MICROBIAL ECOLOGY

Microb Ecol (2000) 39:137–144
DOI: 10.1007/s002480000008

A *Burkholderia* Strain Living Inside the Arbuscular Mycorrhizal Fungus *Gigaspora margarita* Possesses the *vacB* Gene, Which Is Involved in Host Cell Colonization by Bacteria

J.M. Ruiz-Lozano, P. Bonfante

Dipartimento di Biologia Vegetale, Università di Torino and CSMT-CNR, Viale P.A. Mattioli. 25, 10125 Torino, Italy

Received: 28 June 1999; Accepted: 8 November 1999; Online Publication: 29 March 2000

ABSTRACT

The arbuscular mycorrhizal (AM) fungus *Gigaspora margarita* harbors a resident population of endosymbiontic *Burkholderia* in its cytoplasm. Nothing is known about the acquisition of such bacteria and about the molecular bases which allow colonization of the fungus. We wondered whether the intracellular *Burkholderia* strain possesses genetic determinants involved in colonization of a eukaryotic cell. Using degenerated oligonucleotide primers for *vacB,* a gene involved in host cell colonization by pathogenic bacteria, an 842 bp DNA fragment was cloned, sequenced, and identified as a part of the *vacB* gene in *Burkholderia* sp. The insert was used as a probe to screen a fungal library that, because of the presence of intracellular *Burkholderia* cells, was also representative of the bacterial genome. The complete nucleotide sequence of *vacB* and flanking genes was determined. The bacterial origin of this genomic region was established by PCR, using specific *vacB* primers on DNA from Gigasporaceae that did or did not contain cytoplasmic *Burkholderia,* as well as on DNA from other bacteria, including free-living *Burkholderia.* We hypothesize that the *vacB* gene is part of a new genetic region acquired by a rhizospheric *Burkholderia* strain, which became able to establish a symbiotic interaction with the AM fungus *G. margarita.*

Introduction

The Glomales (Zygomycetes) are obligate endosymbionts of plants which associate with the roots of 80% of land plants, establishing the most widespread association between soil-borne fungi and plants. Mycorrhizal fungi also interact in the rhizosphere with bacteria and this create a synergism that, in addition to promoting plant growth and health, may also be significant for rhizosphere ecology [1]. Interactions between Glomales and bacteria range from apparently simple association through surface attachment, to intimate and obligatory symbiosis [23]. Bianciotto et al. [5], in fact, have demonstrated a resident population of endosymbiotic bacteria in the cytoplasm of the arbuscular mycorrhizal (AM) fungus *Gigaspora margarita* (BEG34). Ribosome DNA sequence analysis showed that they belong to the genus

* *Present address:* Departamento de Microbiología y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Prof. Albareda, 1, 18008 Granada, Spain

Correspondence to: P. Bonfante; Fax: +39 011 670 74 59; E-mail: p.bonfante@csmt.to.cnr.it

Burkholderia. Hosny et al. [18] have since used this probe to reveal similar bacterial populations in two *Scutellospora* isolates.

The presence of as many as 250,000 microbial cells in the same fungal cell [5] raises many questions not only as to their origin and acquisition, but also as to the cellular and molecular basis of their colonization. Perotto and Bonfante [23] have suggested the possibility that a rhizospheric bacterial strain once acquired the ability to actively invade the fungal cytoplasm. Colonization determinants common in pathogenic and symbiotic bacteria have been described by Galán and Collmer [10], who found that the so-called type III secretion systems are present in plant and animal pathogenic and symbiotic bacteria. Another important determinant is *vacB,* originally described in *Shigella flexneri* and enteroinvasive *Escherichia coli* (EIEC) as a chromosomal gene required for the expression of virulence genes carried on their large plasmid [35]. Altough VacB was first defined as a virulence factor, it is currently known to be an exoribonuclease RNase R involved in posttranscriptional processing of mRNAs [7]. It enables production of the Vir proteins encoded by *virG, ipa,* region-3, region-4, and region-5 operons, and so modulates the ability of bacteria to adhere and to penetrate cells, and later spread for full virulence expression [35]. Hence, since the capacity of these enteroinvasive bacteria to invade their host cells is dependent on the *vacB* gene function, the aim of the present work was to determine whether the endosymbiotic *Burkholderia* strain living in the AMF *G. margarita* possesses a similar genetic colonization determinant. Elucidation of this fact should shed further light on the possible mechanisms leading to acquisition of the bacterium by the fungus. However, these intracytoplasmic microbes are unculturable by standard culture methods [29] and defy elucidation of their structural genome and functions. Development of a genomic library from *G. margarita* which was also representative of the genome of its intracellular bacteria [37] alleviated this constraint and allowed us to start an investigation of the bacterial genome [26].

Materials and Methods

AM Fungi

Spores of *Gigaspora margarita* Becker and Hall (isolate BEG 34) and *Gigaspora rosea* Nicolson and Schenck (isolates BEG 9, INVAM 185, DAOM 194) were recovered from pot cultures of *Trifolium repens* L. by wet sieving [11]. Spores of *Gigaspora gigantea* (Nicol & Gerd.) Gerdemann & Trappe were isolated from the Compost Utilization Trial at the Rodale Institute Experimental Farm, Kutztown (Pennsylvania, USA). Finally, spores of a *Scutellospora* sp. were collected from sand dunes in Migliarino (Pisa, Italy) by V. Bianciotto. All the spores were rinsed five times with sterile, filtered, and distilled water, surface sterilized with 4% chloramine-T and 300 ppm streptomycin for 30 min, and then rinsed seven times for 1 h (total) with sterile, filtered, and distilled water [5].

Bacterial Strains and Plasmid

Escherichia coli MRA (P2) was used as host strain for the λ gt10 cloning system. *Burkholderia cepacia* MC14, *Burkholderia cepacia* MCl13, *Burkholderia cepacia* PHP7, *Burkholderia vietnamensis* TVV 75, *Agrobacterium tumefaciens* (LBA4404), *Rhizobium meliloti,* and an endosymbiotic *Nostoc* strain [30] were used in PCR with specific primers designed on the *vacB* sequence. Plasmid pGME (Promega, Madison, WI) was used to clone the PCR products obtained with degenerated primers.

Fungal/Bacterial DNA Isolation

Approximately 100 surface-sterilized spores were crushed with a plastic pestle in 300 µl of lysis buffer (50 mM Tris-HCl, pH 8; 25 mM Na-EDTA, 100 mM NaCl, 1% SDS, 0.1% Triton X-100 and 0.1% β-mercaptoethanol) and then treated with proteinase K (final concentration, 50 µg ml^{-1}) at 60°C for 1 h and with DNase-free RNase for 30 min at 37°C. Then, 0.1 vol of potassium acetate 5 M was added. The tube was left on ice for 10 min and then centrifuged for 10 min at 11,000 × *g.* The supernatant was recovered and treated with 1 vol phenol/chloroform (1/1), followed by centrifugation at 11,000 × *g* for 10 min. The new supernatant was collected and the DNA precipitated with 0.1 vol of ammonium acetate (5 M) plus 1 vol of isopropanol, followed by centrifugation for 30 min at 15,000 × *g* at 4°C. The DNA pellet was finally washed with 70% ethanol, air dried, and resuspended in 20 µl of TE buffer. Extreme care was taken to avoid any contamination. All solutions were filter sterilized, and sterile procedures were used throughout the DNA extraction period.

Cloning of the vacB Gene

Several stretches of conserved amino acids were apparent from the compilations of sequences for the VacB protein in bacteria. Two stretches were used to design degenerate oligonucleotide primers as described by Numberg et al. [22] [Fig. 1, nucleotides 1708 to 1727, 5′-TGG GTI GCI ATI GCI GA(T or C) GT-3′; and nucleotides 2530 to 2550, 5′-(A or G)TC IGG (A or G)TA IC(G or T) IC(G or T) IAT IGG-3′]. Using DNA from *G. margarita/Burkholderia* as template, an 842 bp DNA fragment was amplified with these primers and the polymerase chain reaction (PCR). PCR were carried out in a final volume of 50 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 100 pmol of each primer, and 2 units of *Taq* DNA polymerase (Sigma, St. Louis, MO, USA). A PerkinElmer/Cetus DNA Thermal Cycler (0993/8412) was

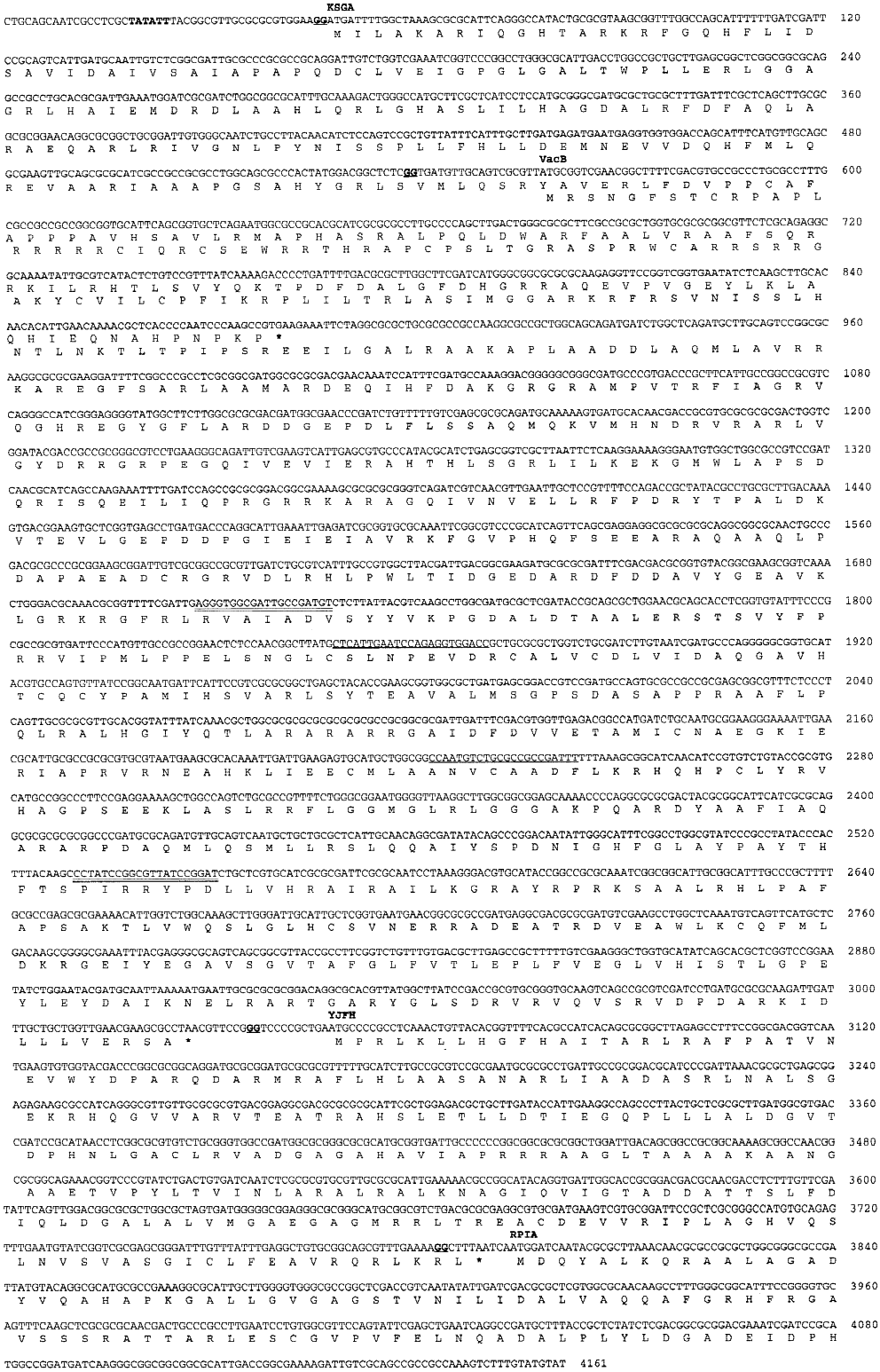

Fig. 1. Nucleotide sequence of the *vacB* region from *Burkholderia* sp. (top line). The second lines show the deduced amino acid sequences for the ORFs. An asterisk indicates stop codon. A possible promoter before *ksgA* origin is in bold. Bold, underlined GGs preceding the first amino acid of a protein may be part of a putative RBS. Sequences corresponding to degenerated PCR primers are double underlined and those from specific primers are single underlined.

employed with the following values: initial denaturation at 95°C for 5 min, followed by 35 denaturation cycles at 95°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min. The amplified DNA was purified following electrophoresis throughout a 1.2% agarose gel with the QIAEX II Gel Extraction Kit (Qiagen), then cloned into pGME plasmid (Promega, Madison, WI) and recombinant plasmids were used to transform competent *E. coil* XL1-Blue cells. Positive clones screened by PCR [14] were subcultured and plasmid DNA isolated according to Sambrook et al. [27]. Sequencing was performed by

the dideoxy-sequencing method [28] using fluorescent dye-linked universal primers (T7 and SP6) and an Applied Biosystems model 370A DNA sequencer (Genome Express Society, Grenoble, France).

Screening of the Genomic Library

A genomic library constructed for *G. margarita* and proven to be also representative of the bacterial genome [37] was used in this study. Approximately 10^4 λ bacteriophage particles were plated on the host *E. coli* MRA (strain P2). After incubation for 7 to 8 h at 37 °C, the colonies were produced and the petri plates placed at 4°C for 1 h to harden the top agarose. They were then overlaid with a Hybond-N^+ nylon membrane (Amersham, UK) and left for 2 min at room temperature, or for 4 min in the case of duplicate filters. DNA was denatured and fixed by autoclaving the filter for 5 min at 120°C. The filters were then hybridized with a probe consisting of the 842 bp DNA fragment, which was labelled using a chemiluminiscent detection system (ECL Direct DNA Labelling and Detection System, Amersham, UK). Hybridization was performed overnight at 42°C using the buffer supplied with the labelling system, and in accordance with manufacturer's instructions. The filters were then washed twice (10 min each) at 55°C with 0.5 × SSC + 0.4% SDS, and twice (5 min each) with 2 × SSC at room temperature. An X-ray film (Hyperfilm ECL, Amersham, UK) was exposed to the membrane for 3 h and developed as recommended by the manufacturer. Positive areas giving hybridization signal with the *Burkholderia* sp.-*vacB* probe were secondary and tertiary screened [27]. For that, they were recovered from the plates with an inverted yellow tip, put into 1 ml SM buffer + 20 µl chloroform, and left at 4°C and gently shaken overnight. Dilutions 10^{-3} and 10^{-4} were plated with the appropriate host bacteria and then used to obtain duplicate filters (as described above) for a new hybridization with the *Burkholderia* sp.–*vacB* probe. The positive areas obtained were recovered from the new plates with an inverted yellow tip and put into 1 ml of SM buffer + 20 µl chloroform. After incubation overnight, the dilutions 10^{-4} and 10^{-5} were used to obtain new filters for hybridization. On these filters, single positive areas could be identified and used for phage DNA extraction (Qiagen) and subsequent sequencing.

Sequencing the Clone DNA and Analyses

Sequencing was performed as described above. Sequences were analyzed with the PC/gene software (IntelliGenetics, Inc. Mountain View, CA, USA) and similarity searches were carried out in the EMBL databank, using the FASTA program from the Wisconsin Package 8 (Genetics Computer Group, WI, USA) or the BLAST software available through the National Center for Biotechnology Information (NCBI). Sequence alignments were performed using the program package Clustal W, also available through the NCBI. After sequence alignments, a phylogenetic tree was constructed using the Neighbour-Joining method from the program package ClustalX.

Design of Specific Primers, PCR, and Southern Blot

The PCR-Plan program from PC/gene software was employed to design the following specific primers on the *Burkholderia* sp.–*vacB* gene: forward, nucleotides 1848 to 1870; 5'-CTCATTGAATC-CAGAGGTGGACC-3', reverse, nucleotides 2222 to 2243; 5'-AAATCGGCGGCGCAGACATTGG-3'. Fasta analysis on these primers did not reveal any significant homology with other known sequences. PCR with these primers, with universal eukaryotic 18S rDNA primers NS1/NS2 [9], or with universal prokaryotic 16S rDNA primers 704f/1495r [21] was carried out as described by Hosny et al. [18] with annealing temperatures of 60 or 55°C, respectively. PCR products obtained with specific *vacB* primers were transferred to nylon membranes by capillarity [27], and Southern blot hybridization was carried out as previously described for the screening of the library.

Table 1. Comparison of the *Burkholderia sp.* and *E. coli* gene products

Protein	% identity	No. of amino acids in deduced protein sequences	
		Burkholderia sp.	*E. coli*
KsgA	48	277	273
VacB	41	822	813
YjfH	47	247	243
RpiA	49[a]	122*	219

[a] Partial sequence.

Nucleotide Sequence Accession Number

The nucleotide sequence shown in Fig. 1 has been deposited in the EMBL database under accession number AJ242786.

Results and Discussion

Cloning and Sequencing of the vacB *Region*

Using DNA from the *Burkholderia*-containing *G. margarita* spores as template, and the degenerated oligo primers mentioned above, an 842 bp DNA fragment of *vacB* gene was amplified by PCR and then cloned. Negative control reactions with DNA from *G. rosea* did not lead to any amplification. This suggested a possible bacterial origin of the amplified DNA fragment on the *G. margarita/Burkholderia* DNA template. The insert was sequenced, identified as a part of the *vacB* gene, and then used as a probe for the screening of the above-mentioned genomic library [37]. The complete nucleotide sequence of the *vacB* gene in *Burkholderia* sp. of *G. margarita* was obtained. Sequencing was extended to determine the flanking genes (Fig. 1). Four open reading frames (ORF) were detected in the sequenced region. All of them were identified on the basis of their amino acid sequence similarity to known *E. coli* gene products (Table 1).

ORF1 encodes a protein of 277 amino acids similar (48% identity) to Kasugamycin dimethyltransferase (KsgA) in *E. coli* [36]. The predicted molecular weight of KsgA protein is 30.6 kDa. ORF2 encodes a protein of 822 amino acids and predicted molecular weight of 91.9 kDa, similar (41% identity) to VacB in EIEC [7, 35]. The third ORF encodes a protein of 247 amino acids and predicted molecular weight of 26.1 kDa, similar (47% identity) to YjfH, a rRNA/tRNA-methyltransferase in *E. coli* [6]. Finally, the partial sequence of a protein similar (49% identity) to a ribose-5-phosphate isomerase A (RpiA) in *E. coli* [19] was detected in ORF4.

Analysis of the vacB Region

The guanine-plus-cytosine content of the sequence was 61%. A possible RNA polymerase binding sequence (TANNNT), known as Pribnow box [16, 25], can be found from nucleotides 19 to 24, in the region preceding the translational start point of *ksgA* gene (Fig. 1). In addition, several GG motifs are found in this region, and all the four genes are preceded by GG 1 to 18 nt in front of the initiating ATG codon. This nucleotide pair is thought to be part of a ribosome binding site [31]. The stop codon usage is TAA or TGA indistinctly, and there was an overlapping region of 323 nucleotides between the *ksgA* and *vacB* genes. No promoter sequence was evident immediately before the *vacB* gene or between *vacB* and *yjfH*. The only possible promoter is found upstream from *ksgA*. Also, no transcriptional terminator was seen downstream from *vacB*. If these predictions are confirmed, together with the fact that the four genes are transcribed in a common direction, this would indicate that *vacB* is part of an operon together with the adjacent genes. This possibility was previously proposed by Cheng et al. [7], who also found that, in *E. coli, vacB* was followed by *yjfH* gene, with a similar organization in the region. In fact, three of the proteins have a function in RNA processing. *ksgA* and *yjfH* genes are rRNA- and rRNA/tRNA-methyltransferases [6, 36], respectively, and *vacB* is a exoribonuclease RNase R [7] involved in rRNA methylation and mRNA posttranscriptional processing. Using PC/gene software, KsgA and VacB have been classified as integral proteins with one predicted transmembrane region each. In contrast, YjfH seems to be a peripheral protein. RPIA could not be analyzed since only a partial sequence is available.

Assessment of the Bacterial Origin of the vacB Region

We cloned the *vacB* gene described here after PCR with degenerated primers on DNA from the fungus/bacterium complex. The genomic library used for sequencing the *vacB* region also contained representatives of the two genomes [37]. Therefore, *vacB* could be derived from the genome of either of the partners. The lack of introns, characteristic of eukaryotic genes, which give discontinuous ORFs, itself suggests a bacterial origin for these genes. However, to demonstrate that the *vacB* gene belongs to the intracellular *Burkholderia* genome, specific primers were designed on the basis of the nucleotide sequence of the *Burkholderia* sp.–*vacB* gene. When these primers were used in PCR (Fig. 2), they successfully amplified the expected fragment from DNA extracted from *G. margarita* spores containing the intracellular *Burkholderia* and from a *Scutellospora* sp. *Scutellospora* is a member of the Gigasporaceae, and the isolate used possesses similar intracellular *Burkholderia* to *G. margarita* (Bianciotto et al., in preparation). In contrast, no amplification occurred on DNA from three different isolates of the related species *G. rosea* or from *Gigaspora gigantea* [3], which are devoid of intracellular bacteria. Positive reactions carried out with the same DNA and primers NS1/NS2 [9] successfully amplified on DNA from all the fungal species tested (data not shown).

PCR with the same *vacB*-specific primers was also performed using DNA of different free-living *Burkholderia* species and from other bacteria able to invade eukaryotic cells during their life cycle. As shown in Fig. 2, two out of three *B. cepacia* isolates led to a successful amplification of the expected DNA fragment. No amplification occurred with *B. vietnamensis,* with *B. cepacia* PHP7, or with any of the other isolates. This lack of amplification may be due to the absence of such a gene in their genome or to a higher degree of genetic divergence with respect to *Burkholderia* sp. from *G. margarita* than those of the two *B. cepacia* species that amplified. Positive reactions were carried out with the same DNA template and universal ribosomic prokaryotic primers 704f/1495r [21], and all the species tested successfully amplified the expected DNA fragment (data not shown).

Southern blot hybridization of the PCR products obtained with the *vacB*-specific primers stated above, using the 842 bp *Burkholderia-vacB* fragment as a probe, confirmed the identity of the amplified DNA in *G. margarita,* in *Scutellospora* sp., and also in *B. cepacia* MCl4 and *B. cepacia* MCl13 (Fig. 2). The same results were obtained by Southern blot with PCR products obtained with the degenerated primers for *vacB* (data not shown).

Phylogenetic Analysis

The nucleotide sequence of the *Burkholderia vacB* gene was compared with that of a number of bacteria. As shown in

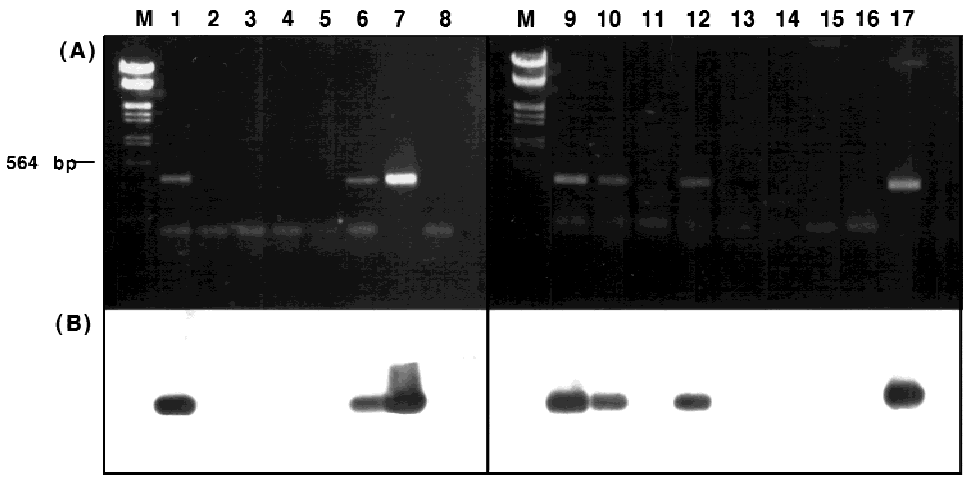

Fig. 2. (A) Electrophoresis on 1.2% agarose gel for PCR products obtained with specific primers designed on *Burkholderia* sp.–*vacB* gene. (B) Southern blot hybridization using the 842 bp *Burkholderia* sp–*vacB* insert as a probe. M, λ marker; lane 1, *G. margarita/Burkholderia;* lane 2, *G. rosea* (BEG 9); lane 3, *G. rosea* (INVAM 185); lane 4, *G. rosea* (DAOM 194); lane 5, *G. gigantea;* lane 6, *Scutellospora* sp./*Burkholderia;* lane 7, plasmid containing *vacB* gene; lane 8, no-DNA control; lane 9, *G. margarita/Burkholderia;* lane 10, *Burkholderia cepacia* CMI4; lane 11, *Burkholderia cepacia* PHP7; lane 12, *Burkholderia cepacia* MCI13; lane 13, *Burkholderia vietnamensis* TVV75; lane 14, *Agrobacterium tumefaciens;* lane 15, *Rhizobium meliloti;* lane 16, *Nostoc* 1–26; lane 17, plasmid containing *vacB* gene; lane 18, no-DNA control.

Fig. 3, *Burkholderia* sp. is clustered with bootstrap values of 100 in a group comprising *Haemophilus influenzae, E. coli,* and *S. flexneri.* The *vacB* gene seems to be widespread not only in enteroinvasive bacteria, but also in pathogens from the genus *Streptococcus, Helicobacter, Chlamydia,* or *Mycoplasma,* which have different target cells. However, two clusters can be distinguished in the tree. *Chlamydia, Mycoplasma,* and *Helicobacter* seem to have a high divergence degree with respect to the group comprising *Burkholderia* sp., as shown by the tracheotomy in the tree.

From an evolutionary point of view, it is of interest to understand how *G. margarita* acquired its cytoplasmic bacterial population. It has been proposed recently that *Burkholderia* spp. are versatile bacteria which can behave as opportunistic pathogens and invade eukaryotic cells [17, 20, 33, 34]. Bacterial cells are continuously undergoing genetic alterations that enable them to face various environmental conditions and colonize new ecological niches. These processes are a key step in their evolution and can convert environmental strains to strains able to form close associations with eukaryotic hosts [15, 32]. Selective pressure then decides whether a specific feature is maintained in the population [24]. The impact of horizontal gene transfer on microbial evolution has become apparent from recent molecular analyses [2, 8, 13]. For example, the SPI-1 pathogenicity island was probably acquired very early in the evolution of *Salmonella* and enabled that genus to invade epithelial cells [2]. In the same way, Sullivan and Ronson [32] reported the evolution of nonsymbiotic rhizobia to symbiotic strains by acquisition of a symbiotic island. They also proposed that this island should contain both genes necessary for its specific function (N_2 fixation), and others contributing to the success of the host–microbe interaction.

We hypothesize that the *vacB* region described here is part of a genetic region acquired by a rhizospheric *Burkholderia* strain, which, together with other genes, enabled this strain to establish a symbiotic interaction with the AM fungus *G. margarita.* The *vacB* gene could fall into the abovementioned category of genes which contribute to the success of the host–microbe interaction [32], being involved in the initial colonization of the fungal cytoplasm.

Interestingly, specific primers for *vacB* also amplified the expected DNA fragment in two free-living *B. cepacia* strains, but not in *B. vietnamensis.* Although all the *Burkholderia* strains used were isolated from the rhizosphere of maize [4, 33], members of the *B. cepacia* branch have been described as opportunistic pathogens that can colonize epithelial eukaryotic cells [17, 20, 33, 34]. In contrast, *B. vietnamensis* is a rhizospheric bacterium [12], so far not related with pathogenicity. These results also support our hypothesis of a pos-

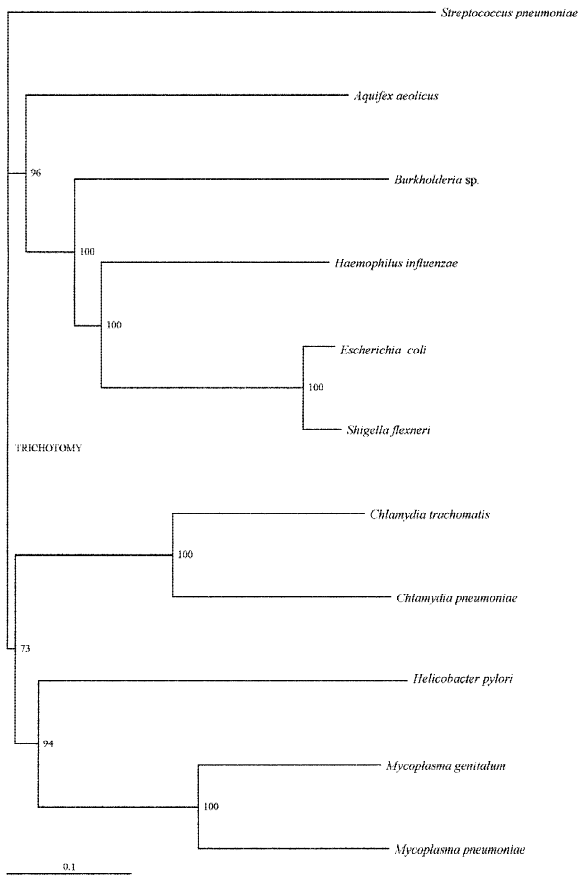

Fig. 3. Phylogenetic tree constructed after alignment of *vacB* sequences from *Burkholderia* sp. and other bacteria. Scale represents estimated number of nucleotide substitutions per sequence position. Accession numbers of sequences used to construct the tree are as follows: *Aquifex aeolicus* AE000769; *Burkholderia* sp. AJ242786; *Chlamydia trachomatis* AE001313; *Chlamydia pneumoniae* AE001635; *Escherichia coli* AE000490; *Haemophilus influenzae* U32767; *Helicobacter pylori* AE000630; *Mycoplasma genitalum* U39691; *Mycoplasma pneumoniae* AE000057; *Shigella flexneri* D11024; and *Streptococcus pneumoniae* AF052209.

sible role of the *vacB* gene described in *Burkholderia* sp. in the initial bacterial colonization of the fungal cytoplasm. Then, the presence of other genes—i.e., *nif* genes reported by Minerdi et al. (in preparation)—in the bacterial genome could have accounted for the establishment of the symbiotic association between the bacteria and the fungus.

Acknowledgments

This work was supported by an EU research training grant to J. M. Ruiz-Lozano (Contract BIO4-CT97-5118), by an EU Biotechnology Project (IMPACT; Contract BIO4-CT96-0027), and by CNR. We thank Dr. A. Bevivino (ENEA-Roma, Italy) and Dr. A. Schuessler (Darmstadt, Germany) for kindly providing us with the free-living *Burkholderia* isolates and the endosymbiotic *Nostoc* strain, respectively.

References

1. Barea JM (1997) Mycorrhiza-bacteria interactions on plant growth promotion. In: Ogoshi A, Kobayashi K, Homma Y, Kodama F, Kondo N, Akino S, (eds) Plant Growth Promoting Rhizobacteria. OECD Press, Paris, pp 150–158
2. Bäumler AJ (1997) The record of horizontal gene transfer in *Salmonella.* Trends Microbiol 5:318–322
3. Bentivenga SP, Morton JB (1995) A monograph of the genus *Gigaspora,* incorporating developmental patterns of morphological characters. Mycologia 87:719–731
4. Bevivino A, S , Sarrocco Damastri C, Tabacchioni S, Cantale C, Chiarini L (1998) Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia:* Effect of seed treatment on diseases suppression and growth promotion of maize. FEMS Microbiol Ecol 27:225–237
5. Bianciotto V, Bandi C, Minerdi D, Sironi M, Ticky HV, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010
6. Burland V, Plunkett G, Sofia HJ, Daniels DL, Blattner FR (1995) Analysis of the *Escherichia coli* genome VI: DNA sequence of the region from 92.8 through 100 minutes. Nucleic Acids Res 23:2105–2119
7. Cheng ZF, Zuo Y, Li Z, Rudd KE, Deutscher M (1998) The *vacB* gene required for virulence in *Shigela flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. J Biol Chem 273:14077–14080
8. Covacci A, Falkow S, Berg DE, Rappuoli R (1997) Did the inheritance of a pathogenicity island modify the virulence of *Helicobacter pylori*? Trends Microbiol 5:205–208
9. Dams E, Hendriks L, Van de Peer Y, Neefs J-M, Smits G, Vandenbempt I, De Wachter R (1988) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 16:87–173
10. Galán JE, Collmer A (1999) Type III secretion machines: Bacterial devices for protein delivery into host cells. Science 284:1322–1328
11. Gerdemann JW (1963) Spores of mycorrhizal Endogone species extracted from soil by wet-sieving and decanting. Trans Br Mycol Soc 46:235–244
12. Gillis M, Tran Van V, Bardin R, Goor M, Hebbar P, Willems A, Segers P, Kersters K, Heulin T, Fernández M (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamensis* sp. nov. from N_2-fixing isolates from rice in Vietnam. Int J Syst Bacteriol 45:274–289

13. Groisman EA, Ochman H (1997) How *Salmonella* became a pathogen. Trends Microbiol 5:343–349
14. Güssov D, Clackson T (1989) Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucleic Acids Res 17:4000
15. Hacker J, Blum-Oehler G, Mühldorfer I, Tschäpe H (1997) Pathogenicity islands of virulent bacteria: Structure, function and impact on microbial evolution. Mol Microbiol 23:1089–1097
16. Hawley DK, McClure WR (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res 11:2237–2255
17. Holmes B (1986) The identification of *Pseudomonas cepacia* and its occurrence in clinical material. J Appl Bacteriol 61:299–314
18. Hosny M, van Tuinen D, Jacquin F, Füller P, Zhao B, Gianinazzi-Pearson V, Franken P (1999) Arbuscular mycorrhizal fungi and bacteria: How to construct prokaryotic DNA-free genomic libraries from the Glomales. FEMS Microbiol Lett 170:425–430
19. Hove-Jensen B, Maigaard M (1993) *Escherichia coli rpiA* gene encoding ribose phosphate isomerase A. J Bacteriol 175:5628–5635
20. Jones AL, DeShazer D, Woods DE (1997) Identification and characterization of the two-component regulatory system involved in invasion of eukaryotic cells and heavy-metal resistance in *Burkholderia pseudomallei.* Infect Immun 65:4972–4977
21. Lane DJ (1991) 16S/23S rRNA sequncing. In: Stackebrandt E, Goodfellow M (eds) Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New York, pp 115–147
22. Numberg JH, Wright DK, Cole GE, Petrovskis EA, Post LE, Compton T, Gilbert JH (1989) Identification of the thymidine kinase gene of feline herpesvirus: Use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. J Virol 63:3240–3249
23. Perotto S, Bonfante P (1997) Bacterial associations with mycorrhizal fungi: Close and distant friends in the rhizosphere. Trends Microbiol 5:496–501
24. Römling U, Schmidt KD, Tümmler B (1997) Large genome rearrangements discovered by the detailed analysis of 21 *Pseudomonas aeruginosa* clone C isolates found in environment and disease habitats. J Mol Biol. 271:386–404
25. Rosenberg M, Court D (1979) Regulatory sequences involved in the promotion and termination or RNA transcription. Annu Rev Genet 13:319–353
26. Ruiz-Lozano JM, Bonfante P (1999) Identification of a P-transporter operon in the genome of a *Burkholderia* strain living inside the arbuscular mycorrhizal fungus *Gigaspora margarita.* J Bacteriol 181:4106–4109
27. Sambrook J, Fritsch EF, Maniatis TA (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. F, Sanger Nicklen S, Coulsen AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
29. Scannerini S, Bonfante P (1991) Bacteria and bacteria-like objects in endomycorrhizal fungi (Glomaceae). In: Margulis L, Fester R (eds) Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis. MIT Press, Cambridge, MA, pp 273–287
30. Schuessler A, Mollenhauer D, Schnepf E, Kluge M (1994) *Geosiphon pyriforme,* an endosymbiotic association of fungus and cyanobacteria: The spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot Acta 107:36–45
31. Stormo GD, Schneider TD, Gold LM (1982) Characterization of translational initiation sites in *E. coli.* Nucleic Acids Res 10:2971–2996
32. Sullivan JT, Ronson CW (1998) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a *phe-tRNA* gene. Proc Natl Acad Sci USA 95:5145–5149
33. Tabacchioni S, Visca P, Chiarini L, Bevivino A, Di Serio C, Fancelli S, Fani R (1995) Molecular characterization of rhizosphere and clinical isolates of *Burkholderia cepacia.* Res Microbiol 146:531–542
34. Tipper J, Ingham E, Cove JH, Todd N, Kerr KG (1998) Survival and multiplication of *Burkholderia cepacia* within respiratory epithelial cells. Clin Microbiol Infec 4:450–459
35. Tobe T, Sasakawa C, Okada N, Honma Y, Yoshikawa M (1992) *vacB,* a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri.* J Bacteriol 174:6359–6367
36. van Buul CP, Knippenberg PH (1985) Nucleotide sequence of the *ksgA* gene of *Escherichia coli:* Comparison of methyltransferases affecting dimethylation of adenosine in ribosomal RNA. Gene 38:65–72
37. van Buuren ML, Lanfranco L, Longato S, Minerdi D, Harrison MJ, Bonfante P (1999) Construction and characterization of genomic libraries of two endomycorrhizal fungi: *Glomus versiforme* and *Gigaspora margarita.* Mycol Res 103:955–960