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ORIGINAL ARTICLE

Phase variation has a role in *Burkholderia ambifaria* niche adaptation

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Members of the *Burkholderia cepacia* complex (Bcc), such as *B. ambifaria*, are effective biocontrol strains, for instance, as plant growth-promoting bacteria; however, Bcc isolates can also cause severe respiratory infections in people suffering from cystic fibrosis (CF). No distinction is known between isolates from environmental and human origins, suggesting that the natural environment is a potential source of infectious Bcc species. While investigating the presence and role of phase variation in *B. ambifaria* HSJ1, an isolate recovered from a CF patient, we identified stable variants that arose spontaneously irrespective of the culture conditions. Phenotypic and proteomic approaches revealed that the transition from wild-type to variant types affects the expression of several putative virulence factors. By using four different infection models (*Drosophila melanogaster*, *Galleria mellonella*, macrophages and *Dictyostelium discoideum*), we showed that the wild-type was more virulent than the variant. It may be noted that the variant showed reduced replication in a human monocyte cell line when compared with the wild-type. On the other hand, the variant of isolate HSJ1 was more competitive in colonizing plant roots than the wild-type. Furthermore, we observed that only clinical *B. ambifaria* isolates generated phase variants, and that these variants showed the same phenotypes as observed with the HSJ1 variant. Finally, we determined that environmental *B. ambifaria* isolates showed traits that were characteristic of variants derived from clinical isolates. Our study therefore suggest that *B. ambifaria* uses phase variation to adapt to drastically different environments: the lung of patients with CF or the rhizosphere.

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Introduction

The *Burkholderia* genus comprises more than 40 species that inhabit remarkably diverse ecological niches, as they have been isolated from soil, rhizosphere, insects, fungi and infected humans. Many *Burkholderia* have developed beneficial interactions with their plant hosts and have considerable ecological significance: several species of *Burkholderia* have proven to be very efficient biocontrol and bioremediation agents (Chiarini *et al.*, 2006). This is especially true for members of the *Burkholderia cepacia* complex (Bcc), which currently comprises 17 closely related genomovars (Vanlaere *et al.*, 2009). For instance, *Burkholderia ambifaria* J82 was registered by the US Environmental Protection Agency for use as a biospecticide and was commercially used for a few years (Parke and Gurian-Sherman, 2001). However, Bcc isolates are now increasingly identified as opportunistic pathogens, notably infecting people suffering from cystic fibrosis (CF) or chronic granulomatous disease (Govan *et al.*, 2007). For this reason, their use as biological control agents is not allowed anymore (Chiarini *et al.*, 2006).

Within the Bcc, *B. ambifaria* (genomovar VII) typically illustrates the duality between isolates with biological control purposes and those that cause infections in humans (Coenye *et al.*, 2001). This dichotomy is indeed underlined by the choice of the name *ambifaria* from the latin name *ambifarius* (having two sides; ambiguous) (Coenye *et al.*, 2001). One of the most investigated biocontrol agents, the sequenced strain *B. ambifaria* AMMD (LMG19182), isolated from the rhizosphere of the pea, shows well-demonstrated antagonistic activity

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against a number of plant pathogenic fungi (Parke *et al.*, 1991). On the other hand, several *B. ambifaria* isolates have been isolated from CF patients (Coenye *et al.*, 2001). *B. ambifaria* is among the most common Bcc species in the plant rhizosphere; however, a minority of Bcc-infected CF patients harbor this genomovar (Ramette *et al.*, 2005; Reik *et al.*, 2005).

No clear distinction is known between Bcc isolates from environmental and human origins, and they seem to be clonally related. For instance, the biocontrol strain *B. ambifaria* AMMD and clinical isolate *B. ambifaria* AU0212 are clonal (Payne *et al.*, 2005). A *B. cepacia* genomovar III isolate recovered from agricultural soil in the mid-Atlantic region of the United States is indistinguishable from an isolate frequently recovered from CF patients of the same region using the fingerprinting method (LiPuma *et al.*, 2002).

Phase variation, or phenotypic switching, is an adaptive process conferring to bacteria the capacity to adapt to fluctuating environmental conditions. Phase variation implies phenotypic changes resulting from genetic or epigenetic alterations at specific genetic loci (Villemur and Déziel, 2005; Wisniewski-Dyé and Vial, 2008). In contrast to spontaneous mutations that occur at a frequency of approximately 10^{-8} mutations per growing cell per generation, phase variation occurs at frequencies higher than 10^{-5} switches per cell per generation and always affects the same phenotype(s) (van der Woude and Baumler, 2004). This phenomenon results in an increased diversity within a bacterial population and favors bacterial fitness. Phase variation has an important adaptive role in the capacity of bacteria to colonize different host anatomical niches and escape host defense mechanisms.

While investigating the presence and role of phase variation in *B. ambifaria* isolates recovered from CF patients, we identified stable, smooth-colony variants showing numerous phenotypic differences when compared with their rough-colony parent. Strikingly, we found that clinically rough isolates are more virulent than their variants, whereas these phase variants are more competitive in rhizosphere colonization. Our data suggest that, by phase variation, *B. ambifaria* adopts two different phenotypic states: the first one is better adapted to the lungs of CF patients and the second is more adapted to plant roots environment.

Materials and methods

Bacterial strains and growth conditions
The *B. ambifaria* strains used in this study are listed in Supplementary Table S1. Unless otherwise stated, strains were grown in tryptic soy broth (TSB, Difco, Detroit, MI, USA) at 37 °C with shaking (240 r.p.m.), or on TSB agar plates containing 0.1% (w/v) Congo red (CRTSB). Specific culture conditions can be found in the Supplementary data.

Phenotypic characterization
Several enzymatic activities were evaluated using API-ZYM strips (bioMerieux, Lyon, France). Siderophore, proteolytic and antifungal activities were measured as described earlier (Vial *et al.*, 2008). Hemolytic activity was assessed on TSB agar supplemented with 5% (v/v) sheep blood. TSB agar containing tributyrin 1% v/v was used to detect lipolytic activity. Cholesterol oxidase activity was assessed using TSB agar plates containing 0.34% Triton X-100 and 0.9 mM cholesterol (Sigma-Aldrich, Oakville, Ontario, Canada) (Doukyu and Aono, 2001). Specific details can be found in the Supplementary data.

The presence of elastase was detected using elastin-Congo red (Sigma) as substrate (Pearson *et al.*, 1997) and phospholipase C activity was detected as described earlier (Esselmann and Liu, 1961). The capacity of *B. ambifaria* to produce exopolysaccharides (EPSs) was determined as described by Zlosnik *et al.* (2008).

Disc diffusion assay was used to test the susceptibility of *B. ambifaria* to H_2O_2. In brief, logarithmic-phase cells (OD_{600} of 3.0) or stationary-phase cells (OD_{600} of 6.0) were spread as a lawn on TSB agar plates and disks with 10 µl of 30% H_2O_2 were applied to the surface. The plates were incubated for 16 h at 37 °C, and then the diameter of the zone of growth inhibition around each disk was measured.

Biofilm formation and abiotic adherence assay
The biofilm formation protocol was performed as before (Déziel *et al.*, 2001), with the following modification: 12×75-mm^2 polystyrene tubes containing TSB were inoculated from overnight TSB cultures and incubated at 37 °C without agitation for 24 h.

The abiotic adherence assay was performed as described by Aubert *et al.* (2008), with the following modifications: approximately 10^5 colony-forming units (CFUs) were added to polystyrene tubes containing 1 ml of NaCl 0.8% and then incubated at 37 °C for 20 min. After four washes with NaCl 0.8%, the bound bacteria were detached from the surface by adding 500 µl of 10 mM EDTA and 1.5 ml of 1% Triton X-100. The adherence of the wild-type and variant was calculated by dividing the output number by the input number. Experiments were performed in triplicate and independently repeated three times.

Sensitivity to antimicrobial peptides
For sensitivity to antimicrobial peptides, bacteria were grown on TSB agar plate for 24 h at 37 °C, and then for 24 h at room temperature. Cells (4×10^5 CFU) were resuspended in 200 µl of 0.1 × phosphate buffered saline containing various concentrations of antimicrobial peptides: 50 or 100 µg ml^{-1} melittin (Sigma), 200 µg ml^{-1} LL-37 (AnaSpec, San Jose, CA, USA) or 1000 µg ml^{-1} polymyxin B (Sigma). Bacteria were then incubated

without agitation at 37 °C for 8 h. After treatment, viable cells were enumerated on TSB agar plates. All assays were conducted in triplicate and repeated independently at least twice. The survival (%) was defined as follows: (CFU of peptide-exposed culture/CFU of nonexposed culture) × 100.

Lipopolysaccharide (LPS) analysis
LPSs were prepared for gel analysis as described earlier (Apicella *et al*., 1994), with slight modifications as outlined in the Supplementary data.

Quantifications of 4-hydroxy-2-alkylquinolines (HAQs) and N-*acyl-*L*-homoserine lactones (AHLs)*
Quantifications of HAQs and AHLs were performed as described earlier (Lépine *et al*., 2003; Vial *et al*., 2008).

Bacterial survival/proliferation and competitive index in human macrophages
Macrophage infection assays were performed using the human acute monocytic leukemia cell line THP-1 as described in Forest *et al*. (2007), with the following modifications: bacteria grown overnight at 37 °C in static conditions in TSB were added to a cell monolayer at a multiplicity of infection of 10. After incubation for 20 min at 37 °C (time 0), the infected cells were washed three times with prewarmed phosphate buffered saline. In order to kill extracellular bacteria, fresh medium containing 125 μg ml^{-1} tetracycline and 1 mg ml^{-1} ceftazidime was added, and cultures were incubated for another 2 h at 37 °C. The number of surviving bacteria (intracellular bacteria) was determined by bacterial plate counting (CFU) on CRTSB at time 0 and at 2, 4, 6, and 8 h after initial inoculation. At 8 h after initial inoculation, the final wash was plated for determining the number of viable extracellular bacteria. The level of phagocytosis was expressed as a percentage of the initial inoculum. All assays were conducted in duplicate and repeated independently at least three times.

For competitive index (CI) in macrophages, a 1:1 mixture of two bacterial strains was added to THP-1-cultured cells at a multiplicity of infection of 10 as described above. The numbers of viable intracellular bacteria were determined 2 and 8 h after infection. Infections were performed in triplicate. The CI in proliferation is defined as the ratio between the two strains in the output (intracellular bacteria recovered 8 h after infection) divided by their ratio in the input (intracellular bacteria after the 2-h treatment with antibiotics) (Segura *et al*., 2004).

Dictyostelium discoideum *plaque assay*
D. discoideum strain DH1-10 was grown in a Petri dish at 21 °C in HL-5 liquid medium containing 15 μg ml^{-1} tetracycline (Cornillon *et al*., 2000; Mercanti *et al*., 2006). Amoebae were collected by centrifugation, washed and resuspended in HL-5 at the concentration of 2×10^5 per 5 μl. Subsequent serial dilutions were carried out in HL-5 to obtain the following concentrations (per 5 μl): 1×10^5, 5×10^4, 2.5×10^4, 1.25×10^4, 6.25×10^3, 3.13×10^3, 1.56×10^3 and 7.8×10^2. *B. ambifaria* strains were grown on CRTSB for 24 h at 37 °C and then for 24 h at 25 °C. Suspensions of bacteria for each strain were plated on HL-5 agar and the bacterial lawns were spotted with 5-μl droplets of the *D. discoideum* serial dilutions. Plates were incubated at 23 °C for 24 h and examined for plaque formation. This experiment was performed four times with HSJ1 and its variant (HSJ1v), and at least two times with all other strains. Similar results were obtained each time.

Infection of Galleria mellonella *larvae*
G. mellonella killing assays were performed as described previously (Seed and Dennis, 2008). In competition experiments, larvae were injected with wild-type and variant in a 1:1 ratio (1×10^4 CFU of wild-type and variant), and the CI was evaluated 48 h after co-inoculation, just before the death of the larvae. To estimate *B. ambifaria* concentration inside the larvae, each of them was surface sterilized (by dipping in ethanol and passing the larvae through a Bunsen flame), crushed and homogenized in 1 ml 0.8% NaCl. The CI is defined as the CFU output (48 h after infection) ratio of HSJ1v in comparison with HSJ1, divided by their ratio in the input (inoculum). This experiment was repeated twice.

Rhizosphere colonization
Pisum sativum seeds were sterilized as described earlier (O'Sullivan *et al*., 2007). After 3 days, germinated seeds were sown in tubes containing Perlite as the solid substrate and 8 mM KNO_3^--supplemented Fahraeus liquid medium (Fahraeus, 1957). Bacterial isolates were inoculated in a 1:1 ratio (10^5 CFU of wild-type and variant). Plants were maintained in a controlled-condition incubator: 12 h of light at 25 °C with 65% relative humidity. Bacteria were recovered from the rhizosphere by vortexing the roots for 2 min in a tube containing 10 ml of 0.8% NaCl with glass beads and by plating on CRTSB with antibiotics (25 μg ml^{-1} gentamycin, 15 μg ml^{-1} polymyxin B, 20 μg ml^{-1} cycloheximide). Roots were sampled 2, 5, 10 and 15 days after inoculation. The CI for colonization was defined as the CFU output (day 15 after inoculation) ratio of HSJ1v in comparison with HSJ1, divided by the CFU input (inoculum) ratio of variant to wild-type.

Results

Phase variation in B. ambifaria *HSJ1*
Phase variation was observed in the *B. ambifaria* strain HSJ1 isolated at the Sainte-Justine Hospital

(Montréal, Canada) from a patient suffering from CF. When a single colony of strain HSJ1 was plated on CRTSB agar, two colonial forms developed (Figure 1). Most colonies (wild-type phenotype) were rough, often wrinkled and bound Congo red, giving them a red appearance. The other form of colony (variant phenotype) was translucent, smoother and unable to bind Congo red. To verify the stability of the two phenotypes, single colonies were individually grown overnight in TSB and plated on CRTSB. The wild-type (HSJ1) continued to produce variant colonies, whereas the variant (HSJ1v) remained stable: no reversion to the wild-type or appearance of another phenotype was detected. Variant colonies arose spontaneously at low frequencies, irrespective of the culture conditions used (that is, growth medium and carbon source). The frequency of occurrence of variant colonies was 1.37×10^{-4} ($\pm 0.85 \times 10^{-4}$) per cell per generation in the conditions tested. Identical profiles were obtained using polymerase chain reaction/restriction-fragment length polymorphism of the 16S ribosomal DNA region. This result confirms that HSJ1v is not a co-cultivated bacterium, but is instead derived from the wild-type strain HSJ1.

Pleiotropic differences of the B. ambifaria *HSJ1 variant*

The growth rates of the wild-type and of the variant were determined to be the same in TSB and in M63, with the following carbon sources: dextrose, mannitol, fructose and galactose (Supplementary Figure S1a and b and data not shown). Both types were unable to use lactose or maltose. Interestingly, inferior growth of HSJ1 was observed on saccharose, xylose, sorbitol and glycerol when compared with HSJ1v (Supplementary Figure S1).

Many physiological and biochemical characteristics of the variant HSJ1v were different from those of the wild-type HSJ1 (Table 1 and Figure 1). The variant type showed pleiotropic phenotypic losses, such as the absence of hemolytic and antifungal activities. On the other hand, HSJ1v type was frankly mucoid (+++) on yeast extract-mannitol agar, whereas HSJ1 was only partially mucoid (+) (Table 1). Furthermore, the variant produced more siderophores than the wild-type, suggesting that HSJ1v possesses a greater ability for iron scavenging than HSJ1. Indeed, superior growth of the variant was observed in a minimal medium supplemented with an iron-chelating agent (Supplementary Figure S1). Moreover, an increase in the proportion of variant was observed when iron was limiting (Supplementary Figure S2).

Secretomic studies on the two phenotypic types revealed three major secreted proteins that were absent from HSJ1v (Supplementary Figure S3). Two spots (WT1 and WT2) were identified as zinc metallopeptidases ZmpB and ZmpA (Bamb_4475 and Bamb_3836, according to the annotation of sequenced *B. ambifaria* strain AMMD) associated

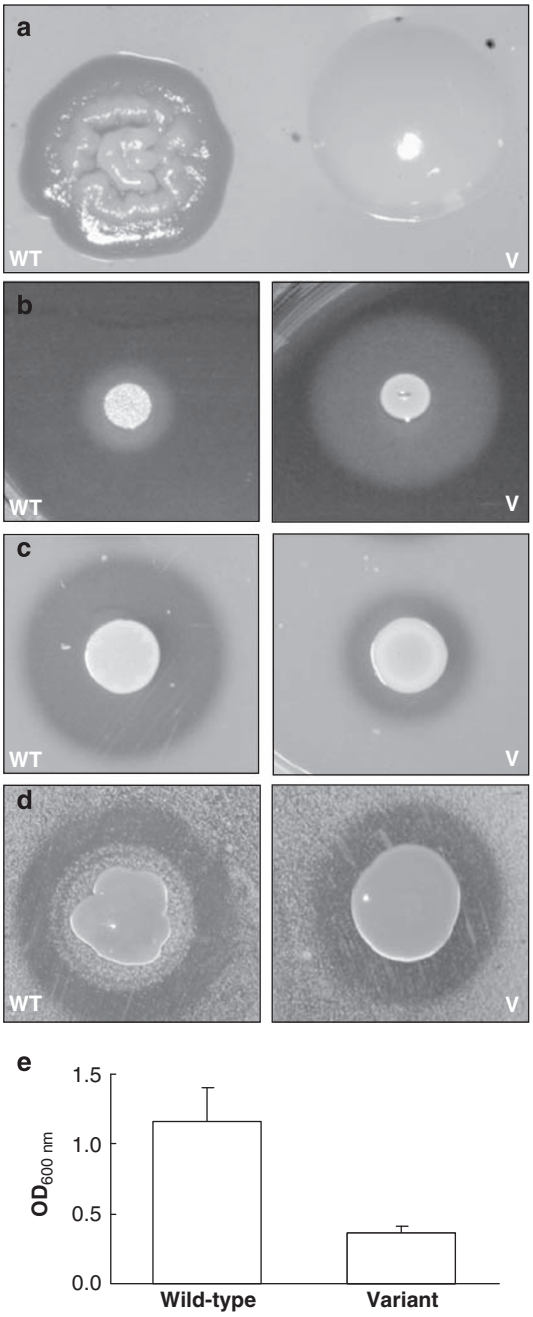

Figure 1 Phenotypic comparison between *B. ambifaria* wild-type (W) and variant (V). (**a**) Colony distinction between *B. ambifaria* HSJ1 wild-type and variant on Congo red tryptic soy broth (CRTSB) medium. Plates were incubated for 24 h at 37 °C and then kept for 72 h at room temperature. (**b**) Siderophore production on chrome azurol S (CAS) agar. The orange halo surrounding the colony indicates the release of siderophores. (**c**) Proteolytic activity on agar plate containing 1% skim milk powder. (**d**) Cholesterol oxidase activity on TSB agar plate containing 0.9 mM cholesterol. The presence of a turbid halo around the colony indicates cholesterol oxidase activity. (**e**) Biofilm assay. Biofilms developed in polystyrene tubes were stained with crystal violet and quantified by spectrophotometry (A_{600}) after solubilization of the dye in ethanol. Error bars represent the standard deviations. Abbreviations: WT, *B. ambifaria* HSJ1 wild-type; V, *B. ambifaria* HSJ1 variant. A full colour version of this figure is available at The *ISME* Journal online.

Table 1 Physiological and biochemical characteristics of *B. ambifaria* HSJ1 wild-type in comparison with its phase variant, HSJ1v

Characteristics	*Wild-type*	*Variant*
Proteolytic activity	571(±102) mm²	158(±31) mm²
Hemolytic activity	103(±21) mm²	ND
Lipase activity[a]	431(±120) mm²	377(±76) mm²
Phospholipase C activity	+	+
Antifungal activity		
Candida albicans	632(±94) mm²	ND
C. glabrata	762(±60) mm²	ND
Cryptococcus neoformans	833(±107) mm²	ND
Elastase	ND	ND
Siderophore production	199(±34) mm²	1620(±64) mm²
H_2O_2 sensitivity		
Stationary-phase cells	24.3(±0.6) mm	22.7(±0.6) mm
Log-phase cells	26(±1) mm	25.3(±1.5) mm
Congo red binding	+	−
EPS production[b]	+	+++
β-Glucosidase activity[c]	−	+

Abbreviations: EPS, exopolysaccharide; ND, not detected.
[a]Lipase activity was also tested using API-ZYM strips, and Rhodamine B-olive oil agar plates. These tests gave similar results for the wild-type and the variant.
[b]The capacity of *B. ambifaria* to elaborate EPS was determined as described by Zlosnik *et al.* (2008).
[c]Results were obtained with API-ZYM strips, and for the other 19 substrates tested, no difference between the strains was observed.

with the protease activity of *B. cenocepacia* (Kooi *et al.*, 2006). Accordingly, HSJ1v showed significantly less proteolytic activity on skim milk than the wild-type (Table 1 and Figure 1c). Spot WT3 was identified as an FAD-dependent cholesterol oxidase (Bamb_6465) (Doukyu and Aono, 2001). Absence of the corresponding activity in HSJ1v was confirmed on cholesterol-containing agar plates (Figure 1d).

When inoculated into TSB and incubated at 37 °C without shaking, HSJ1v grew suspended in the broth, whereas the wild-type formed aggregates, mostly at the air–liquid interface. Accordingly, the wild-type formed more biofilm than the variant (Figure 1e). Moreover, HSJ1 was 1.6-fold more adherent to abiotic surfaces than HSJ1v. Bacterial motility mechanisms are known to have an important role in biofilm formation. However, swimming activity was similar for HSJ1 and HSJ1v on tryptone swim plate and we were unable to detect any swarming motility for both types (data not shown).

Eight wild-type colonies and eight variant colonies were randomly chosen from several CRTSB plates and screened for antifungal, proteolytic and hemolytic activities. No variability between the different wild-type colonies was observed and each variant colony showed the same phenotypes, suggesting that all the wild-type and variant colonies show a consistent phenotype pattern. Moreover, in a wide range of conditions tested (temperature, carbon source and oxidative stress), no reversion from variant to wild-type was observed.

The variant type is unable to produce 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs)

The production of extracellular proteases, siderophores and of an unknown antifungal compound is under quorum-sensing control in *B. ambifaria* (Zhou *et al.*, 2003; Wopperer *et al.*, 2006). As these quorum-sensing-regulated phenotypes are altered in the variant, we hypothesized that quorum-sensing might be affected. We thus quantified the production of octanoyl-homoserine lactone, C_8-HSL, the predominant quorum-sensing signal produced by *B. ambifaria* (Zhou *et al.*, 2003; Vial *et al.*, 2008). However, no significant difference was observed between the wild-type and the variant: for instance, during exponential phase ($OD_{600} = 4.00$), concentrations were 1.43 mg ml^{-1} for HSJ1 versus 1.29 mg ml^{-1} for HSJ1v.

Recently, we have reported that the wild-type HSJ1 strain produces HAQ analogues bearing a methyl group at the third position: HMAQs (Vial *et al.*, 2008). HAQs, such as the *Pseudomonas* quinolone signal, are implicated in the regulation of virulence genes in *P. aeruginosa* (Dubern and Diggle, 2008). We thus analyzed several randomly chosen variant colonies and found that none were producing HMAQs. We therefore compared the phenotypes of an HMAQ-negative *hmqA*$^-$ mutant with those of HSJ1 and HSJ1v. However, despite the absence of HMAQ production in HSJ1v, an *hmqA*$^-$ mutant showed mostly wild-type features (data not shown). Furthermore, we have recently described that the *hmqA*$^-$ mutant shows greater antifungal and proteolytic activity than the wild-type (and consequently more than the variant) (Vial *et al.*, 2008). Taken together, these results indicate an absence of correlation between loss of HMAQ production and the variant phenotype.

Sensitivity to antimicrobial peptides and modification of the LPS profile

By disk diffusion assay, we observed that the wild-type and its variant present essentially the same antibiogram (Supplementary Table S2). However, as Bcc isolates are highly resistant to antimicrobial peptides, we challenged the HSJ1 wild-type and variant with antimicrobial peptide in suspension, followed by enumeration of viable cells. Using this precise method, we analyzed further the response to three antimicrobial peptides. Figure 2a shows that the survival of the wild-type is not affected by the presence of 1000 μg ml^{-1} polymyxin B or 200 μg ml^{-1} LL-37, a human cathelicidin. In contrast, HSJ1v type shows only 60% of survival in the presence of these compounds, and this increased sensitivity of the variant is even more pronounced with the honeybee polypeptide mellitin (Figure 2b).

The altered appearance of variant colonies suggests changes in the cell wall, possibly in LPS structure. Such differences are known to affect the response to antimicrobial peptides (Rosenfeld and

Shai, 2006). As shown in Figure 2c, tricine SDS-polyacrylamide gel electrophoresis analysis revealed modifications in the O-antigen and lipid A-core regions.

The wild-type is more virulent in several infection models

Certain Bcc strains can invade and survive within respiratory epithelial cells and macrophages (Valvano *et al.*, 2005). The levels of phagocytosis and intracellular replication after infection with the wild-type and the variant were thus evaluated using THP-1 cells. Although HSJ1v is more amenable

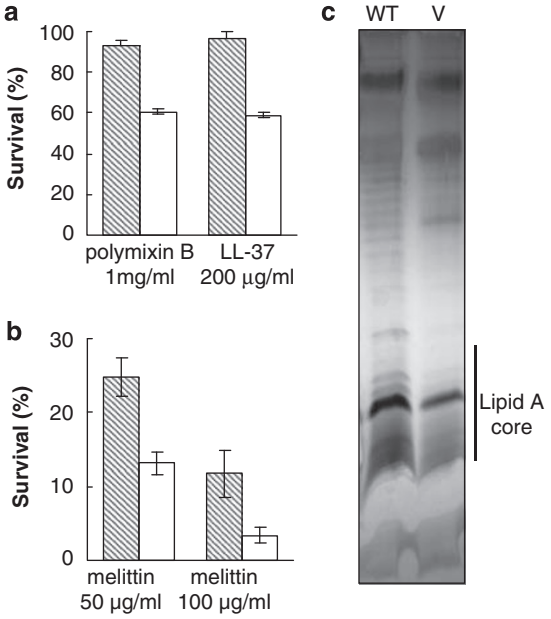

Figure 2 Antimicrobial peptides assay and analysis of lipopolysaccharides (LPSs). (**a**) The graph shows antimicrobial peptide survival of *B. ambifaria* wild-type (hatched bars) and variant (white bars). Antimicrobial peptide survival was calculated from the ratio of exposed cells to nonexposed cells. (**b**) Analysis of LPSs by tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Abbreviations: WT, *B. ambifaria* wild-type; V, *B. ambifaria* variant.

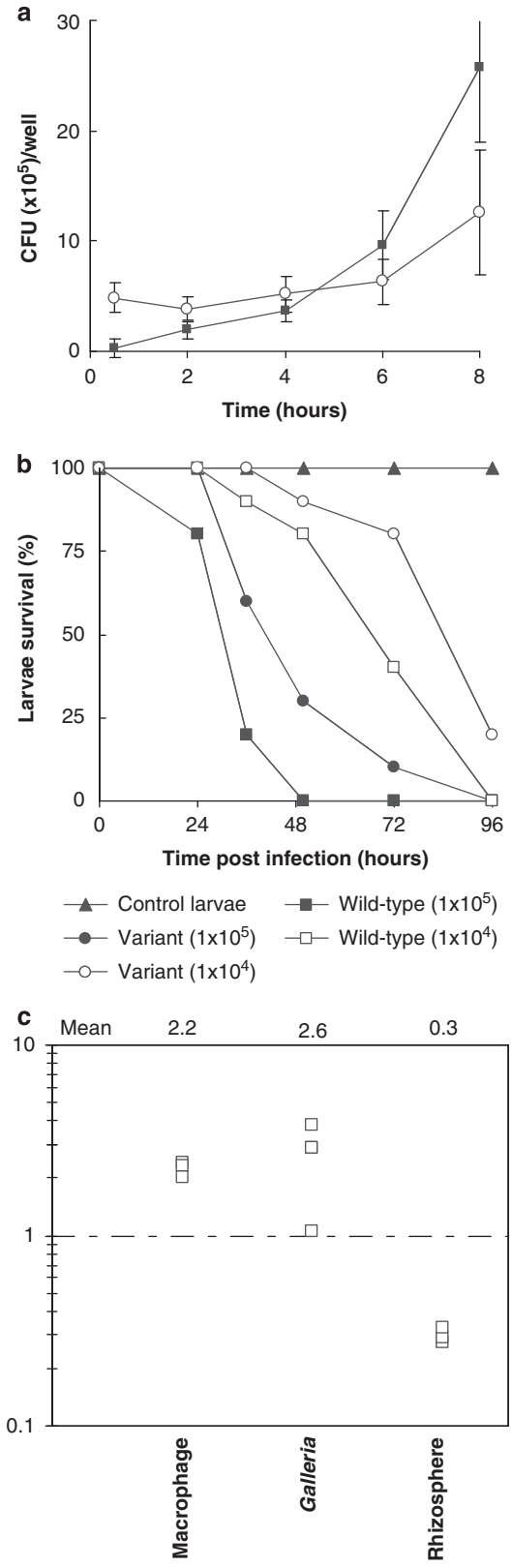

Figure 3 Comparison of the virulence of *B. ambifaria* wild-type HSJ1 and variant. (**a**) Quantification of *B. ambifaria* HSJ1 wild-type (■) and variant (○) within human acute monocytic leukemia cell line (THP-1) macrophages. The results are expressed as the means ± s.d. of the replicate experiments. (**b**) *G. mellonella* larvae survival over time infected with *B. ambifaria* HSJ1 wild-type and variant. The larvae were injected with bacteria (1×10^6 or 1×10^5) and monitored for their survival. Each data set is representative of a single trial with the specified strain ($N = 10$). (**c**) Competitive index (CI) analysis of *B. ambifaria* wild type and variant. CI in proliferation in THP-1 macrophages is defined as the ratio between the two strains in the output (intracellular bacteria recovered 8 h after infection) divided by their ratio in the input (intracellular bacteria after the 2-h treatment with antibiotics). CI in *G. mellonella* is defined as the colony-forming unit (CFU) output (48 h after infection) ratio of the variant in comparison with the wild-type strain, divided by the CFU input ratio (inoculum) of mutant to wild-type; CI for colonization of the rhizosphere of *P. sativum* is defined as the CFU output (day 15 after inoculation) ratio of the variant in comparison with the wild-type strain, divided by the CFU input (inoculum) ratio of variant to wild-type. The wild-type is more competitive than the variant when the CI is greater than 1.

to phagocytosis, with a twofold higher level (30.7 ± 4.1%) compared with HSJ1 (15.9 ± 2.8%), it shows a much reduced ability to replicate in macrophages (Figure 3a). Importantly, during these experiments, no difference in the frequency of phase variation between the wild-type inoculum and the population recovered from the macrophages 8 h after infection was observed.

The wax moth, *G. mellonella*, was recently reported as a useful surrogate host for determining the virulence of the Bcc, notably *B. ambifaria* (Seed and Dennis, 2008). When compared with wild-type, HSJ1v shows reproductively less virulence towards *G. mellonella* larvae (Figure 3b). This result was also confirmed using the *Drosophila* pricking model (Supplementary Figure S4).

Finally, the virulence of *B. ambifaria* wild type and variant was quantitatively characterized by spotting serial dilutions of *D. discoideum* on bacterial lawns (Figure 4). The social amoeba *D. discoideum* is a suitable host for studying the virulence of bacterial pathogens, including the Bcc (Aubert *et al.*, 2008). *D. discoideum* forms clear plaques on HSJ1v variant lawns at dilutions containing at least 25 000 amoebae. However, on wild-type lawns, *D. dictyostelium* is unable to produce clear plaques, even at the highest concentration of amoebae. This result suggests that the wild-type, in contrast with the variant, is able to resist *D. discoideum* predation.

To better define the difference of the wild-type and the variant in intracellular replication abilities within THP-1 cell lines, we produced a co-infection to generate a CI in proliferation (Segura *et al.*, 2004). A mean value of 2.2 for the CI confirmed the better survival and replication of the wild-type within THP-1 cells (Figure 3d). A CI performed during *G. mellonella* larvae infection further established that the wild-type is also better adapted to survive and replicate in this host (Figure 3d).

Several *G. mellonella* larvae and *Drosophila melanogaster* flies were infected with *B. ambifaria* variant, and at different times after the inoculation, we evaluated the presence of revertant or occurrence of another phenotype on CRTSB. In these conditions, neither reversion to wild-type nor appearance of new phenotypes was detected. These results showed that the variant type was stable in all conditions tested.

The variant of B. ambifaria *HSJ1 is more competitive in rhizosphere colonization*

As several *B. ambifaria* environmental strains have been isolated from the rhizosphere of the pea (*Pisum sativum*) (Coenye *et al.*, 2001), this plant was used as our model to investigate the rhizosphere-colonizing competence of HSJ1 wild-type and variant. Our results showed that HSJ1v was more competitive in colonizing the rhizosphere (Figures 3c and 5). At 15 days after co-inoculation, the proportion of variants in the rhizospheric population nearly reached 80%. In the control medium, no significant difference in the proportion of the two populations was observed. These results highlight a central difference in competitiveness between the wild-type and the variant in colonizing the rhizosphere because when the wild-type was inoculated alone no increase in the

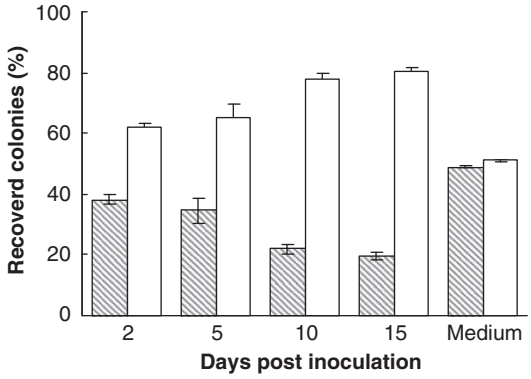

Figure 5 Competitive root colonization by *B. ambifaria* wild type and variant. Plants were inoculated 1:1 with the wild-type and the mutant. Results are shown as means ± s.d. Hatched bars and white bars represent the percentage of colonies recovered from the wild-type strain and the variant strain, respectively. Medium represents the percentage of colonies recovered from Fahraeus (FP) mineral medium inoculated and stored 15 days in the same condition as the roots.

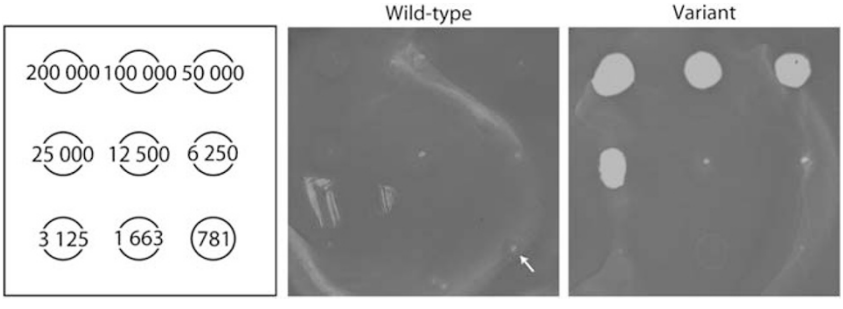

Figure 4 Plaque assay with *D. discoideum*. The bacterial lawn was spotted with 5-µl droplets of the serial dilutions of *D. discoideum*, and the ability of *D. discoideum* to produce clearing plaques in the bacterial lawn was recorded after 24 h at 23 °C. Small holes, such as the one pointed by the arrow, are not clearing plaques but only scratches made when amoebae were deposited on the bacterial lawn.

variant proportion was observed (data not shown). The difference observed in competitiveness during colonization did not seem to be the consequence of a better root attachment of the variant (Supplementary Figure S5).

Occurrence of phase variation in the B. ambifaria *genomovar*

To ascertain that *B. ambifaria* phase variation is not solely restricted to isolate HSJ1, *B. ambifaria* isolates recovered from CF patients ($n=7$) and from environmental sources ($n=6$) were grown overnight in TSB medium and plated on CRTSB (Supplementary Table S1). We found that clinical isolates formed rough, wrinkled colonies binding Congo red, the phenotype typical of HSJ1 wild-type. Moreover, all clinical isolates generated variants at a frequency of 10^{-4} variants per cell per generation (Supplementary Table S3). These variants were translucent and unable to bind Congo red, similar to HSJ1v. Moreover, all the isolates recovered from CF patients produced variant colonies with the same phenotypes observed with HSJ1v: absence of hemolytic, antifungal or cholesterol oxidase activities (Table 2). Only clinical isolates were able to produce HMAQs, with the exception of CEP0516. Virulence of the clinical isolates and their variants was investigated using the *D. discoideum* plaque assay. We observed, such as with HSJ1, that the amoebae were unable to form clear plaques on the wild-type clinical isolates lawns, in contrast with their variants (Table 2).

On the other hand, all isolates recovered from environmental sources failed to bind Congo red, showed only one type of colony on CRTSB, even after several subcultures, and showed common features with the variants derived from the clinical isolates (Table 2). Notably, environmental isolates were frankly mucoid on yeast extract-mannitol agar and did not produce HMAQs. With one exception, they did not show antifungal and cholesterol oxidase activities, similar to the variants derived from clinical isolates. Finally, most environmental isolates were susceptible to predation by the amoebae, similar to the variants (Table 2).

Discussion

B. ambifaria *uses phase variation for niche adaptation*

Bcc isolates that occur in the environment are also capable of infecting humans and it is widely accepted that differentiation between the environmental and clinical isolates of Bcc is unrealistic (Govan *et al.*, 2000). To the best of our knowledge, this is the first report that distinguish between two distinct phenotypic states inside a genomovar of the *B. cepacia* complex. The first phenotypic type of *B. ambifaria* is composed of isolates adapted to clinical infections or interactions with eukaryotic hosts and are recovered from patients suffering from CF. The second type is associated with environmental isolates and is better adapted to the rhizosphere. We suggest that this distinction mediated by phase

Table 2 Physiological and biochemical characteristics of *Burkholderia ambifaria* clinical strains WT, variants and environmental strains

	Mucoidy[a]	*Hemolysis*	*Antifungal*[b] *activity*	*Cholesterol oxidase activity*	*HMAQ production*	Dictyostelium discoideum *assay*
CEP0516 WT (CF)	+	+	+	+	−	*R*[c]
CEP0516 variant	++++	−	−	−	−	12 500
CEP0617 WT (CF)	++	+	+	+	+	*R*
CEP0617 variant	+++	−	−	−	−	6250
CEP0958 WT (CF)	+	+	+	+	+	*R*
CEP0958 variant	++++	−	−	−	−	50 000
CEP0996 WT (CF)	−	+	+	+	+	*R*
CEP0996 variant	−	−	−	−	−	12 500
AU0212 WT (CF)	−	+	+	−	+	*R*
AU0212 variant	++	−	−	−	−	12 500
AU4157 WT (CF)	++	+	+	+	+	*R*
AU4157 variant	+++	−	−	−	−	25 000
AU8235 WT (CF)	++	+	+	+	+	*R*
AU8235 variant	++	−	−	−	−	25 000
HI3590 (Env)	++++	−	−	−	−	25 000
HI3687 (Env)	++++	−	−	−	−	6250
AMMD (Env)	+++	−	−	−	−	25 000
PHP7 (Env)	+++	−	−	−	−	25 000
LMG17828 (Env)	++++	−	−	−	−	*R*
IOP40-10 (Env)	+++	−	+	+	−	*R*

Abbreviations: CF, cystic fibrosis; Env, environment; EPS, exopolysaccharide; HMAQ, 4-hydroxy-3-methyl-2-alkylquinolines; WT, wild-type.
[a]The capacity of *B. ambifaria* to elaborate EPS was determined as described by Zlosnik *et al.* (2008).
[b]Results obtained with *C. glabrata*. Similar results were obtained with *C. albicans* and *C. neoformans*.
[c]*R* indicates that *D. discoideum* was unable to form clear plaques, even at the highest concentration of amoebae (200 000). The value indicated is the lowest number of *D. discoideum* cells on the plate leading to the formation of a visible plaque. This experiment was performed on two independent occasions with similar results.

variation allows the adaptation of a *B. ambifaria* strain to drastically different environments: the lungs of patients with CF or the plant roots environment.

A striking phenomenon observed with *B. ambifaria* is that only clinical isolates generate phase variants, under laboratory conditions. Most importantly, these variants show features typical of isolates recovered from the environment, suggesting that phase variants from clinical isolates are in fact rhizosphere colonizers. An extensive number of *B. ambifaria* isolates would be required to confirm this hypothesis.

Features of B. ambifaria *clinical isolates*

As the virulence of Bcc strains can vary depending on the model host used, it was appropriate to test the virulence of HSJ1 and HSJ1v using several models. A number of infection models have earlier been tested with Bcc members, including alfalfa seedlings, *G. mellonella* and *Caenorhabditis elegans* (Bernier *et al.*, 2003; Cardona *et al.*, 2005; Seed and Dennis, 2008). By using four infection models (*D. melanogaster*, *G. mellonella*, macrophages and *D. discoideum*), we clearly showed that the wild-type is more virulent than the variant. Interestingly, our study using *D. discoideum* uncovers an intriguing distinction between the two phenotypic states in *B. ambifaria*: amoebae are unable to form clear plaques in lawns of clinical isolates, whereas most environmental isolates and all the variants are permissive to *D. discoideum*. Further investigations are required for a better understanding of clinical isolate resistance to amoebae.

Direct evidence of intracellular survival of the Bcc species has been shown in airway epithelial cells, in murine macrophages, in human monocytic cells and in amoebae (Marolda *et al.*, 1999; Saini *et al.*, 1999; Savoia and Zucca, 2007). The capacity of Bcc strains to survive and replicate within eukaryotic cells may explain their persistence in the respiratory system (Valvano *et al.*, 2005). However, the replication ability of *Burkholderia* inside cell lines is known to vary between isolates or genomovars (Saini *et al.*, 1999; Sajjan *et al.*, 2006). In this study we showed that both *B. ambifaria* HSJ1 wild-type and variant can replicate within phagosomes, but HSJ1v clearly displayed reduced replication in the human monocyte cell line compared with HSJ1. Our results are consistent with those of Savoia and Zucca (2007), who observed replication of a *B. ambifaria* clinical strain in murine macrophages but no significant replication for an environmental strain.

Numerous factors involved in the virulence of Bcc strains have been identified, especially in *B. cenocepacia* (Kooi *et al.*, 2006; Aubert *et al.*, 2008; Sajjan *et al.*, 2008). In our study, phenotypic and proteomic approaches reveal that transition from wild-type to variant affects the expression of several putative virulence factors, for instance, biofilm formation or protease activity. Indeed, HSJ1 shows greater protease activity than HSJ1v, probably because of low ZmpA and ZmpB expression in HSJ1v. In *B. cenocepacia*, *zmpA*$^-$ *zmpB*$^-$ mutants were less virulent in a rat agar bead chronic infection model (Kooi *et al.*, 2006). These proteins are probably also involved in the virulence of *B. ambifaria*, as they have a very high degree of homology inside the Bcc (for example, 93% identity between *B. ambifaria* and *B. cenocepacia*). In the same way, cholesterol oxidation, which is detected only in the wild-type, could be involved in the virulence of HSJ1. Indeed, cholesterol oxidase is required for mice infection by *Mycobacterium tuberculosis* (Brzostek *et al.*, 2007).

A remarkable difference between the two subgroups of *B. ambifaria* is the production of HMAQs, which is synthesized by clinical isolates. These compounds are also produced by the human pathogen *B. pseudomallei* (Vial *et al.*, 2008). Their role in *B. ambifaria* is still under investigation. In *P. aeruginosa*, some of the HAQs are implicated in the regulation of multiple virulence factors (Dubern and Diggle, 2008). Therefore, HMAQs could also be involved in the virulence of clinical isolates, especially during infections in CF patients.

Bcc isolates are resistant to a wide variety of antimicrobial compounds, including antimicrobial peptides (Mahenthiralingam *et al.*, 2005; Loutet *et al.*, 2006). In the Bcc, the complete lipid A-core oligosaccharide is required for resisting polymyxin B and melittin (Loutet *et al.*, 2006). The modification of the LPS profile that we observed between the wild-type and the variant might explain their difference in sensitivity to antimicrobial peptides.

All these differences between the two phenotypic states could explain that clinical isolates are more virulent to animal hosts, and possibly better adapted to the lungs of CF patients.

Adaptation of the variant to the rhizosphere

Our data show that the variant is more competitive in colonizing the rhizosphere than the wild-type. Interestingly, in *P. fluorescens* F113, phenotypic variation occurs during rhizosphere colonization (Martinez-Granero *et al.*, 2006). For *B. ambifaria* HSJ1, an increase in the proportion of variants generated by the wild-type during the colonization of the pea rhizosphere was not observed. However, it is conceivable that the rhizosphere of other plants could stimulate the frequency of phase variation.

Using API-ZYM strips, we detected that HSJ1v possesses a stronger β-glucosidase activity when compared with HSJ1 (Table 1). β-Glucosidases belong to a heterogeneous group of enzymes that catalyze the hydrolysis of cellobiose and related β-glucosides. Furthermore, only the variant can grow with saccharose and xylose as carbon sources. Finally, HSJ1v grows faster than HSJ1 when polyols (glycerol and sorbitol) are used as carbon sources.

The HSJ1 variant is nutritionally more versatile than the clinical wild-type and consequently probably better adapted to the rhizosphere. Indeed, these sugars recovered from plant root exudates could be used as carbon source.

The variants and the environmental isolates of *B. ambifaria* produce more EPSs than the clinical isolates, a result that is in agreement with the data from Zlosnik *et al.* (2008). In that study, 92.5% of the *B. ambifaria* environmental strains were frankly mucoid, whereas the clinical strain CEP0996 was not. Interestingly, *B. cenocepacia* strains, the most virulent species of the Bcc, are generally non-mucoid (Zlosnik *et al.*, 2008). This would suggest that EPS production is not a feature of CF lungs colonization, in contrast with *P. aeruginosa*. The role of EPSs in plant–*Burkholderia* interaction is not well documented. However, onion tissues induce EPS production from *Burkholderia*, probably through the carbohydrates produced by the onion (Bartholdson *et al.*, 2008). In some plant–bacterial associations (for example, *Rhizobium*), EPSs are involved in the attachment of bacteria to the roots. EPS from the Bcc and notably from *B. ambifaria* could be involved in bacterial–plant interactions.

B. ambifaria, and the other Bcc members, exert beneficial effects on plant growth by preventing the deleterious functions of pathogenic microorganisms, generally by producing antibiotics or by competing for nutrients such as iron (Compant *et al.*, 2008). The variant HSJ1v expresses a much greater ability for iron scavenging than HSJ1. In contrast, only the wild-type HSJ1 produces an antifungal compound *in vitro*. Surprisingly, this activity is absent from variants and from most environmental strains. Indeed, *B. ambifaria* is well characterized as a biocontrol agent: *B. ambifaria* AMMD is known to show antagonistic activity against the plant pathogens *Pythium* and *Aphanomyces euteiches* (Parke *et al.*, 1991). We have confirmed that *B. ambifaria* AMMD possesses the capacity to inhibit the growth of *Pythium* (data not shown), suggesting the presence of several compounds responsible for the antifungal activity in *B. ambifaria*.

Phase variation in B. ambifaria

Phase variants of *B. ambifaria* arise at frequencies of 10^{-4} per cell per generation, which is similar to those reported for variants in many bacteria (Wisniewski-Dyé and Vial, 2008). However, in most reports, the variants are unstable and revert to the original phenotype. In our case, variants of all the clinical *B. ambifaria* isolates showed stable phenotypes over multiple subculturing or culture conditions in the laboratory. Notably, even after interactions with eukaryotic hosts (for instance, in the *G. mellonella* or macrophage cell line) no reversion could be detected. Remarkably, for the phytopathogen *Ralstonia solanacearum*, a phenotypic conversion from mucoid to non-mucoid state was evident *in vitro*, but revertants could only be detected *ex planta* or in the presence of tomato root exudates (Poussier *et al.*, 2003). In the case of *B. ambifaria*, reversion to the wild-type might be restricted to very specific niche conditions, as, for instance, typically obtained in CF lungs. Thus, it is conceivable that environmental isolates of *B. ambifaria* would represent the primary source of infection of CF patients.

Phase variation mechanisms can involve a genome modification (for example, gene conversion and site-specific inversion) or epigenetic mechanisms such as differential methylation. In *B. cenocepacia* strain K56-2, a putative LysR transcriptional regulator (BCAS0225) was responsible for the shiny colony morphology and several shiny colony variants harbor spontaneous mutation in this gene. Interestingly, a highly conserved homologue of this gene is present in *B. ambifaria* (Bamb_6152 for *B. ambifaria* AMMD, >90% identity in amino acid sequences with BCAS0225). However, and in contrast with *B. cenocepacia*, no difference was detected between the gene sequence for the wild-type and for the variant, suggesting that this LysR regulator is not involved in the regulation of phase variation in *B. ambifaria* (data not shown).

Finally, it must be mentioned that until now, *B. ambifaria* has been essentially isolated from the plant rhizosphere and from CF lung infections. From the perspective we used here, the wild-type phenotype is the clinical form of the bacterium. Alternatively, because the variant type we identified is apparently the regular state in the environment, the latter could be considered closer to the actual wild type from the environmental point of view.

Conclusion

Currently, phase variation is viewed as a mechanism to evade immune system or for adaptation to different sites during host interaction (van der Woude and Baumler, 2004). The case of *B. ambifaria* appears unprecedented as it suggests that an opportunistic pathogen is able to generate a variant form that is less virulent to animal hosts but better adapted to the rhizosphere. In other words, a phase variation phenomenon occurs in *B. ambifaria* for niche adaptation.

Phase variation is likely not limited to *B. ambifaria* within the Bcc. For instance, shiny colony variants, isolated from *B. cenocepacia* strain K56-2, are less virulent in a chronic lung infection system and in the alfalfa model (Bernier *et al.*, 2008). These variants, as is the case for *B. ambifaria*, could be preferentially adapted to the rhizosphere and show beneficial properties for plant growth.

Further studies are needed to decipher the mechanism used by *B. ambifaria* for phase variation, notably to understand the phenotypical stability of the variants. This could allow the development of

genetically stable variants, which would combine desirable biocontrol properties with a reduced risk to cause human infections.

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