

Selection of nitrogen-fixing deficient *Burkholderia vietnamiensis* strains by cystic fibrosis patients: involvement of *nif* gene deletions and auxotrophic mutations

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Summary

Burkholderia vietnamiensis* is the third most prevalent species of the *Burkholderia cepacia* complex (Bcc) found in cystic fibrosis (CF) patients. Its ability at fixing nitrogen makes it one of the main Bcc species showing strong filiations with environmental reservoirs. In this study, 83% (29 over 35) of the *B. vietnamiensis* CF isolates and 100% of the environmental ones (over 29) were found expressing the dinitrogenase complex (encoded by the *nif* cluster) which is essential in N₂ fixation. Among the deficient strains, two were found growing with ammonium chloride suggesting that they were defective in N₂ fixation, and four with amino acids supplements suggesting that they were harbouring auxotrophic mutations. To get insights about the genetic events that led to the emergence of the N₂-fixing defective strains, a genetic analysis of *B. vietnamiensis* nitrogen-fixing property was undertaken. A 40-kb-long *nif* cluster and *nif

regulatory genes were identified within the *B. vietnamiensis* strain G4 genome sequence, and analysed. Transposon mutagenesis and *nifH* genetic marker exchanges showed the *nif* cluster and several other genes like *gltB* (encoding a subunit of the glutamate synthase) to play a key role in *B. vietnamiensis* ability at growing in nitrogen-free media. *nif* cluster DNA probings of restricted genomic DNA blots showed a full deletion of the *nif* cluster for one of the N₂-fixing defective strain while the other one showed a genetic organization similar to the one of the G4 strain. For 17% of *B. vietnamiensis* clinical strains, CF lungs appeared to have favoured the selection of mutations or deletions leading to N₂-fixing deficiencies.

Introduction

The *Burkholderia* genus is part of the *Betaproteobacteria* and consists of more than 30 bacterial species. It includes a tight phylogenetic complex of more than 10 species which is named the *Burkholderia cepacia* complex (Bcc). Most of these Bcc species can infect cystic fibrosis (CF) patients. They can also infect immunocompromised individuals or patients with chronic granulomatous disease (for review of Bcc infections see Mahenthiralingam *et al.*, 2005). *Burkholderia vietnamiensis* is the third most prevalent species of the Bcc found among CF patients (LiPuma, 2005). The most damaging and prevalent Bcc species in CF patients are *B. cenocepacia* and *B. multivorans*. *Burkholderia vietnamiensis* was also reported in bacteraemia (Magalhães *et al.*, 2003) and a neck abscess (Gillis *et al.*, 1995). Other *Burkholderia* species like *B. gladioli* and *B. pseudomallei* were also recorded in CF patients (Bauernfeind *et al.*, 1998; O'Carroll *et al.*, 2003). *Burkholderia pseudomallei* is the aetiological agent of melioidosis (Brygoo, 1953).

Several *Burkholderia* species including those of the Bcc are well known for their potential benefits in agriculture and the chemical industries. *Burkholderia* spp. can also be nitrogen fixers, converting N₂ into NH₃. Only one species of the Bcc, *B. vietnamiensis*, is, these days, considered able to fix nitrogen (Perin *et al.*, 2006). However, the literature published prior to the definition of the Bcc

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and *B. vietnamiensis* grouped several diazotrophs into *B. cepacia* (Bevivino *et al.*, 1994), leading to some confusion regarding the distribution of the N₂-fixing property in the Bcc. Plant growth can be promoted by nitrogen fixation (e.g. Somers *et al.*, 2004).

Diazotrophs like *B. vietnamiensis* produce a nitrogenase complex that can catalyse the reductive breakage of N₂ into NH₃. The nitrogenase complex is made up of two main components; a dinitrogenase and a dinitrogenase reductase. The first one is a heterotetramer with a molybdenum–iron cofactor, and is encoded by the *nifD* and *nifK* genes. The latter is a dimer encoded by *nifH*. The reduction of one molecule of N₂ by the nitrogenase complex was shown to require at least 16 ATP (Dixon and Kahn, 2004). This is considered one of the most energy-demanding reaction. In consequence, the synthesis of the nitrogenase complex is stringently regulated. Among the diazotrophs investigated thus far, several regulatory networks involved in N₂ fixation have been described. In proteobacteria, NifA acts with the σ^{54} transcriptional factor to regulate transcription of the *nifHDK* operon (Dixon and Kahn, 2004). *nifA* expression and activity of the NifA protein are regulated by several factors that can vary from one species to another. They mainly concern nitrogen availability, redox and oxygen. The nitrogenase complex is oxygen labile (for review see Dixon and Kahn, 2004). Genes involved in nitrogen fixation are mostly found in a single genomic region, named the *nif* cluster.

The ability of *B. vietnamiensis* at fixing nitrogen makes it one of the main Bcc species showing strong filiations with environmental reservoirs. *Burkholderia vietnamiensis* can thus be considered an excellent model to address the problems of community-acquired strains and their acclimation to the CF lung environment. Studying this Bcc species could also bring light into the understanding of the selective forces that drive the evolution of these bacteria in CF lungs, and that can lead to an emergence of more fit clonal complexes. However, there is so far no evidence of any particular genetic adaptation or drift among *B. vietnamiensis* suggesting an increased fitness for CF lungs. In fact, nitrogen fixation is still considered a key character in the allocation of Bcc strains to the *B. vietnamiensis* group (Gillis *et al.*, 1995), suggesting this property to be always conserved whatever the origin of the strain. Nevertheless, at the time of this latter study, despite the fact that some clinical isolates were analysed, no CF *B. vietnamiensis* isolate was available. In this work, we postulated that if more adapted *B. vietnamiensis* CF lung isolates were emerging, nitrogen fixation should be one of the main activities affected by the genetic processes involved. Secretions and sputum from CF lungs are rich in amino acids (Barth and Pitt, 1996; Thomas *et al.*, 2000; von Bredow *et al.*, 2001), and, in consequence, the nitrogenase complex should not be

expressed and could be counter-selected. However, outside CF lungs, in soil and the rhizosphere, nitrogen sources being scarce, *B. vietnamiensis* isolates should have conserved their nitrogen-fixing ability. Here, we thus present an investigation of the nitrogen-fixing abilities of a large collection of *B. vietnamiensis* isolates coming from CF patients, non-CF clinical specimens, soil and the rhizosphere of various plants. This study shows a loss of nitrogen-fixing activity by several CF strains because of the deletion or mutation of *nif* genes or genes involved in amino acids biosynthesis. No environmental strain showed a loss of nitrogen-fixation activity. The *B. vietnamiensis nif* cluster is described for the first time in this paper.

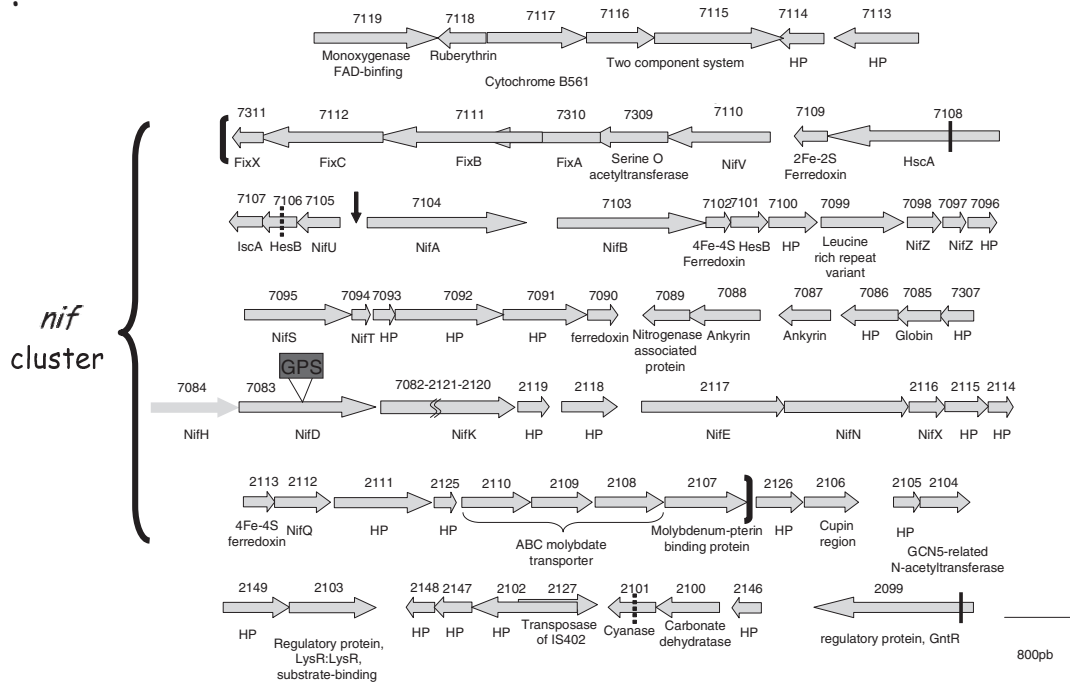
Results and discussion

The genetics of nitrogen fixation in B. vietnamiensis

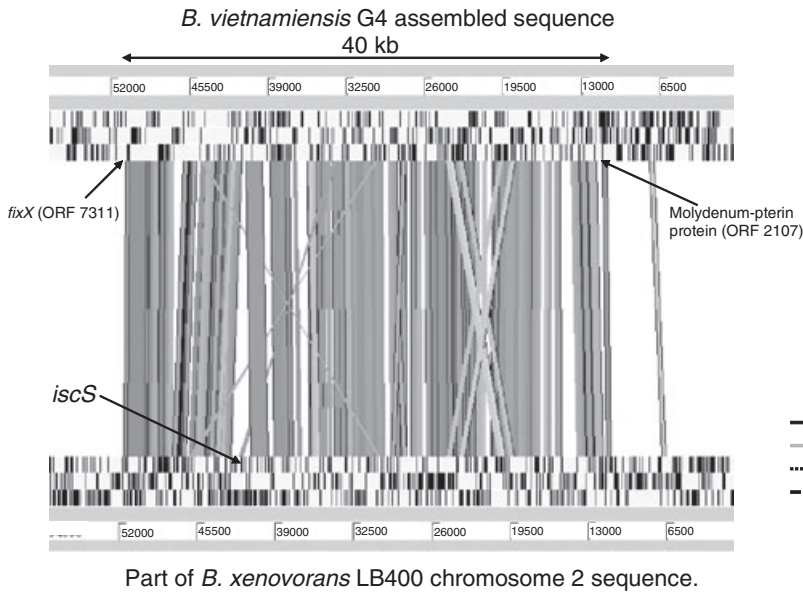
The genetics of nitrogen fixation has never been investigated in *Burkholderia vietnamiensis*. Three analyses were thus performed prior to investigate the processes involved in the emergence of nitrogen-fixing deficient *B. vietnamiensis*: (i) an annotation of the genes of the *B. vietnamiensis* G4 draft genome sequence likely to be involved in nitrogen fixation, with inferences regarding their regulation, (ii) phylogenetic and synteny analyses of the *nif* clusters among the *Burkholderia* genus, and (iii) the mutagenesis of some of the genes involved in nitrogen fixation. Figure 1A presents the annotation of the *B. vietnamiensis* G4 genetic determinants involved in nitrogen fixation. The physical organization of these genes shows similarities with the one of other nitrogen fixers. *Burkholderia vietnamiensis* G4 contains a 40 kb *nif* cluster grouping more than 50 genes involved in the synthesis of the dinitrogenase complex and its genetic regulation. This cluster shows the classical *nifHDK* and *nifENX* genetic organizations. Synteny with *B. xenovorans nif* cluster is conserved from position 1748546 to 1788938 on chromosome 2, with a single indel related to the presence of a putative *iscS* gene in the *B. xenovorans nif* cluster (Fig. 1A and B). Outside the *nifHDK* and *nifENX* organizations, synteny is poorly conserved with *nif* clusters of non-Burkholderial genomes; several rearrangements can be observed.

A *nifA* gene, encoding an enhancer binding protein, is encoded by the *B. vietnamiensis* strain G4 *nif* cluster. NifA is the main regulatory protein of the *nif* cluster, and it has a modular domain structure. *Burkholderia vietnamiensis* NifA shows three domains, GAF, a AAA+ ATPase, and a helix–turn–helix domain, which are involved in DNA binding and interaction with the σ^{54} RNA polymerase holoenzyme. Our inferences about *B. vietnamiensis* G4 genes likely playing a role in nitrogen fixation suggest that

A :



B :



C :

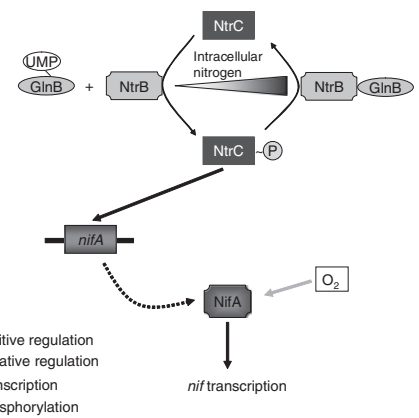


Fig. 1. Organization of the nitrogen-fixation genetic (*nif*) cluster of the *B. vietnamiensis* G4 genome. A. ORFs and their putative deduced proteins of the *B. vietnamiensis* G4 *nif* cluster and its closest flanking sequences. The ORFs sequencing project annotation numbers are indicated. The GPS insertion site in *nifD* is indicated. A black arrow indicates the position of *iscS* found in *B. xenovorans* LB400 genome but not detected in the G4 genome. Vertical bars indicate borders of the L3E cosmid, while dotted bars indicate those of the L9C cosmid. Waved bars indicate a gap in the partially assembled *B. vietnamiensis* G4 genome release (26/01/2005 version). Brackets indicate *nif* cluster boundaries. B. Graphic representation of the Artemis Comparison Tool (using TBLASTX) of the synteny between *nif* clusters of *B. vietnamiensis* G4 (from ORF 2099 to 7119) and *B. xenovorans* LB400 chromosome 2 (accession #NC_007952). C. Inferred *nif* regulation cascade in *B. vietnamiensis*.

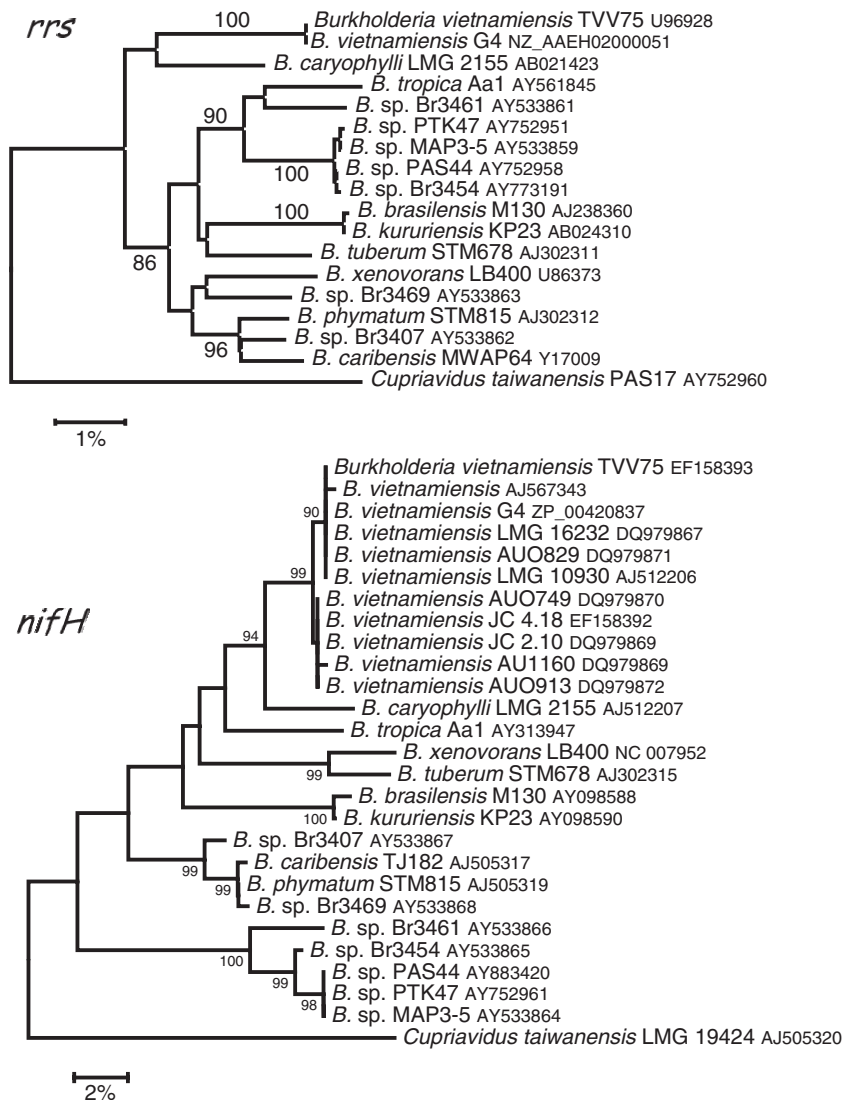


Fig. 2. Nitrogen-fixing *Burkholderia* species *rrs* (16S rDNA) and *nifH* Neighbour-Joining phylogenetic trees. The sequences were retrieved from the GenBank database or were obtained in this work. Distances are proportional to evolutionary divergences expressed in substitutions per 100 sites. Gap-containing sites were removed by pairs. Bootstrap values higher than 85% are given. Accession numbers of the sequences obtained in this work are as follow: DQ979867 (LMG 16232), DQ979868 (JC2.10), DQ979869 (AU1160), DQ979870 (AU0749), DQ979871 (AU0829) and DQ979872 (AU0913).

NifA activity would be directly responsive to oxygen, and would not involve a NifL oxygen sensor. In fact, no *nifL* encoding gene could be found in *B. vietnamiensis* G4. However, four PAS containing deduced proteins (oxygen sensors) were detected in *B. vietnamiensis* G4 including two histidine kinases (numbered ORFs 1771 and 4216) and two response regulators (numbered ORFs 5496 and 6748). One of these PAS containing proteins was found to be a NtrB orthologue. NtrB is part of a two-component system that senses the cell nitrogen status. *Burkholderia vietnamiensis* G4 NtrB is likely to play a role in *nifA* regulation of nitrogen fixation according to nitrogen availability but could also be involved in a regulatory process responding to both nitrogen and oxygen status. The gene encoding the response regulator interacting with NtrB, named *ntrC* (ORF 4215), was also found in *B. vietnamiensis* G4 genome. The *glnB* (named ORF 3159) and *glnK* (named ORF 4433) genes, producing PII

signal-transduction proteins interacting, respectively, with NtrB and AmtB (involved in nitrogen transport) in function of the nitrogen status of the cell, were also found in the *B. vietnamiensis* G4 genome. PII proteins are uridylylated in the absence of nitrogen. Uridylylated GlnB-PII proteins cannot interact with NtrB, which can then phosphorylate the NtrC response regulator that will favour *nifA* expression and nitrogen fixation (see Dixon and Kahn, 2004). The *ntrBC* and *glnBK* genes were not found at a close physical proximity of the *nif* cluster. An inferred regulatory cascade of *B. vietnamiensis* G4 *nif* transcription is shown in Fig. 1C.

A comparative phylogenetic analysis of the *nifH* nitrogenase complex structural gene with the one of the *rrs* gene encoding the 16S rRNA among the *Burkholderia* genus was performed and showed some discrepancies between the inferred phylogenetic trees (Fig. 2). Two main differences are the position of the *B. tropica* and

B. xenovorans sequences. In the *rrs* tree, the *B. tropica* sequence is closely related to a group of sequences from unspecified strains (supported by 90% of the bootstrap replicates) but, in the *nifH* tree, its sequence is more closely related to *B. vietnamiensis* and *B. caryophylli* sequences. In the *nifH* tree, the *B. xenovorans* LB400 sequence is grouping with the one of *B. tuberum* (99% of the bootstrap replicates) but, in the *rrs* tree, sequences from these two species are not closely associated. It thus appears that the *nif* cluster could be transferred laterally from one species to another. It is noteworthy that the *nif* cluster is found in about 25% of the *Burkholderia* species.

To confirm the involvement of the *nif* cluster in *B. vietnamiensis* nitrogen-fixation activity, a library of 1000 Tn-insertional mutants of strain TVV75^T was built. These insertional mutants were overlaid onto nitrogen-free medium. Eight mutants showed reduced or no growth on nitrogen-free medium. ARA (acetylene reduction activity) test showed three (named 4B8, 7E4 and 9D8) of these not to have a nitrogenase activity, and five (named 5C11, 5G1, 2E10, 1E4 and 9E5) to have a reduced activity likely due to a much decreased doubling time. The Tn-containing genomic region of each mutant showing no nitrogenase activity was rescued and cloned into *Escherichia coli* S17λpir. The Tn-insertion region was sequenced. The mutant 4B8 had an insertion in the *gltB* gene (matching position 1051 of the cds encoding the GltB of ZP_00426623) encoding the large subunit of the dimeric glutamate synthase (GOGAT) protein. This enzyme is part of the ammonium assimilation pathway involving glutamine synthase. Depending on the bacterial species considered, the assimilation of nitrogen sources by GOGAT mutants appeared variable (Bani *et al.*, 1980; Mandal and Ghosh, 1993). Here, the *B. vietnamiensis* GOGAT mutant (named 4B8) was found to grow onto nitrogen-free medium supplemented with 0.5% glutamate or 0.5% glutamine but not 5 mM NH₄Cl, confirming the role of this enzyme in the assimilation of either ammonium derived from fixed and non-fixed nitrogen sources. The second mutant (named 9D8) was found to have an insertion in a permease gene (matching position 1422 of the cds encoding the permease of ZP_00420273), and the third one (named 7E4) had an insertion in a *rrl* gene encoding the 23S rRNA (matching position 1908 of *B. vietnamiensis* G4 *rrl*). Growth of these mutants was tested on minimal medium supplemented with NH₄Cl. These two mutants could not grow with this supplement suggesting that they were more likely affected in pathways involved in amino acids biosynthesis than nitrogen fixation. No mutant containing an insertion in the *nif* cluster was obtained.

To obtain a *nif* mutant, a genetic marker exchange strategy was finally used. A *B. vietnamiensis* cosmid library was built, and two *nif*-containing cosmid clones

(named L3E and L9C) were selected. DNA sequencing of the cosmid inserts' ends was performed and allowed to position the inserts on the map of *B. vietnamiensis* G4 genome (Fig. 1A). *In vitro* Tn-mutagenesis was performed on cosmid L3E, and a *nifD* insertional mutant was selected by PCR screenings and DNA probings (see *Experimental procedures*). A 6 kb *ScaI* fragment of the mutagenized cosmid clone was subcloned into the pBluescript vector. This new construct was used to select Kn-r gene cassette exchanges into three *B. vietnamiensis* strains (LMG 6998, LMG 10823 and CL) *nifD* gene sequences. The selected mutants were shown not growing onto semisolid nitrogen-free medium and not having any nitrogenase activity detectable by ARA. The *B. vietnamiensis* strain LMG6998*nifD* mutant was used for further analysis. This mutant was found growing onto semisolid nitrogen-free medium supplemented with NH₄Cl (Table 1). These results clearly demonstrated the role of the *nif* cluster in *B. vietnamiensis* nitrogen-fixation activity. However, the data obtained through the analysis of the library of insertional mutants also showed that several other genes are essential in the assimilation of fixed nitrogen, and that several genetic events could thus explain a loss of nitrogen-fixation activity. However, the natural occurrence of nitrogen-fixation deficient strains among free-living nitrogen fixers had thus far not been documented.

Loss of nitrogen-fixation activity and nif genes by B. vietnamiensis from CF patients

A panel of 66 *B. vietnamiensis* strains was selected for this study (Table 2). This panel included 35 clinical strains from North American and French CF patients, and two strains from non-CF patients. All the other strains were environmental isolates coming mainly from the rhizosphere of plants like rice and teosinte (the wild relative of maize) but some soil and wastewater isolates were also included. The taxonomic status of all these strains was confirmed by the *recA* PCR-RFLP analysis elaborated by Mahenthalingam and colleagues (2002). The 66 strains used in this work were selected from a larger collection of strains in which some clones were found identical by *SpeI* pulsed field gel electrophoresis (PFGE) genotypings. *SpeI* PFGE analyses showed a very important genetic diversity among our panel of strains. All *SpeI* PFGE profiles showed more than seven band differences. The ARA assay was used to detect expression of the nitrogenase complex under nitrogen-free conditions. After 10 days of incubation at 28°C, four growth phenotypes on semisolid nitrogen-free medium were observed for the 66 strains tested (Fig. 3): (i) bacterial growth just below the top of the agar, (ii) growth inside the agar at a depth of more than 2 mm, (iii) diffused growth inside the agar at a depth of more than 2 mm, and (iv) no growth. Sixty out of 66

Table 1. Growth of *B. vietnamiensis* strains in semisolid defined medium.

Strain	LB (tryptone yeast extract)	Nitrogen-free medium	Nitrogen-free medium supplemented with												
			One amino acid								Pair of amino acids				
			NH ₄ ⁺	His	Tyr	Ser	Asp	Glu	Gln	Met	Ser				
											Glu	Gln	Asp		
TVV75	+	+	+	+	+	+	+	+	+	+	+	nd	nd	nd	nd
TVV75 <i>gltB::Tn5</i>	+	-	-	nd	nd	nd	nd	+	+	+	+	nd	nd	nd	nd
LMG 6998 <i>nifD::Tn7</i>	+	-	+	+	+	+	+	+	+	+	+	nd	nd	nd	nd
CH-D (Δnif)	+	-	+	+	+	+	+	+	+	+	+	nd	nd	nd	nd
AU0913	+	-	+	+	+	+	+	+	+	+	+	+	nd	nd	nd
JC 4.18	+	-	-	-	+	-	-	-	-	-	-	-	nd	nd	nd
JC 2.10	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
AU0749	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-
AU1160	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-

Nitrogen-free medium was supplemented with either 5 mM NH₄Cl or 0.5% of each amino acid (presented using the three letter code). Presence (+) or absence (-) of growth after 72 h (nitrogen-free medium) or 24 h (supplemented nitrogen-free medium or LB medium). nd, not determined.

Table 2. *B. vietnamiensis* strains used in this study, and their acetylene reduction activity (ARA).

Strains names	Origin	Species	<i>nifH</i> PCR	ARA	<i>nifH</i> probing
AU0109 ^a , AU0319 ^a , AU1085 ^a , AU1102 ^a , AU1124 ^a , AU1150 ^a , AU1152 ^a , AU1344 ^a , AU1402 ^a , AU1431 ^a , AU1453 ^a , AU1506 ^a , AU1546 ^a , AU1627 ^a , AU1674 ^a , AU1707 ^a , NAP ^e	CF patient	<i>B. vietnamiensis</i>	nd	+	nd
AU0829 ^a , CL ^e , DEG-AUR10.7 ^a , 8. 10 ^d , LMG 16233, LMG 16228, LMG 16230, DM 1.44 ^d , DM 2.51 ^d , DM 3.10 ^d , LMG 16234, MG 16232	CF patient	<i>B. vietnamiensis</i>	+	+	+
LMG 6998, LMG 6999	Blood, abscess	<i>B. vietnamiensis</i>	nd	+	nd
MMI-344 ^b , CCE-201 ^b , TTe-1912 ^c , LMG 10824, MMI-1577 ^b , CCE-115 ^b , TTe-2114 ^c , MTe-7351 ^c , MTe-73513 ^c , MTe-7381 ^c , TTe-1810 ^c	Soil, rhizosphere	<i>B. vietnamiensis</i>	nd	+	nd
CCE-101 ^b , MMI-353 ^b , MMI-1547 ^b , CCE-312 ^b , MMI-1486 ^b , TTe-184 ^c , MTe-7362 ^c , LMG 10928, LMG 11349, LMG 11347, LMG 10932, TVV75 ^f (LMG 10929 ^f)	Soil, rhizosphere	<i>B. vietnamiensis</i>	+	+	+
CCE-21 ^b , CCE-303 ^b , TTe- 1812 ^c , LMG 10823	Soil, rhizosphere	<i>B. vietnamiensis</i>	+	+	nd
ATCC 29424, ATCC 53617(G4)	Soil, waste water	<i>B. vietnamiensis</i>	+	+	+
AU0749 ^a , AU0913 ^a , AU1160 ^a	CF patient	<i>B. vietnamiensis</i>	+	-	+
JC 2.10 ^d	CF patient	<i>B. vietnamiensis</i>	+	-	+
JC 4.18 ^d	CF patient	<i>B. vietnamiensis</i>	+	-	+
CH-D ^e	CF patient	<i>B. vietnamiensis</i>	-	-	-
AU1458 ^a , JC 7.79 ^d	CF patient	<i>B. multivorans</i>	-	-	-
ATCC 17616	Polluted soil	<i>B. multivorans</i>	-	-	-
ATCC 17759	Soil	<i>B. cepacia</i>	-	-	-
DM 4.20 ^d	CF patient	<i>B. stabilis</i>	-	-	-

Results of the *nifH* DNA probings and PCR screenings are indicated.

Strains were from:

- Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan.
- Estrada-de Los Santos and colleagues (2001).
- J. Caballero-Mellado collection.
- French Observatoire cepacia.
- E.Mahenthalingam collection.

Strains coming from a same patient are indicated by black circles and squares. Strain JC 7.79 (black circle) was obtained after lung transplantation. Species determination was performed according to Mahenthalingam and colleagues (2002). *nifH* PCR, ARA and *nifH* probings were as described in *Experimental procedures*. Strains with the same pattern are grouped. ARA⁻ strains are boxed. nd, not determined.

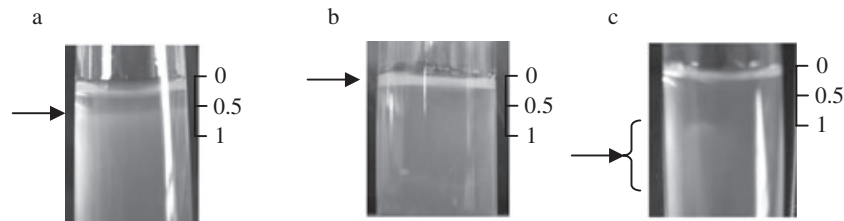


Fig. 3. Growth patterns of *B. vietnamiensis* strains in nitrogen-free semisolid medium.

A. > 2 mm depth growing strains, AU0319, AU1085, AU1102, AU1124, AU1152, AU1402, AU1453, AU1674, MMI-353, MMI-1486, MMI-344, MTe-7351, MTe-73513, MTe-7362, ATCC 29424, NAP, LMG 16230, DM 2.51, DM 3.10, LMG 16234.
 B. Subsurface growing strains, AU1150, AU1344, AU1431, AU1506, AU1627, AU1707, CCE-101, MMI-1547, CCE-201, CCE-211, CCE-303, CCE-312, TTe-184, TTe- 1810, TTe-2114, LMG 10929, CL, DEG-AUR10.7, 8.10, LMG 6998, LMG 6999, LMG 10823, LMG 10824, LMG 16228, LMG 16232, LMG 11349, LMG 11347, LMG 10932, LMG 10928.
 C. Delayed diffuse growth: AU0109, AU0829, AU1546, ATCC 53617, DM 1.44, LMG 16233. The following strains showed no growth, CH-D, AU0749, AU0913, AU1160, JC 2.10, JC 4.18. Scale is indicated in cm.

strains growing in this semisolid nitrogen-free medium were shown to express the nitrogenase complex by the ARA assay (Table 2). Six strains (AU0749, AU0913, AU1160, JC 2.10, JC 4.18 and CH-D) recovered from CF patients were unable to fix nitrogen (Table 2). The proportion of nitrogen fixers among CF and environmental strains was found significantly different ($P < 0.05$). Growth differences in the semisolid nitrogen-free medium are likely due to differences in protection of the nitrogenase complex against oxygen. A *nifH* PCR was used to verify the presence of the *nif* cluster among most of these strains (Table 2). All strains tested that were growing under nitrogen-free conditions were found to harbour a *nifH* sequence. A single strain, *B. vietnamiensis* CH-D, out of the six that were unable to grow under nitrogen-free conditions, did not yield a *nifH* gene PCR product. This datum was further confirmed by *nifH* DNA probing of a *B. vietnamiensis* CH-D DNA blot (Table 2; Fig. 4).

The six *B. vietnamiensis* strains showing no growth onto semisolid nitrogen-free medium were supplemented with various nitrogen sources such as NH_4Cl and amino acids (Table 1). These supplements were defined according to Barth and Pitt (1995) who previously observed auxotrophies among a collection of Bcc strains from undefined species. These tests allowed to identify two natural nitrogen-fixing deficient (CH-D and AU0913) mutants which could grow on any of the nitrogenous supplements used. These strains had a behaviour very similar to the ones of the *nif* mutants produced by the genetic marker exchange experiments described in the previous section (Table 1). These strains were considered natural *nif* mutants because all enzymatic transformations of NH_4Cl into amino acids appeared possible. The other four natural nitrogen-fixing deficient mutants were found to grow with supplements of specific amino acids suggesting mutations in amino acids biosynthesis or transformation pathways. One strain, JC 4.18, was found to require a tyrosine supplement, two strains,

AU0749 and AU1160, methionine supplements, and strain JC 2.10, to require a combination of amino acids i.e. serine with glutamate or glutamine or aspartate (Table 1).

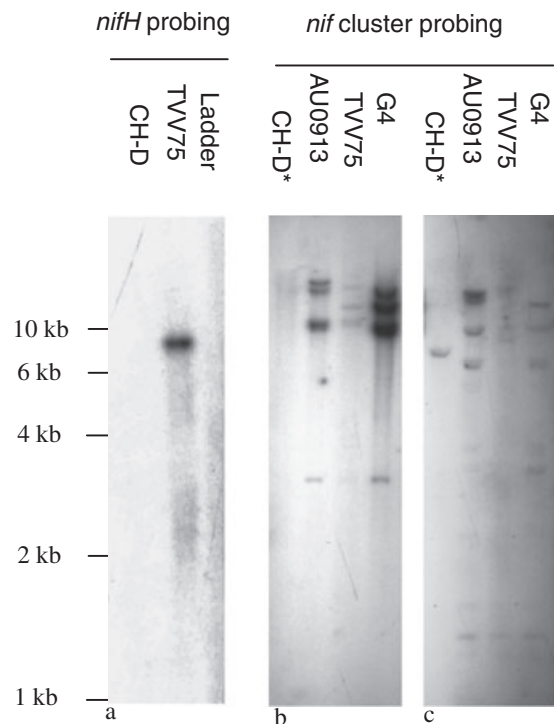


Fig. 4. *nif* probeings of restricted *B. vietnamiensis* total DNA blots. DNA probeings were performed as described in *Experimental procedures*. After agarose gel electrophoresis, restricted DNAs were transferred onto nylon membranes and hybridized with (A) ^{32}P -labelled *nifH* or (B and C) the L3E pCPP47 ^{32}P -labelled cosmid clone containing about 80% of the *nif* cluster of *B. vietnamiensis* strain TVV75 (see Fig. 1). *These hybridization patterns were the same as those obtained with ^{32}P -labelled pCPP47 without an insert. A, EcoRI-, B, BglII-, C, ClaI-restricted total DNAs. Position of some DNA fragments of the Smart Ladder is indicated.

In order to gain insights about the genetic disorders that could have played a role in the emergence of *B. vietnamiensis nif* mutants, DNA probes using the L3E cosmid (encoding about 80% of the *nif* cluster, see Fig. 1) were performed on restricted genomic DNA blots of these strains. The hybridization profiles (Fig. 4) confirmed loss of the *nif* cluster by the *B. vietnamiensis* CH-D strain, and showed strain AU0913 to have a *nif* cluster similar to the one of strain G4, with little band variations. The loss of nitrogen-fixation activity by *B. vietnamiensis* AU0913 thus remains unexplained. In the previous section, lateral transfers of *nifH* genes were suggested to might have occurred between certain *Burkholderia* species. The origin of the *nif* clusters among *B. vietnamiensis* was thus verified. *nifH* sequences were obtained for a selection of *B. vietnamiensis* strains and introduced in the *Burkholderia nifH* data set built during this work. Phylogenetic analysis showed all *B. vietnamiensis nifH* sequences to be part of a single phyletic cluster and to show very few nucleotide changes (at least 99% identity). This analysis was in line with the *recA* PCR-RFLP identification of the strains used in this work. However, the fact that *B. vietnamiensis* CH-D did not harbour any part of the *nif* cluster led us to further investigate its taxonomic status by DNA sequencing of its *recA* gene sequence. Phylogenetic analysis of this sequence confirmed the *recA* PCR RFLP analysis, and clearly clustered this sequence with *recA* sequences from well-established *B. vietnamiensis* strains, including the type strain (data not shown).

Concluding remarks

Nitrogen fixation is a complex process requiring several genes either directly involved in the synthesis of the dinitrogenase complex, its regulation or the transformation of fixed nitrogen into various essential nitrogenous molecules. NifA-, NtrB- and GlnB-driven regulatory processes are likely playing a key role in the expression of *B. vietnamiensis nif* gene cluster. This cluster could be delimited, in this work, by the use of comparative genomics approaches, and was found to be made of about 40 kb. Our data suggest that the CF lung environment can favour the loss of this genetic cluster. Other genetic disorders affecting *B. vietnamiensis* N₂-fixing ability also appeared to be selected by the CF lungs. The high amino acids content of these lungs could be the physiological parameter leading to the emergence of these genetic rearrangements, and observed *B. vietnamiensis* variants. Sputum amino acid content of CF patients was found to be high during infective exacerbations and to correlate with pulmonary disease severity (Thomas *et al.*, 2000). This parameter was previously found to increase the proportion of auxotrophs to prototrophs during infective exacerbation (Taylor *et al.*, 1993). Here, two out of six

B. vietnamiensis nitrogen-fixing deficient strains, with distinct Spel PFGE profiles, came from a same patient, and could be supplemented with tyrosine, or a mixture of two amino acids. These data suggest that at the time these strains were collected, the patient could have been undergoing infective exacerbation that would have selected these variants. This would need to be further investigated. Interestingly, N₂-fixing deficient strains recovered in this work came from three independent cohorts, suggesting that CF lungs could repeatedly lead to the emergence of such variants. Overall, these results and observations tend to suggest that CF lungs can favour the selection of genetic variants among community-acquired *B. vietnamiensis*, and that some of these variants will likely be affected in their nitrogen-fixing ability. These variants appear physiologically more dependent upon their CF host than their mother strain, and are unlikely to be highly competitive in the rhizosphere of most plants.

One of our future lines of research regarding this work will be to investigate the mechanisms involved in the emergence of these 'natural' nitrogen-fixing deficient strains selected by CF patients. IS elements could have played a role in their emergence. In fact, an IS element, IS402, was found physically close to *B. vietnamiensis* G4 *nif* cluster, and could have been the site for genetic recombination events that led to *nif* deletions.

Experimental procedures

Burkholderia vietnamiensis strains used and the ARA assay

Burkholderia vietnamiensis strains used in this study are presented in Table 2. *Burkholderia cepacia*, *B. multivorans* and *B. stabilis* strains were used as negative controls throughout this work (Table 2). These strains were grown at 30°C on Luria-Bertani's Broth (LB) (Bertani, 1951) or LB containing 1.6% agar (LA). The taxonomic affiliation of these strains was confirmed by *recA* PCR-RFLP analyses (Mahenthalingam *et al.*, 2002). A Spel PFGE analysis of the genomic content of the *B. vietnamiensis* strains was performed according to Romling and colleagues (1997). Pulsed field gel electrophoreses were run for 11 h with a pulsed field of 5–25 s, followed by pulses of 5–60 s for 13 h. Voltage was kept at 5 V cm⁻¹. PFGE profiles were compared using the Gel compare software (Applied Maths). This analysis prevented over-representation of certain genotypes in the *B. vietnamiensis* strains panel of this study. The ARA assay was performed as described by Estrada de los Santos and colleagues (2001). The nitrogen-free medium used was slightly modified by replacing azealic acid by malic acid, and by not including bromothymol blue; the pH was adjusted to 6.5. Inoculation of this medium was performed with late log phase growing cells in LB, washed twice with 0.8% NaCl. Statistical comparison of the proportion of nitrogen-fixing strains in our collection of CF and environmental strains was performed using the Z-test ($P < 0.05$) (Wassertheil-Smoller, 1995).

DNA manipulations

PCR amplifications (25 µl) were done according to the *Taq* polymerase manufacturer (Invitrogen, Cergy Pontoise, France) with 1.25 µl of DMSO. 2.5 µl of stationary phase cells was incubated for 10 min at 95°C and used directly in the PCRs as the source of DNA matrices. *nifH* PCR primers were as follow: NifH1: GGCAARTCSACCACGTGC; NifH2: TGCTGNACGTGTTGTCGC. *recA* PCR primers are presented in Mahenthiralingam and colleagues (2002). PCR cycles were as follow: (i) 95°C for 5 min, (ii) 95°C for 45 s, 57°C for 45 s, 72°C for 45 s (35 cycles), (iii) 72°C for 5 min. PCR products were sequenced by Genome Express (Meylan, France).

For DNA blot analyses, genomic DNAs were extracted according to Pitcher and colleagues (1989) and resuspended in 300 µl of water. These DNAs (5 µg) were digested by 1 U µg⁻¹ *Clal* or *BglII* (Fermentas, St Leon-Rot, Germany) for 16 h at 37°C. DNA blots were performed using Genescreen Plus (PerkinElmer Life Science, Boston, USA) nylon membranes and the procedure described by Sambrook and colleagues (1989). *nifH* PCR products and L3E cosmid were labelled using the random priming labelling kit (Amersham-Pharmacia, Orsay, France). DNA hybridizations and nylon membrane washes were carried out according to the membrane manufacturer. Membranes were hybridized and washed at 65°C. Autoradiography was performed according to Sambrook and colleagues (1989).

Transposon mutagenesis

Burkholderia vietnamiensis mutagenesis of TVV75 was performed using a transposome conferring resistance to kanamycin (Epicentre, UK) and introduced by electroporation. Electrocompetent *B. vietnamiensis* cells were obtained as follow: 250 ml of LB broths of late-exponential phase growing cells was centrifuged for 15 min at 2600 RCF and subsequently washed three times with 30 ml ice cold 0.5 M sucrose. Cells were resuspended in 100 µl 0.5 M sucrose and kept on ice until use. Electroporation was performed using the Bio-Rad Gene Pulser II set at 50 µF, 800 Ω, 2.5 kV 1 µl of transposome was mixed with 100 µl of electrocompetent cells. These preparations were then introduced into cold 0.2 cm electroporation cuvettes and were electroporated. After electroporation, cells were incubated 1 h in LB broth, at 37°C, and were then plated on LA containing 200 µg ml⁻¹ kanamycin. Transposon rescue was performed according to manufacturer and using *E. coli* S17λpir. Sequencing of the rescued plasmid was performed by Genome Express (Meylan, France).

Genetic marker exchange

Burkholderia vietnamiensis LMG 6998, LMG 10823 and CL were electroporated with plasmid pAM20 encoding part of the *nif* cluster and harbouring a Tn7 insertion in *nifD*. Transformants were plated on LA supplemented with 100 µg ml⁻¹ of kanamycin, and incubated 2 days at 30°C. Growing bacterial colonies were tested for nitrogen fixation (ARA assay) to verify inactivation of the targeted *nif* gene. Plasmid pAM20 was obtained as follow: a *B. vietnamiensis* TVV75 genomic

DNA library was built using the pCPP47 cosmid vector (Bauer and Collmer, 1997) in *E. coli* XL1 Blue MR. Among the gene library, a *nif* harbouring cosmid clone, named pAM18, was detected by *nifH* gene PCR amplification. pAM18 insertional mutagenesis was performed using the GPS system (Biolabs, Ozyme, Saint Quentin en Yvelines, France). pAM18 insertional mutants were electroporated into *E. coli* 10G Elite (from Lucigen) and plated on LA supplemented with 50 µg µl⁻¹ kanamycin. Two hundred clones were retrieved. Pools of 12 clones were grown in LB supplemented with 50 µg µl⁻¹ kanamycin, and their plasmid content was extracted and digested with *Scal*. Pools showing a band shift from pAM18 were further investigated. Individual cosmid clones from each positive pool were extracted, digested with *Scal*, analysed by electrophoresis, and transferred onto nylon membranes. *nifH* and GPS radioactive probes (labelled with Fermentas Hexa-Label DNA labelling kit) were hybridized on these cosmid blots. Cosmid clones showing hybridization signals of the same size were sequenced using a GPS Tn primer. pAM19 showed a GPS Tn insertion in *nifD*. pAM19 was digested with *Scal* and the 6 kb fragment containing *nifD*-GPS was subcloned into the *EcoRV* site of pBluescript SKII (Invitrogen, Cergy Pontoise, France), leading to pAM20. pAM20 was analysed by *EcoRI*/*HindIII* digestions because the GPS Tn contains a unique *HindIII* site.

Molecular phylogenetic analyses

DNA sequences obtained in this work (*nifH*) or from GenBank (*nifH* and *rrs*) were aligned using CLUSTALW (Thompson *et al.*, 1994). Evolutionary distances between sequence pairs, the inferred phylogenetic trees, and bootstrap values were all computed through the Mega3 graphic tool (Kumar *et al.*, 2004). Phylogenetic trees were built using the Neighbour-Joining method (Saitou and Nei, 1987). Bootstrap replicates were performed using 1000 replicates. Gap containing sites were not included in the analyses.

The synteny analysis was performed using the Artemis Comparison Tool (Carver *et al.*, 2005), configured with TBLASTX input files (executable retrieved from the NCBI web site). Default per cent identity cut-offs were kept.

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