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ORIGINAL ARTICLE

Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones

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Abstract

Aims: Cyanobacteria-deprived lichens of the species *Canoparmelia caroliniana*, *Canoparmelia crozalsiana*, *Canoparmelia texana*, *Parmotrema sancti-angeli* and *Parmotrema tinctorum* were screened for the presence of chemo-organotrophic nitrogen-fixing bacteria.

Methods and Results: Fifty-three lichen samples subjected to enrichment selection using a nitrogen-free minimal medium were positive for acetylene reduction. Seventeen isolates, able to fix nitrogen, belonged to Gamma-proteobacteria group and were identified as: *Acinetobacter* sp., *Pantoea* sp., *Pseudomonas* sp., *Pseudomonas stutzeri*, *Serratia marcescens* and *Stenotrophomonas maltophilia*, according to 16S rRNA gene sequences and biochemical tests. The excretion of amino acid and phytohormone and the ability of mineral phosphate solubilization were determined in 14 isolates. All isolates were able to release amino acids and 3-indoleacetic acid. About 64% of the isolates solubilized phosphates and 30% released ethylene.

Conclusions: These data confirm sparse evidence from the literature on the occurrence of chemo-organotrophic nitrogen-fixing bacteria in cyanobacteria-deprived lichens; the isolates presented physiologic features which might benefit the host if they are expressed when the bacteria are harboured by lichens.

Significance and Impact of the Study: Chemo-organotrophic nitrogen-fixing bacteria were isolated from a high percentage (72·6%) of cyanobacteria-deprived lichens. All isolates presented important physiological characteristics, some of which are being described here for the first time.

Introduction

Lichenized fungi are widespread in nature and represent about 20% of all fungi species (Hawksworth 1988). They may be characterized by the types of associations between the fungi (mycobiont) and their photosynthetic partners (photobionts). About 85% of the lichenized fungi comprise associations with green micro-algae, 10% with cyanobacteria and 4% with both cyanobacteria and green algae (Tschermak-Woess 1988). In this mutual partnership, the mycobiont provides a suitable environment for the photobionts, enabling gas exchange with the atmosphere, water and organic nutrient supply, and protection against drying. Green algae, in exchange, provide a source

of organic matter via photosynthesis and cyanobacteria provide a combined nitrogenated source via nitrogen fixation (Honegger 1998).

Despite the success of this nutritional strategy, relatively little information is available regarding nitrogen cycling in lichens (Brown 1992; Miller and Brown 1999), and these experiments were all conducted using lichens associated with nitrogen-fixing cyanobacteria. Lichen-associated cyanobacteria have also been shown to have enhanced nitrogen fixation rates compared with nonassociated cells (Meeks 1998). $^{15}N_2$ experiments using *Peltigera aphthosa* proved that almost all nitrogen fixed by cyanobacteria is transferred to the fungi (Rai 1988). Alternatively, cyanobacteria-deprived lichens may harbour chemo-organotrophic nitrogen-fixing bacteria.

Nitrogen-fixing bacteria have been traditionally reported in symbiotic associations with higher plants, such as the classical case of rhizobia and legumes, and more recently as endophytes (James 2000; Elbeltagy *et al.* 2001), but sparse data are available for other types of interactions, such as with prokaryotes, fungi or lichens. For lichens, in particular, reports from the previous decades have dwelt mainly on the aspects of isolation of diverse groups of bacteria, including nitrogen-fixing bacteria (Uphof 1925a,b, 1926; Suessenguth 1926; Kolumbe 1927; Iskina 1938; Krasil'nikov 1949; Scott 1956; Panosyan and Nikogosyan 1966; Genkel' and Plotnikova 1973). In some cases, epiphytic bacteria have been shown to metabolically interact with the fungi in the production of phenolic compounds (Blanco *et al.* 2002).

Free-living and endophytic chemo-organotrophic, diazotrophic bacteria are able to release nitrogenated compounds, including amino acids, vitamins (González-Lopes *et al.* 1983), and phytohormone (Bastián *et al.* 1998; Thuler *et al.* 2003b) as seen under *in vitro* growth conditions. This feature is potentially useful in possible interactions between the bacteria and other organisms.

In the current study, five species of cyanobacteria-deprived lichens which are common in São Paulo State (Brazil), i.e. *Canoparmelia caroliniana*, *Canoparmelia crozalsiana*, *Canoparmelia texana*, *Parmotrema sancti-angeli* and *Parmotrema tinctorum*, were analysed for the presence of chemo-organotrophic nitrogen-fixing bacteria. In order to detect N_2 fixation, a standard acetylene reduction assay (ARA) was used; the presence of *nif*H genes, responsible for the production of the nitrogenase Fe protein subunit, was also screened by molecular probing. Isolates were characterized taxonomically by analyses of 16S rRNA gene sequences and by phenotypical data. Physiological tests such as amino acid and phytohormone release, and phosphate solubilization, were performed in order to determine the potential contribution of the bacteria to the environment through the release of different substances.

Material and methods

Sampling of lichens and selective enrichment of nitrogen-fixing bacteria

In total, 73 samples of five different species of cyanobacteria-deprived lichenized fungi were analysed (Table 1). Samples of *C. caroliniana* (Nyl.) Elix & Hale, *C. crozalsiana* (de Lesd.) Elix & Hale, *C. texana* (Tuck.) Elix & Hale, *P. sancti-angeli* (Lynge) Hale and *P. tinctorum* (Despr. ex Nyl.) Hale were collected from a preserved area of the Atlantic Rain Forest, located in the Botanical Garden of São Paulo (São Paulo, Brazil). Intact and physiologically active individuals were carefully removed from tree surfaces, using sterile scalpels, and transported to the laboratory, stored in sterile paper bags at room temperature. Specimens were then immersed into sterile distilled water in Petri dishes for 1 min and 2 cm^2 pieces were cut using sterile forceps and scalpel. These samples were macerated in a sterile mortar and 5-μl aliquots were inoculated into 10-ml glass vials containing 4 ml of nitrogen-free NFb semi-solid (0·18% agar) medium (Baldani *et al.* 1997), supplemented with 30 $\mu g\ ml^{-1}$ nystatin (Sigma Aldrich). Vials were sealed with rubber stoppers, crimped and incubated at 30°C for 72 h prior to headspace ARAs. Five replicate vials were prepared for each lichen specimen processed.

Acetylene reduction assays

Nitrogen fixation of enrichment cultures and of bacterial isolates were determined by following a standard assay described previously (Turner and Gibson 1980). All replicates of the enrichment cultures or, alternatively, vials inoculated with pure cultures, were injected with 1 ml of pure acetylene into the overhead space (10% of vial volume) and incubated at 30°C for 24 h. Acetylene reduction was detected by gas chromatographic analysis of overhead samples by using a Shimadzu C-R5A-S system (Kyoto, Japan), fitted with a Porapack N column and a hydrogen flame ionization detector (FID). Non-inoculated vials with NFb medium (Döbereiner 1980) were used as control.

Isolation of nitrogen-fixing bacteria

Enrichment cultures that showed acetylene reduction were streaked onto solid NFb medium (1·5% agar) supplemented with 20 $\mu g\ ml^{-1}$ yeast extract, in order to isolate single colonies of nitrogen-fixing bacteria. Pure cultures were re-inoculated onto NFb semi-solid medium to confirm nitrogen-fixing ability by the ARA. The bacterial strains isolated in this study are deposited in the Brazilian

Table 1 Detection and identification of nitrogen-fixing bacteria taken from samples from cyanobacteria-deprived lichenized fungi

Lichens	ARA-positive cultures/lichen samples tested	Identification of the bacterial isolates	Reaction with *nif*H gene probe	Genbank accession	Similarity (%) with the closest phylogenetic neighbour (Genbank accession)
C. caroliniana	7/17	*Ste. maltophilia* Li 4 (=CBMAI 458)*	+	DQ250137	830/835 bp (99), *Ste. maltophilia* (AJ131114)
		Ste. maltophilia Li 56 (=CBMAI 471)*†	+	DQ250150	625/644 bp (97), *Ste. maltophilia* (AJ131114)
C. crozalsiana	10/10	*Pantoea* sp. Li 35 (=CBMAI 465)*†	n.d.	DQ250144	895/896 bp (99), *Pantoea* sp. (AJ002811)
		Ser. marcescens Li 62 (=CBMAI 473)*	n.d.	DQ250152	1168/1170 bp (99), *Ser. marcescens* (AY732377)
C. texana	7/11	*Ser. marcescens* Li 61 (=CBMAI 472)*‡	+	DQ250151	559/561 (99), *Ser. marcescens* (AY043388)
		Pseudomonas stutzeri Li 8 (=CBMAI 460)*†‡	+	DQ250139	1038/1038 bp (100), *Pseudomonas stutzeri* (AJ288151)
P. sancti-angeli	19/23	*Acinetobacter calcoaceticus* Li 32 (=CBMAI 464)*†‡	n.d.	DQ250143	935/935 bp (100), *Acinetobacter* sp. (AJ303010)
		Ser. marcescens Li 7† (=CBMAI 459)*	+	DQ250138	1174/1174 (100), *Ser. marcescens* (AY827577)
		Ser. marcescens Li 78* (=CBMAI 474)*	+	DQ250153	1183/1183 (100) *Ser. marcescens* (AY827577)
		Ser. marcescens Li 31* (=CBMAI 463)*	n.d.	DQ250142	1190/1190 (100), *Ser. marcescens* (AY827577)
P. tinctorum	10/12	*Pantoea* sp. Li 43 (=CBMAI 467)*†‡	+	DQ250146	633/633 bp (100), *Pantoea* sp. (AJ002811)
		Pseudomonas sp. Li 46 (=CBMAI 468)*†	+	DQ250147	873/875 bp (99), *Pseudomonas* sp. (AF468451)
		Ste. maltophilia Li 41 (=CBMAI 466)*	+	DQ250145	843/846 bp (99), *Ste. maltophilia* (AJ131114)
		Ste. maltophilia Li 9 (=CBMAI 461)*‡	+	DQ250140	935/937 bp (99), *Ste. maltophilia* (AY360340)
		Acinetobacter sp. Li 25 (=CBMAI 462)*‡	n.d.	DQ250141	570/573 (99), *Acinetobacter* sp. (AY956937)
		Ser. marcescens Li 51 (=CBMAI 469)*	n.d.	DQ250148	700/715 (97), *Ser. marcescens* (AY043388)
		Ser. marcescens Li 52 (=CBMAI 470)*	n.d.	DQ250149	1157/1157 (100), *Ser. marcescens* (AY732377)

CBMAI accession numbers refer to strains deposited at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI, Coleção Brasileira de Microrganismos de Ambiente e Indústria, Campinas, SP, Brazil; http://www.cpqba/unicamp/br/cbmai). n.d.: not determined (these isolates could not be recovered from preservation vials after storage, therefore were not available for further studies).
Bacteria identified by *16S rRNA gene sequence analysis, †classical biochemical tests and/or the ‡VITEK system.

Collection of Industrial and Environmental Microorganisms (CBMAI/CPQBA-UNICAMP).

Detection of *nif*H genes

Genomic bacterial DNAs were isolated by using the 'Wizard Genomic DNA Purification Kit' (Promega, Madison, WI, USA; Cat. A 1120), according to the manufacturer's instructions. DNAs were spotted onto Hybond N+ membranes (ECL System; Amersham Pharmacia, Piscataway, NJ, USA), as recommended by the manufacturer's protocol. Dot-blot hybridizations were carried out using a 705-bp probe of *Azospirillum brasilense* $Sp7^T$ *nif*H gene (Genbank accession number M64344), amplified by PCR using primers PPf (5′-GCAAGTCCACCACCTCC-3′) and PPr (5′-TCGCGTGGACCTTGTTG-3′) (Reinhardt 1999). Probe labelling and hybridizations were performed according to the ECL System (Amersham Pharmacia) recommended protocol, and hybridization was carried out at 60°C for 16 h, without formamide.

16S rRNA sequencing and analysis

Amplification of the 16S rRNA gene was performed by using 30 ng of genomic DNA in 25-μl reactions containing 2 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs (each),

0·3 μmol l^{-1} of primers 27f and 1401r (Lane 1991), and 2 U *Taq* DNA polymerase (Gibco BRL, Carlsbad, CA, USA), in the recommended buffer. PCRs were performed in an MJ PTC-100 thermocycler. The thermal programme consisted of (i) 94°C for 2 min, (ii) 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, and (iii) final extension for 10 min at 72°C. Sequencing reactions were performed using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBace (Amersham Pharmacia), following the manufacturer's instructions, and analysed in an MegaBace 1000 sequencer (Amersham Pharmacia). Sequences were compared with 16S rRNA gene sequences available at Genbank (Altschul *et al.* 1997) and the RDP (http://rdp8.cme.msu.edu/html/) in order to assess related taxa. Selected sequences were aligned and trees were constructed by using the Jukes–Cantor algorithm (Jukes and Cantor 1969) and neighbour-joining algorithm (Saitou and Nei 1987), as implemented in the MEGA software (Kumar *et al.* 2001). Bootstrap analyses were performed according to Felsenstein (1985).

Phenotypic characterization

The morphological and biochemical features of the bacterial strains were analysed using standard protocols (Balows *et al.* 1991) and the Vitek Gram-negative identification system (BioMerieux Vitek, Inc., Hazelwood, MO, USA).

To determine the production of amino acids, 3-indoleacetic acid (IAA) and ethylene, as well as mineral phosphate solubilization, the following culture media were employed: (i) NFb medium, added with 1·32 g l^{-1} ammonium sulfate as a combined nitrogen source (NFb_{AS}), was used for amino acid production; to promote the growth of the *Stenotrophomonas* strains 0·4 g l^{-1} methionine were added (Wolf *et al.* 2002) to NFb_{AS}; (ii) nutrient broth added with 0·5 g l^{-1} tryptophan (NB_{TRP}) (Zimmer and Bothe 1988), was used for IAA production and (iii) NFb_{AS}, added with 0·4 g l^{-1} methionine ($NFb_{AS+\ met}$) (Arshad and Frankenberger 1998), was used for ethylene production. Mineral phosphate solubilization activity was assayed on Pi solid medium containing (in g l^{-1}) $Ca_3(PO_4)_2$, 0·8; NH_4Cl, 5; NaCl, 1; $MgSO_4{\cdot}7H_2O$, 1; glucose, 10; agar, 15; pH 7·2. For bacteria of the *Stenotrophomonas* genus sucrose was used instead of glucose.

Culture conditions

To determine amino acid production, cultures were prepared in 250-ml Erlenmeyer flasks containing 100 ml NFb_{AS} liquid medium and grown for 200 h, at 30°C and 250 rev min^{-1}. Prior to analysis of amino acids production by HPLC, sample cultures were centrifuged (18 000 ***g***, for 10 min, at 4°C) and filtered through Millipore membranes (0·20 μm). The supernatants were submitted to derivatization with ophtalaldehide (Astarita *et al.* 2004).

Mineral phosphate solubilization activity was assayed in plates. The strains were inoculated on Pi solid medium and incubated at 30°C. The zone of clearance around the colony was observed after 48 h.

3-Indoleacetic acid (IAA) assay was developed in 15-ml tubes with 5 ml of NFb_{TRP} (Thuler *et al.* 2003a) and grown at 30°C without agitation. The cultures were centrifuged (18 000 ***g***, for 5 min) and the supernatants were submitted to Salkowsky method, modified as follows: twice the volume of Salkowsky reagent (0·01 mol l^{-1} $FeCl_2$ in 37·5% H_2SO_4) was added to each bacterial culture. IAA was visually detected by colour development (Ehmann 1977).

To test ethylene production, cultures were prepared in 15-ml penicillin flask containing 8 ml of $NFb_{AS+\ met}$ medium. The flasks were fitted with rubber plugs tightened with metal cowls and then incubated for 9 days at 30°C without agitation. In order to measure ethylene production, 1 ml of aerial phase was withdrawn from the culture flask and analysed in a gas chromatograph (Shimadzu GC-14A) with a PORAPAK-N 80/100 – INOX column operated isothermally at 70°C with nitrogen as the gas carrier and an FID. Pure ethylene (White Martins) was used as a standard. Studies of all analysed substances were performed in triplicate.

Results

The nature of the enrichment and selective isolation procedures employed, i.e. the use of a combined nitrogen-deprived, organic carbon-based solid medium, favoured obtaining of nitrogen-fixing chemo-organotrophic bacterial consortia from all five lichenized fungi tested. From 73 analysed samples, a total of 53 enrichment cultures were positive for acetylene reduction. However, diazotrophic bacteria were only isolated from a small number of cultures. Seventeen of these isolates were identified (Table 1).

According to phenotypic features and 16S rRNA gene sequences, all isolates recovered belonged to the Gammaproteobacteria group, including both fermentative and nonfermentative organisms (Table 1). Bacteria identified in this study (Fig. 2) belong to the following taxa: *Stenotrophomonas maltophilia*, *Pseudomonas* spp., *Pantoea* sp., *Serratia marcescens* and *Acinetobacter calcoaceticus*.

Table 1 shows that only one species was isolated from *C. caroliniana*. Two genera were isolated from *C. crozalsiana*, *C. texana* and *P. sancti-angeli*, and five different genera were isolated from *P. tinctorum*, the lichen that presented the greatest diversity of bacteria.

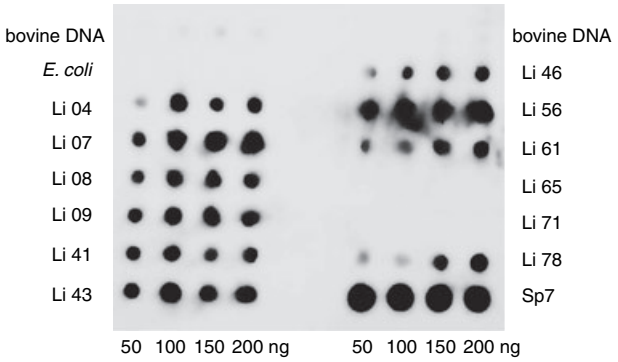

Figure 1 Dot-blot hybridization of genomic DNA from bacterial isolates with the *nif*H gene probe from *Azospirillum brasilense* Sp7^T.

Positive hybridization signals with an *nif*H probe from *Azospirillum brasilense* Sp7^T (Fig. 1) were observed for 10 of 12 ARA-positive strains analysed (Table 1), demonstrating the presence of *nif*H-related genes in their genomes. Two ARA-positive isolates, strains Li 65 and Li 71 (not identified), did not react with the probe under the hybridization conditions used.

The ability to excrete amino acids was observed in all isolated strains. In all 14 tested strains amino acids were detected in the culture media. The results of amino acids production by the tested isolates are shown in Table 2. Different genera excreted different amino acids. *Pantoea* sp. excreted the greatest amount and variety of amino acids, followed by *Acinetobacter calcoaceticus*; *Pseudomonas* sp. excreted the lowest concentration. Not one single amino acid was released by all of the bacterial strains analysed. *Serratia marcescens* and *Pantoea* sp. strains showed the greatest uniformity of types of amino acids they released.

The ability of the 14 isolates to solubilize phosphate is shown in Table 3. *Pantoea* sp., *Acinetobacter calcoaceticus* and *Ser. marcescens* presented phosphate-solubilizing activity. IAA was detected in supernatant samples of all 14 tested isolates, as shown in Table 3. Three of the four isolates of *Ste. maltophilia* were able to produce ethylene in media supplemented with methionine. Only one isolate of *Ser. marcescens* excreted ethylene (Table 3).

Discussion

For the purpose of isolating chemo-organotrophic nitrogen-fixing bacteria from cyanobacteria-deprived lichens, the use of selective enrichment conditions employing a nitrogen-free medium proved to be successful. Previous trials aiming at direct detection of nitrogen fixation in lichen samples were not successful, possibly because of several factors, such as reduced metabolic and nitrogen-fixing activities of the bacteria and/or small numbers of bacteria in the samples, and low sensitivity of the ARA. Failure to detect and isolate nitrogen-fixing bacteria from the cyanobacteria-deprived lichens *Parmelia* sp., *Platismatia* sp., *Alectoria sarmentosa* (Arch.) Arch., *Spherophorus globosus* (Huds.) and *Hypogymnia imshaugii* Krog was reported by Caldwell *et al.* (1979).

Difficulty to recover a higher number of nitrogen-fixing pure cultures from the enrichments suggests that the isolation conditions used in our study may have been rather restrictive and did not allow for the cultivation of a potentially wider diversity of nitrogen-fixing micro-organisms associated to the lichens. The initial enrichment

Table 2 Amino acids liberated by nitrogen-fixing bacteria isolated from cyanobacteria-deprived lichenized fungi

Bacteria	Ala	Asn	Asp	Gln	Gaba	Glu	His	Ile	Leu	Met	Phe	Thre	Tyr
Ste. maltophilia (CBMAI 458)	–	+	++	–	–	++	–	++	–	–	+	+	+
Pseudomonas stutzeri (CBMAI 460)	–	–	+	++	–	–	+	–	–	+++	–	–	–
Ste. maltophilia (CBMAI 461)	–	+	++	–	–	++	–	++	–	–	+	+	+
Acinetobacter calcoaceticus (CBMAI 464)	+	+	++	–	–	++	–	–	–	++++	++	–	+
Pantoea sp. (CBMAI 465)	–	++	+	–	–	+	++	+++	++++	++++	+++	–	+++
Ste. maltophilia (CBMAI 466)	–	–	+	–	–	++	–	–	–	–	+	–	+
Pantoea sp. (CBMAI 467)	–	++	–	–	–	+	+	+++	++++	+++	+++	–	+++
Pseudomonas sp. (CBMAI 468)	–	–	++	–	+++	+	–	–	–	++++	–	–	–
Ste. maltophilia (CBMAI 471)	–	+	+	–	–	++	–	++	–	–	+	–	+
Ser. marcescens (CBMAI 459)	–	–		–	–	–	–	++	++	++++	–	–	+
Ser. marcescens (CBMAI 469)	–	–		–	–	–	–	++	++	++++	–	–	+
Ser. marcescens (CBMAI 472)	–	–		–	–	–	–	+++	+++	++++	–	–	+
Ser. marcescens (CBMAI 473)	–	–		–	–	–	–	++	++	++++	–	–	+
Ser. marcescens (CBMAI 474)	–	–		–	–	–	–	++	+++	++++	+	–	+

The number of '+' signs indicates variation intervals in the concentration of amino acids, in μmol l^{-1}, as follows: + (3·3–13·2); ++ (13·3–58·5); +++ (58·6–237·5); ++++ (237·6–972·1).

Table 3 Phosphate solubilization and 3-indoleacetic acid (IAA) and ethylene release by nitrogen-fixing bacteria isolated from cyanobacteria-deprived lichenized fungi

	Phosphate solubilization*	IAA†	Ethylene‡
Ste. maltophilia (CBMAI 458)	–	+	+
Pseudomonas stutzeri (CBMAI 460)	–	+	–
Ste. maltophilia (CBMAI 461)	–	+	+
Acinetobacter calcoaceticus (CBMAI 464)	+	+	–
Pantoea sp. (CBMAI 465)	+	+	–
Ste. maltophilia (CBMAI 466)	–	+	–
Pantoea sp. (CBMAI 467)	+	+	–
Pseudomonas sp. (CBMAI 468)	–	+	–
Ste. maltophilia (CBMAI 471)	–	+	+
Ser. marcescens (CBMAI 459)	+	+	–
Ser. marcescens (CBMAI 469)	+	+	–
Ser. marcescens (CBMAI 472)	+	+	+
Ser. marcescens (CBMAI 473)	+	+	–
Ser. marcescens (CBMAI 474)	+	+	–

*'+' signs indicate solubilization halo diameters between 1·5 and 3 cm.
†'+' signs indicate production of IAA (qualitative).
‡'+' signs indicate ethylene values between 0·44 and 0·71 nmol produced by 9-day cultures.

culture had material from macerated lichens and, thus, represented a more complex nutritional composition compared with the NFb media used in subsequent cultures and in the isolation media.

The bacteria isolated in this study might be located in the inner or outer structures of the lichen. Recent studies have shown that unicellular bacteria are indeed associated to the inner section of lichens, as demonstrated in electron micrographs of *Baeomyces rufus* (Asta *et al.* 2001), suggesting that they may also play a role in the creation and progressive transformation of the lichen's structure. However, the location of the chemo-organotrophic diazotrophic bacteria isolated in the course of this study cannot be determined. It is not possible to separate bacterial cells located on the outer surface of the lichen, from cells located inside it, by using a chemical disinfection method employed for plant material. It must be noted that lichen structures are permeable to the penetration of solutions, and the use of a disinfectant would kill bacterial, algae and fungi cells.

All identified bacteria are widespread in nature and commonly isolated from soil, plant surfaces and water samples (Balows *et al.* 1992). The various bacterial genera were unevenly distributed among the different lichens studied, suggesting there is no required and/or specific association between lichens and those bacteria; the most common genus was *Serratia*, isolated in all lichen species, except in *C. caroliniana*. Although *Pseudomonas* sp. has diversified metabolic capabilities that enable it to survive extreme physical and chemical conditions (Lindow 1992), some of which may be present in lichens, this genus was found in only 40% of the lichens analysed.

Nitrogen fixation was previously reported for *Ste. maltophilia*, *Pseudomonas* sp, *Pantoea* sp. and *Ser. marcescens* (Gyaneshwar *et al.* 2001; Desnoues *et al.* 2003; Loiret *et al.* 2004; Park *et al.* 2005, respectively), but this is the first time it is reported for strains related to the genus *Acinetobacter*.

The cultures isolated from the resulting enrichment consortia were submitted to further metabolic characterizations and to an evaluation for the presence of *nif*H genes. The presence of known nitrogen fixation systems in the bacterial isolates recovered from the lichens studied was further investigated by using an *nif*H gene probe derived from *Azospirillum brasilense* $Sp7^T$. This gene was selected because it encodes the Fe-protein of the conventional molybdenum nitrogenase (*nifHDK*) (Ueda *et al.* 1995), and is highly conserved (Zehr and Capone 1996), thus allowing the detection of another homologue under low-stringency conditions. Positive acetylene reduction and growth in nitrogen-deprived NFb medium were indicative of active nitrogenase complexes in these bacteria. The occurrence of ARA-positive/*nif*H-negative isolates among the strains tested (Li 65 and Li 71) is comparable with reports of Stolzfus *et al.* (1997), who described endophytic ARA-positive bacterial strains that did not show hybridization signal with an *nif*D gene probe. These authors suggested that the bacteria analysed could have highly divergent *nif*D genes, or even alternative *nif* systems.

An important and heretofore not described characteristic detected in all isolated strains is their ability to release amino acids into the environment. Thuler *et al.* (2003a,b) have shown that *Beijerinckia derxii* (a free-living diazotrophic) and *Azospirillum* sp. (an endophytic diazotrophic)

excreted variable amounts of different amino acids, reaching the highest level of 21·7 μmol l^{-1} of glutamate for *B. derxii*. Pati *et al.* (1994) described the release of various amino acids by three different phyllospheric isolates: *Beijerinckia indica*, *Azotobacter chroococcum* and *Corynebacterium* sp. Although the bacteria isolated in the present study excreted no more than eight different amino acids, their concentrations were always much greater than those observed in *Azospirillum* sp. (Thuler *et al.* 2003a) grown under the same conditions. The same bacteria also produced greater concentrations when compared with other bacteria grown in different media. If these bacteria are in contact with lichens they may directly contribute to the nutrition of those lichens by releasing amino acids available to them. The excretion of amino acids by bacteria into the environment was described by Ruinen (1965). To this day, there is very little information about the mechanism(s) of this release and its possible benefits to the bacterium.

Another interesting feature observed in the present study is the high percentage of phosphate-solubilizing bacteria. Phosphorus (P) is one of the major essential macronutrients. In nature, large portions of inorganic phosphates are immobilized and become unavailable to organisms (Nautiyal 1999). Micro-organisms play a central role in the natural phosphorus cycle by releasing organic acids that are responsible for mineral phosphate solubilization (Rodriguez and Fraga 1999). The phosphate solubilization activity is consistent with one of the physiological characteristics of these bacteria, e.g. the ability to release organic acids (Holt *et al.* 1994). *Stenotrophomonas maltophilia*, *Pseudomonas stutzeri* and *Pseudomonas* sp. were not able to solubilize phosphates, although De Freitas *et al.* (1997) reported *Ste. maltophilia* as a phosphate-solubilizing rhizobacteria. *Pseudomonas* sp and *Pantoea* sp. have already been described as phosphate-solubilizing bacteria (Rodriguez and Fraga 1999; Verma *et al.* 2001 respectively). This paper describes for the first time this property in isolates of *Ser. marcescens* and *Acinetobacter calcoaceticus*.

Several micro-organisms, including soil, epiphytic and tissue-colonizing bacteria have been found to synthesize IAA, an auxin identical to that found in plants (Patten and Glick 1996). IAA influences plant processes, such as cell elongation, division and differentiation (Garcia-Tabares *et al.* 1987). IAA excretion was found to be a feature common to all the isolates presented herein. Release of IAA has already been described for the following: *Ste. maltophilia* (Park *et al.* 2005), *Pseudomonas* (Costacurta and Vanderleyden 1995), *Pantoea* (Beattie and Lindow 1999), *Acinetobacter* (Huddedar *et al.* 2002). The production of IAA by the genus *Serratia* is described here for the first time.

Ethylene is a phytohormone, produced by higher plants as well as by micro-organisms. Ethylene mediates many plant processes, from germination of seeds to senescence of organs, as well as plants' responses to environmental stress (Davies 1995). Ethylene production was observed in three of four strains of *Ste. maltophilia* and in one strain of *Ser. marcescens*. Ethylene production by *Pseudomonas* was described by Berner *et al.* (1999) and by Freebairn and Buddenhagen (1964). No references to the production of ethylene by *Pantoea*, *Acinetobacter*, *Stenotrophomonas* and *Serratia* were found in the literature.

Analysis of 16S rRNA gene sequence data from *Ste. maltophilia* isolates demonstrated that these were related to the sequence of the type strain LMG 958^T (Fig. 2). It is not possible to ascertain at this point the accuracy of the taxonomic assignment of isolates to *Ste. maltophilia*, because data from the literature clearly indicate that this taxon is heterogeneous, comprising several genomic species which may share nearly identical 16S rRNA gene sequences (Hauben *et al.* 1999). In addition, these new taxa are not represented in the databases of the phenotypic identification systems used (Vitek Gram-negative identification system, BioMerieux Vitek, Inc.).

The association of lichenized fungi and phototrophic nitrogen-fixing cyanobacteria has been studied and is well known (Rai 1988; Meeks 1998). However, alternative mechanisms for obtaining nitrogen in cyanobacteria-deprived lichens, in contrast, have been poorly investigated. The few reports from early studies on the isolation of nitrogen-fixing bacteria from lichens did not establish conclusive data about the contribution of these bacteria to lichen metabolism (Uphof 1925a,b, 1926; Suessenguth 1926; Kolumbe 1927; Iskina 1938; Krasil'nikov 1949; Scott 1956; Panosyan and Nikogosyan 1966; Genkel' and Plotnikova 1973). To our knowledge, this is the first wide range survey of chemo-organotrophic nitrogen-fixing bacteria in lichens. Further studies aimed at determining the abundance, distribution and specific location of culturable and unculturable Gamma-proteobacteria in various lichenized fungi species are necessary to shed light into the role of these bacteria in lichen biology.

The bacteria isolated in the present study show the potential for establishing relationships with lichens and higher plants. Their ecological role would be linked to the nutritional needs of lichens. Considering that lichens usually live under severe conditions of restricted nutrient availability, hosting heterotrophic bacteria that possess the characteristics hereby described may turn to be of great advantage to the lichens, something similar to the role played by endophytic bacteria (Strobel and Daisy 2003). The observation that all the isolated bacteria can excrete amino acids and phytohormone, and can solubilize

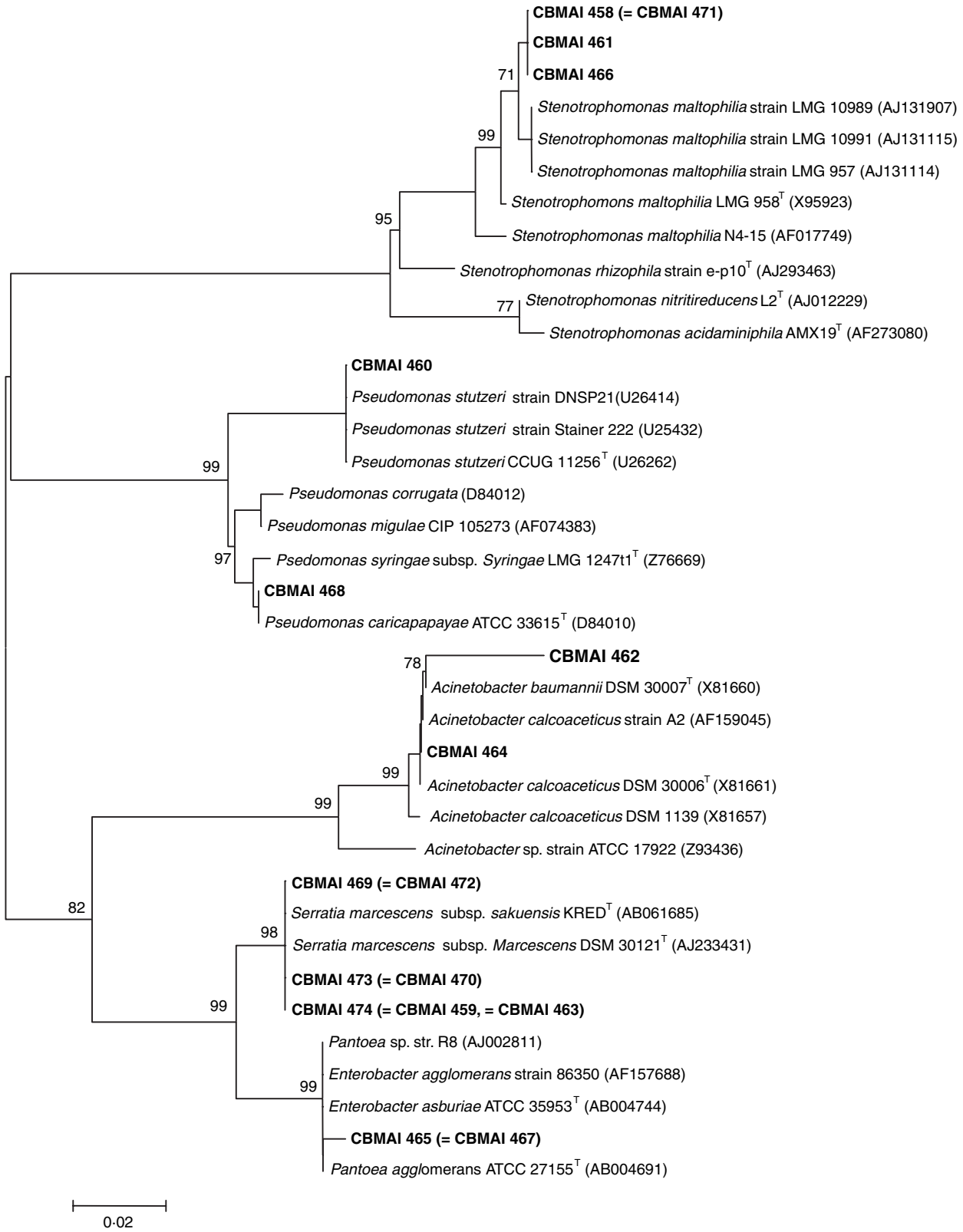

Figure 2 Phylogenetic relationship of the bacterial isolates based on the analysis of 16S rRNA gene sequences (Jukes–Cantor algorithm and Neighbour-Joining tree). Bootstrap support (1000 replicate runs) greater than 60% are indicated in branches.

phosphates, is relevant. The excreted amino acids may be absorbed by the fungi or algae that make up the lichen and incorporated into their carbon skeleton, sparing the lichen the expense of synthesizing its own amino acids. This characteristic is similar to that of endophytic bacteria, as in the case of *Azospirillum* sp. (Thuler *et al.* 2003a). The limited availability of soluble phosphates is one of the great obstacles to the colonization of nutritionally poor environments. The solubilization of phosphates by bacteria would be a powerful tool to overcome this hurdle. This type of benefit can be attributed to bacteria isolated from lichens, similarly to what *Pseudomonas* sp. and *Pantoea* sp. (Rodriguez and Fraga 1999; Verma *et al.* 2001) can do for higher plants when they are found within the plants or in their rhyzosphere. Although the influence of phytohormone on algae and fungi is unknown, they may play an important role in regulating the growth of higher plants if the phytohormone-producing bacteria are transferred from lichens into the soil or the rhyzosphere.

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