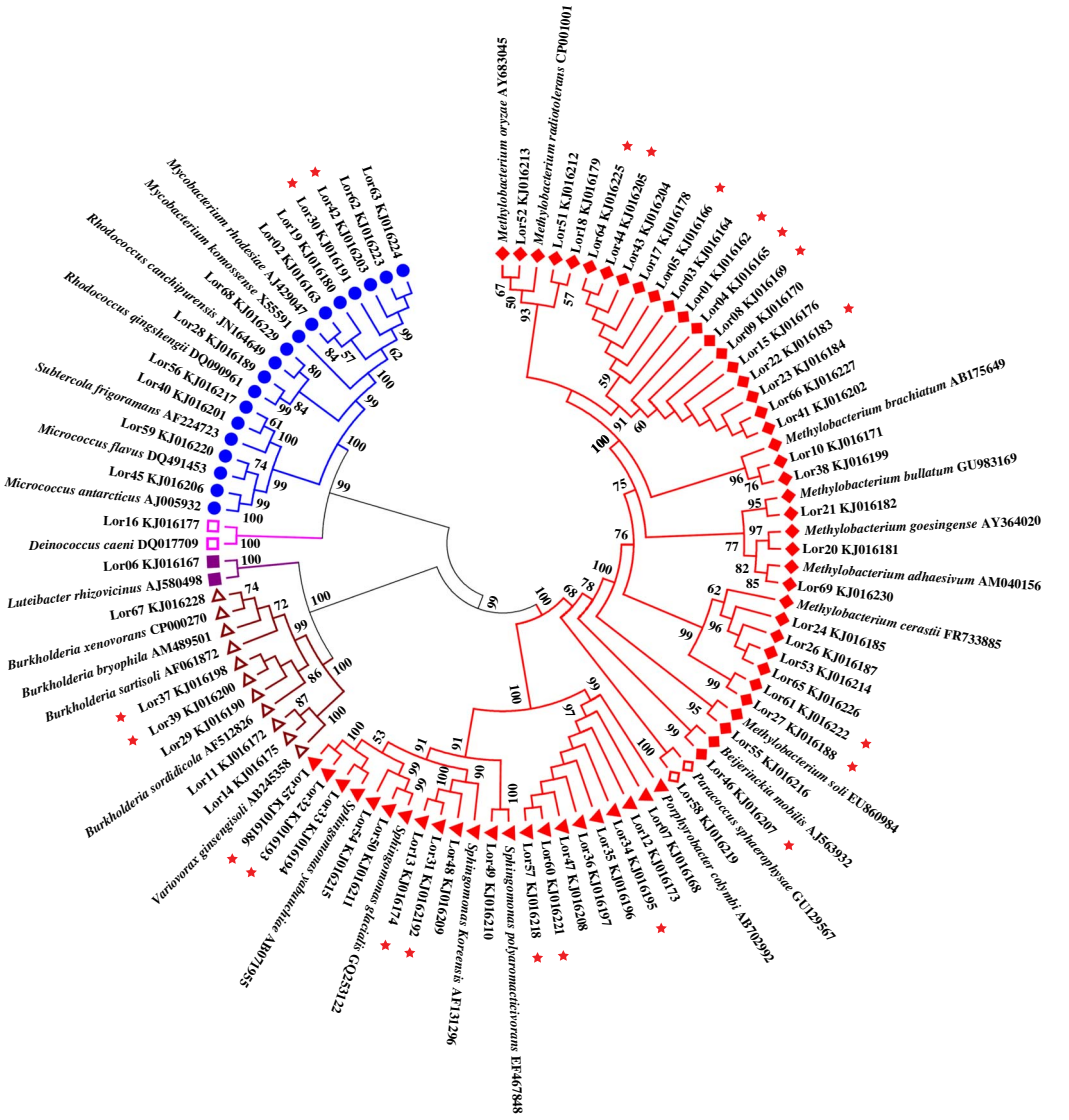

FIG. 3. Unrooted phylogenetic tree for bacterial isolates from *Lobaria retigera*. ◆ = *Rhizobiales*, ◇ = *Rhodobacterales*, ▲ = *Sphingomonadales*, △ = Betaproteobacteria, ■ = Gammaproteobacteria, □ = Deinococci, and ● = Actinobacteria. ★ = nifH gene positive. In colour online.

Umbilicaria esculenta, 93 from *Parmelia omphalodes* and 69 from *Lobaria retigera*) were obtained from the three lichens. After incubating for two weeks, it seemed that all 234 bacterial isolates grew better on the water medium plus DMSO and lichen extract than on the water medium plus DMSO (with more bacterial colonies on the plates), while none of the bacterial isolates were observed growing on the water only medium. Their 16S rDNA were then sequenced and the presence of the nifH gene was also detected (Figs 1–3).

Rhizobiales is the most predominant order among the isolates from *U. esculenta*, *P. omphalodes*, and *L. retigera*. In addition to the 13 known families, *Rhizobiales* includes

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LAR1 which was detected from lichens by part sequences of 16S rDNA (Grube *et al.* 2009; Hodkinson & Lutzoni 2009; Hodkinson 2011; Hodkinson *et al.* 2012). The phylogenetic tree (Fig. 4) based on the part sequences of 16S rDNA shows that several isolates of *Rhizobiales* are members of LAR1 which also includes many members detected from various hosts in addition to lichens. These isolates belong to two well-supported subclades of LAR1. In total, 67 isolates were positive for amplification of the nifH gene (*U. esculenta*: 21, Fig. 1; *P. omphalodes*: 25, Fig. 2; *L. retigera*: 21, Fig. 3) suggesting that these isolates are able to fix nitrogen. Since not all N_2-fixing bacteria are positive for the nifH gene (Islam *et al.* 2012), there may be more than 67 N_2-fixing bacteriobionts isolated from the three lichens.

The better growth on the water medium plus DMSO and lichen extract than on the water medium plus DMSO reveals that, although bacterial isolates could utilize DMSO, the lichen extrolite extracts provided more nutrients. In addition, results for the water only medium indicate that agar did not provide any nutrition for bacteria. Culture-independent studies showed that *Rhizobiales* and Alphaproteobacteria are the predominant order and class among lichen bacteriobionts (Cardinale *et al.* 2008, 2012; Grube *et al.* 2009; Bates *et al.* 2011; Mushegian *et al.* 2011; Schneider *et al.* 2011; Hodkinson *et al.* 2012). Unfortunately, very few species in these groups have been successfully isolated and cultured from lichens on media with conventional carbon sources, regardless of whether the media were nitrogen-free or nitrogen-containing (Grube *et al.* 2012). However, when lichen extrolites were used as the nutrient source, these predominant bacteriobionts were readily isolated from these lichen species, even including the previously uncultured lineage LAR1 which appears to commonly occur in lichens (Hodkinson & Lutzoni 2009; Bates *et al.* 2011; Hodkinson 2011; Hodkinson *et al.* 2012). The results of this study show that lichen compounds might be more appropriate than other carbon sources for isolating and culturing bacteriobionts.

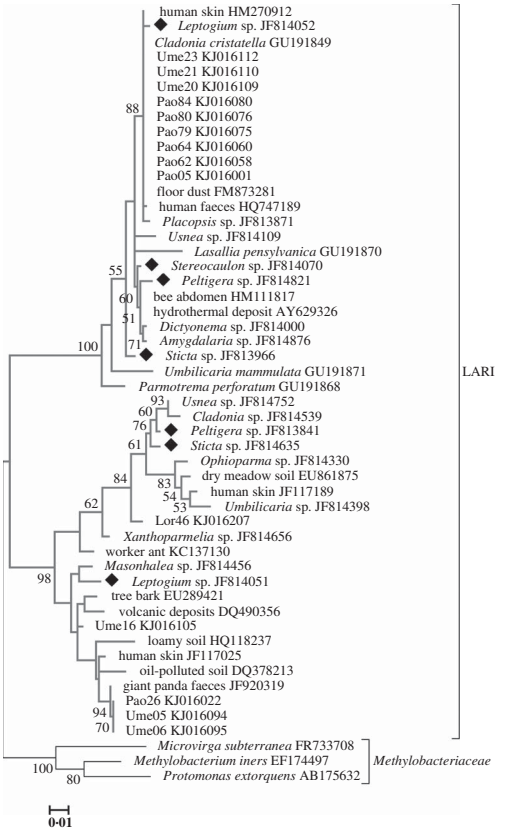

FIG. 4. Phylogenetic tree for LAR1. ◆ = samples from cyanolichens or chlorolichens with cephalodia. Outgroup is represented by 3 species of *Methylobacteriaceae*.

Most of the nifH gene-positive isolates belong to *Rhizobiales*. These results suggest that many bacteriobionts have the potential for N_2 fixation in the lichen thallus, consistent with previous reports (Liba *et al.* 2006; Seneviratne & Indrasena 2006; Grube *et al.* 2009). A large number of N_2-fixing isolates, including the members of LAR1, were isolated from all three lichen species. This demonstrates that both chlorolichens and cyanolichens harbour N_2-fixing bacteriobionts, which possibly use lichen compounds as the carbon source, and that bacteriobionts with N_2-fixing and lichen compound-digesting abilities might potentially help mycobionts transmute part of the spare carbon (lichen extrolites) into available nitrogen.

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