Arabidopsis thaliana and Pisum sativum models demonstrate that root colonization is an intrinsic trait of Burkholderia cepacia complex bacteria

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Burkholderia cepacia complex (Bcc) bacteria possess biotechnologically useful properties that contrast with their opportunistic pathogenicity. The rhizosphere fitness of Bcc bacteria is central to their biocontrol and bioremediation activities. However, it is not known whether this differs between species or between environmental and clinical strains. We investigated the ability of 26 Bcc strains representing nine different species to colonize the roots of Arabidopsis thaliana and Pisum sativum (pea). Viable counts, scanning electron microscopy and bioluminescence imaging were used to assess root colonization, with Bcc bacteria achieving mean (±SEM) levels of $2.49 \pm 0.23 \times 10^6$ and $5.16 \pm 1.87 \times 10^6$ c.f.u. per centimetre of root on the A. thaliana and P. sativum models, respectively. The A. thaliana rhizocompetence model was able to reveal loss of colonization phenotypes in Burkholderia vietnamiensis G4 transposon mutants that had only previously been observed in competition experiments on the P. sativum model. Different Bcc species colonized each plant model at different rates, and no statistical difference in root colonization was observed between isolates of clinical or environmental origin. Loss of the virulence-associated third chromosomal replicon (1 Mb DNA) did not alter Bcc root colonization on A. thaliana. In summary, Bcc bacteria possess intrinsic root colonization abilities irrespective of their species or source. As Bcc rhizocompetence does not require their third chromosomal replicon, the possibility of using synthetic biology approaches to engineer virulence-attenuated biotechnological strains is tractable.

INTRODUCTION

The Burkholderia cepacia complex (Bcc) is a group of genetically related but phenotypically diverse bacteria found in many terrestrial and freshwater habitats (Compant et al., 2008b; Parke & Gurian-Sherman, 2001). Taxonomic reclassification of genomovars within the Bcc initially led to nine Bcc species being formally named, Burkholderia cepacia, Burkholderia multivorans, Burkholderia cenocepacia, Burkholderia stabilis, Burkholderia vietnamiensis, Burkholderia dolosa, Burkholderia ambifaria, Burkholderia anathina and Burkholderia pyrrocinia, with eight additional species recently added to the complex (LiPuma, 2010). The multifaceted nature of the Bcc bacteria is evident in their ability to occupy a diverse range of ecological niches and also to mediate a variety of biotechnologically useful processes such as bioremediation and biological control (Coenye & Vandamme, 2003). In contrast to these beneficial roles, Bcc species are also opportunistic pathogens with a particular predilection to cause lung infections in people with cystic fibrosis (CF) (LiPuma, 2010).

Multiple root-colonizing Bcc strains have also been used as biological control agents of fungi and nematodes in economically important crop plants (Compant et al., 2008b; Parke & Gurian-Sherman, 2001). Biocontrol ability has been associated with strains of B. ambifaria (e.g. AMMD), B. cenocepacia (e.g. M36), B. cepacia (e.g. ATCC 49709), B. pyrrocinia (e.g. BC11) and B. vietnamiensis (TVV75) (Chiarini et al., 2006). Bcc bacteria may also promote plant growth by acting as biofertilizers, with different mechanisms such as altering plant phytohormone homeostasis or enhancing mineral nutrient acquisition linked to this trait (Vial et al., 2011). B. vietnamiensis strains may also fix

Abbreviations: Bcc, Burkholderia cepacia complex; c3, chromosome 3; CF, cystic fibrosis; SEM, scanning electron microscopy; STM, signature-tagged mutagenesis.

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atmospheric nitrogen (Chiarini et al., 2006), while other non-Bcc Burkholderia, such as Burkholderia phymatum, were demonstrated to be the first betaproteobacteria to nodulate leguminous plants (Suárez-Moreno et al., 2012). Another non-Bcc species, Burkholderia phytophirans PSIJN, can also confer tolerance to plant abiotic stress (Theocharis et al., 2012).

Similarities and differences between clinical and environmental Bcc strains have been investigated, but no apparent taxonomic or phenotypic distinctions have been found (Chiarini et al., 2006; Mahenthiralingam et al., 2008). Bcc population studies using MLST found no differentiation at the nucleotide level between strains recovered from clinical and environmental origins, and indicated that >20% of clinical isolates were indistinguishable from environmental isolates (Baldwin et al., 2007). Moreover, clonality has also been demonstrated between strains retrieved independently from both CF patients and the environment (Baldwin et al., 2007; LiPuma et al., 2002; Payne et al., 2005).

Despite a lack of obvious distinguishing characteristics, there is good evidence that different Bcc species are not equally distributed in nature or infection. While B. cenocepaya and B. multivorans are by far the most prevalent species in CF infections (accounting for >68% of infections), isolation of other species such as B. ambifaria, B. pyrrocinia, B. anthina or B. stabilis is more limited (<3% cases) (LiPuma, 2010). B. ambifaria and B. cenocepaya are among the most common Bcc species that can be cultivated from the rhizosphere, followed by B. cepacia, B. vietnamiensis and B. pyrrocinia, whereas others such as B. multivorans, B. dolosa or B. anthina have been rarely found in association with plant roots (Pirone et al., 2005; Ramette et al., 2005).

The phenotypic versatility of Bcc species is underpinned by their large genomes (6–9 Mb), which are unusually arranged across three chromosomal replicons (Agnoli et al., 2012; Mahenthiralingam et al., 2005). Bcc rhizocompetence and environmental fitness has been attributed to multiple genetic determinants such as amino acid biosynthesis, general metabolism, transport, oxidative stress response or gene regulation (O’Sullivan et al., 2007). It has also been proposed that mechanisms responsible for colonization of roots and human lungs may be similar (Berg et al., 2005), although recent studies indicate little overlap between the major genes required for the two processes (Hunt et al., 2004; O’Sullivan et al., 2007). Agnoli et al. (2012) carried out a series of tests on Bcc chromosome 3 (c3) null mutants and concluded that c3 should not be regarded as an essential genomic element, but rather as a large plasmid that encodes virulence, secondary metabolism and accessory functions. Further identification of environmental fitness traits will enhance our knowledge of Bcc adaptation to the rhizosphere as one of the biotechnologically influential habitats of Burkholderia species (Suárez-Moreno et al., 2012). We developed two complementary rhizosphere colonization systems to address rhizocompetence on an agriculturally important species, Pisum sativum (pea), and a model plant, Arabidopsis thaliana. Root growth into agar-immobilized bacteria was used as a novel means to inoculate the A. thaliana rhizosphere, allowing this plant species to be used for the first time to model active bacteria–root interactions, as opposed to passive colonization processes modelled in other systems (Conn et al., 2008; Digonnet et al., 2012; Dong et al., 2003; Timmusk & Wagner, 1999). The microcosms were used to compare Bcc species fitness in relation to their taxonomic identity, clinical or environmental origin, and the presence or absence of the third chromosome.

**METHODS**

**Bacterial strains.** A panel of 26 Bcc strains representing nine of the first formally named and most characterized Bcc species (LiPuma, 2010) was examined (Table 1). Bcc strains were drawn from published strain panels (Coenye et al., 2003; Mahenthiralingam et al., 2000) and the Cardiff University Collection (Table 1; Mahenthiralingam et al., 2008). MLST (Baldwin et al., 2005) was used to genotype all isolates, and at the strain level the collection comprised 16 environmental and 11 clinical strains (the ST122 isolates, AU1054 and HI2424 were recovered from both sources; Table 1; LiPuma et al., 2002). An additional set of third chromosome null mutant derivatives (Agnoli et al., 2012) were also analysed for five Bcc strains (Table 1). Two B. vietnamiensis G4 signature-tagged mutagenesis (STM) mutant strains (O’Sullivan et al., 2007), a pea root hypo-colonizing mutant (3-E9) and a hyper-colonizing mutant (3-E6) were also tested. Four non-Bcc bacteria were used as controls: enteric Escherichia coli NCTC 12241 and skin commensal Staphylococcus aureus NCTC 12981 as species not normally associated with a rhizosphere lifestyle; and Pseudomonas fluorescens SBW25 (Bailey et al., 1995) and B. phytophirans PSIJN (Compart et al., 2008a) as two well-characterized rhizocompetent species.

**A. thaliana root colonization model.** A. thaliana ecotype Columbia (Col-0, N1092) seeds were surface-disinfected, germinated and grown on Murashige & Skoog basal salts medium [MS plates: 1 × MS agar 1% (w/v), pH 5.7] as previously described (Vidal-Quist et al., 2013). Equal root size seedlings were then transferred to square 12 × 12 cm MS plates (six seedlings per plate) and allowed to grow for an additional week, during which time the roots attained the right size for inoculation (primary root tip approximately 1.5–2 cm away from the lower edge of the plate; Fig. 1a).

Fresh bacterial cultures were prepared by growth in Luria–Bertani broth (LB; Sigma Aldrich; originally designated Lysogeny Broth) at 30°C for 16–18 h. Cultures were centrifuged and cell pellets were washed twice in sterile 10% PBS, before being resuspended in 10% PBS at an optical density of 5 U at 600 nm. Cell suspensions were incubated at 32°C for 5 min and diluted 1:1 with 0.8% molten low melting point agarose (LMP; Promega) made in 10% PBS and cooled to the same temperature (this was slightly higher than the growth temperature to keep the agar molten). The suspension (800 μl) was applied on top of the MS medium at the lower 1 cm area of the plate and allowed to solidify, avoiding any contact with the root system (Fig. 1a). Viable count analysis demonstrated that this inoculation protocol correlated with an inoculum density range of 2–4 × 10^6 cfu cm^-2. Root growth was monitored throughout the experiment, with contact with the inoculated surface occurring 12–24 h after application.

Plants were incubated for 8 days post-inoculation at the same climatic regime described above, then 2 cm standardized root sections were aseptically excised 2 cm away from the inoculum front (see Fig. 1a). The root sections were homogenized and serially diluted for bacterial viable count determination as described previously.
Pea root colonization model. Root inoculations were performed with *P. sativum* var. *sativum* (Early Onward Peas; Suttons) as previously described (O’Sullivan et al., 2007). Pea seeds were sterilized using ethanol/bleach washes and germinated on 0.6% basal salts medium (BSM) agar for 3–4 days in the dark at 25 °C. Freshly germinated pea seeds were then aseptically planted into silica sand microcosms (30 g sand in a 2.5 cm diameter × 20 cm long boiling tube; O’Sullivan et al., 2007). Bcc strains were revived from stock cultures and inoculation cultures prepared by growth in tryptone soya broth (TSB; Oxoid) at 30 °C for 16–18 h. A standardized inoculum of approximately 10<sup>2</sup>–10<sup>5</sup> c.f.u. ml<sup>−1</sup> was prepared by diluting these cultures in TSB to an optical density of 1 U at 600 nm, and then further diluting 1:100 in BSM (Hareland et al., 1975); the exact bacterial density was determined from viable counts on tryptone soya agar (TSA) containing 300 U polymixin B sulphate (TSA/P) ml<sup>−1</sup> after 1–2 days. The sand around the germinated pea seeds was inoculated with 4 ml of the standardized bacterial suspensions, and microcosm tubes were resealed with sterile cotton wool and incubated at 25 °C for 7 days in the dark and 7 days in the light, with the addition of 2 ml BSM after the first 7 days.

Whole root systems were harvested aseptically after 14 days of incubation, and rinsed twice in 30 ml BSM to remove loosely adhering sand and bacteria. One-centimetre root sections were excised near the seed for each plant processed, and vortexed twice for 30 s in 1 ml fresh BSM in 1.5 ml microtubes. The level of root colonization attained by each Bcc strain was assessed by a viable counting approach. One-centimetre root sections were macerated for 30 s in 1 ml fresh BSM with a sterile pellet pestle. The resulting root homogenate was serially diluted and incubated on TSA/P to determine viable counts of bacteria colonizing the root [c.f.u. (cm root)<sup>−1</sup>]. Viable counts for each Bcc strain were recorded from at least four root sections, with a maximum of two root sections being taken from each plant.

**Bioluminescent labelling of *Burkholderia***. *B. vietnamiensis* strain G4 (Table 1) was transformed with plasmid pBBR1MCS-LITE (Parveen et al., 2001), containing the lux-CDABE operon of *Photobacterium* *luminescens*. Competent cells were generated as follows: cells were revived from stock cultures and grown on TSA overnight, and a thin layer of cells was then spread on solid supoptimal broth medium (SOB) supplemented with 10 mM MgCl<sub>2</sub> and 10 mM MgSO<sub>4</sub>, and incubated for 6 h at 37 °C. The resulting culture was swabbed into ice-cold, sterile 0.5 M sucrose with 10% glycerol and its light emission stability determined by monitoring bioluminescence imaging.

Scanning electron microscopy (SEM). SEM was used to visualize selected Bcc strains adhering to the root surface in the pea model (SEM was performed for at least one strain per Bcc species). Root sections were fixed by immersion in 2.5% electron microscopy-grade glutaraldehyde (Agar Scientific) for 1 h at room temperature, and rinsed twice for 5 s in 0.05 M sodium cacodylate buffer. Roots were post-fixed in aqueous osmium tetroxide (1%, v/v; Agar Scientific) for 1 h at room temperature, before being dehydrated in a series of ethanol solutions of increasing concentration (50%, 70%, 90%, 3×100%; 10 min each). Critical point drying was achieved using liquid CO<sub>2</sub> (Balzers CPD030). Root sections were gold-coated (EMScope gold sputter-coater) and viewed under a Philips XL20 scanning electron microscope operated at 25 kV.

A qualitative scoring system was developed to allow visual quantification of the level of surface root colonization achieved by 14 of the Bcc strains (see Table 1). Four regions of root of equal size were chosen at random and thoroughly examined by SEM. They were scored on a colonization scale according to the quantity of bacterial cells observed adhering to the root surface. The following colonization scores were recorded: '0' when no bacteria were observed at any of four regions; '1' when 1–25 bacteria were observed in at least one region of root; '2' when 25–100 bacteria were observed in at least one region, but bacteria were not necessarily present at all four regions; '3' if 25–100 bacteria were observed in at least one region, with bacteria observed at all regions examined; and '4' when bacteria were too numerous to count in at least one region, and bacteria were present at all regions.

**Statistical analysis**. Statistical analyses were performed on log<sub>10</sub>-transformed data using the statistical package GraphPad Prism version 5.00 (GraphPad Software). Parametric tests were performed where transformation resulted in normalized data and homogenized variances; non-parametric tests were otherwise carried out. Statistical tests used, including significance (P-value), test-statistic and degrees of freedom (d.f.) are listed as appropriate. The mean pea colonization data were compared using Mann–Whitney’s test, and Kruskal–Wallis and Dunn’s multiple comparison post-test for comparisons of more than two strains. The mean *A. thaliana* colonization data were compared using t-tests or one-way ANOVA and Tukey’s multiple comparison post-test for comparison of more than two strains.

**RESULTS**

**Development of the *A. thaliana* root colonization microcosm**

The *A. thaliana* root colonization gnotobiotic microcosm was initially developed using *B. vietnamiensis* G4lx, a bioluminescent reporter strain carrying a *lux*-CDABE plasmid, to enable real-time tracking of the movement of bacteria (Fig. 1). As the *A. thaliana* growth model was carried out in the absence of antibiotic selection, the *B. vietnamiensis* G4lx bioluminescence reporter was only used for qualitative characterization of root colonization (net light emission was reduced by approximately 70% after 20 generations of growth without antibiotic). Different ways of inoculating *A. thaliana* roots with bacteria were
<table>
<thead>
<tr>
<th><strong>Burkholderia</strong> species</th>
<th>Strain designation*</th>
<th>Sequence type†</th>
<th>Original source‡</th>
<th>Comments and references pertinent to strain§</th>
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<td><strong>B. cepacia</strong> (I)</td>
<td>LMG 18821</td>
<td>5</td>
<td>Clin</td>
<td>CF patient respirator; Mahenthiralingam <em>et al.</em> (2000)</td>
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<td>Type strain; isolated from sour skin disease of onions (USA); genome sequenced strain (2509276048); Mahenthiralingam <em>et al.</em> (2000). High numbers of bacteria observed on the pea root surface by SEM (score=4)</td>
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<td>30</td>
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<td>38</td>
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Table 1. cont.

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*Culture collection abbreviations: LMG, Belgium Co-ordinated Collection of Micro-organisms Laboratorium voor Microbiologie, Universiteit Gent; ATCC, American type Culture Collection; BCC, Burkholderia cepacia Cardiff collection (Mahenthiralingam et al., 2008).
†Sequence type, genotype from the MLST database (Baldwin et al., 2005).
‡Clin, clinical origin; Env, environmental source.
§Genome sequenced strains were identified by their Taxon Object ID – Integrated Microbial Genomes – JGI (http://img.jgi.doe.gov/cgi-bin/w/main.cgi).
evaluated. Application of bacterial suspensions (50 μl) directly onto the roots led to very rapid, passive movement of the fluid along the root surface and produced variable inoculation and colonization densities. An inoculation system that modelled active bacterial root colonization was therefore explored. Resuspension of B. vietnamiensis G4 in soft low melting temperature agarose to form a semi-solid matrix did not prevent the rapid passive movement along roots. A system of bacterial inoculation in a soft agar strip laid along the lower edge of the plate, 0.5–1 cm below the A. thaliana root tips, was subsequently evaluated (Fig. 1).

Light emission was monitored on the day of inoculation when the roots were not in contact with the inoculum, at day 1 after contact with the bacterial inoculum had been made (Fig. 1b), and at day 8 after active root colonization by the bacteria had occurred (Fig. 1b). Inoculated bacterial cells remained immobilized within the soft agar inoculated area and only moved to other regions of the plate by active interaction with the root system. Although light emission from the B. vietnamiensis G4lx reporter was variable across seedlings (Fig. 1b), reproducible levels of bacterial colonization were obtained when sections from groups of 12 seedlings were examined by viable count analysis [mean (±SD) log10 c.f.u. per root section = 5.57 ± 0.18].

To evaluate the performance of the A. thaliana model in terms of identifying differences in colonization associated with isogenic Bcc derivatives, two B. vietnamiensis G4 mutants characterized in a signature-tagged transposon mutagenesis study of pea rhizosphere colonization (O’Sullivan et al., 2007) were examined. Mutant 3-E9 was a hypo-colonizing derivative which had lost rhizocompetence, while mutant 3-E6 possessed a hyper-colonizing phenotype in a pea root colonization model (O’Sullivan et al., 2007). In the A. thaliana model, mutant 3-E9 also showed a significant hypo-colonization phenotype, with a 50-fold reduction in mean bacterial counts compared with the wild-type strain G4 (t-test; P<0.0001, t=7.45, d.f.=31). In comparison, pea hyper-colonization mutant 3-E6 showed a significant 28-fold increase of A. thaliana root colonization.

Fig. 1. A. thaliana rhizosphere colonization model. (a) A. thaliana seedlings were grown for 2 weeks vertically on MS medium until primary root tips were 1.5–2 cm above the lower edge of the plate (see lower section of a day 0 plate). At this point a standardized suspension of B. vietnamiensis G4 in soft agar was applied on top of the MS medium, along the edge of the plate, ensuring that the root tips were not touched (red dotted line, ‘inoculation area’). After 12–24 h of further incubation the roots contacted the bacterial inoculum and allowed active rhizocolonization to be initiated if the strain was competent. Plants were incubated for 8 days and 2 cm standardized root sections were aseptically excised (yellow dotted line, ‘sampling area’) and homogenized for viable counts analysis. (b) A. thaliana inoculation with bioluminescent strain B. vietnamiensis G4lx, after 1 and 8 days of incubation, is shown. Light emission images were overlaid onto the white light image to reveal the active colonization of the plant root systems by strain G4lx after contact with the soft agar inoculum was made. The media between the plant roots at the sampling area showed no sign of luminescence (filled circles) and remained sterile throughout the microcosm experiments.
with respect to wild-type G4 \((t\text{-test}; P=0.0491, t=2.06, \text{d.f.}=28)\). Given this reproducibility and comparable performance to a validated model (O’Sullivan et al., 2007), root penetration into bacteria immobilized in soft agar was adopted as a standardized \(A.\ thaliana\) active colonization model to evaluate further Bcc strains.

**Development of the pea root colonization microcosm**

The pea microcosm used had previously proved successful for identification of \(B.\ vietnamiensis\) G4 transposon mutants with altered rhizosphere colonization phenotypes (O’Sullivan et al., 2007). The effect of inoculation density was investigated by seeding a series of microcosms with serial dilutions of strain G4 ranging from \(10^0\) to \(10^7\) c.f.u. ml\(^{-1}\). After 14 days, no significant difference between the mean colonization level attained, \(1.80 \pm 0.25 \times 10^0\) c.f.u. \((\text{root cm})^{-1}\), were observed in relation to inoculation density (Kruskal–Wallis test; \(P=0.4994, H=5.35, \text{d.f.}=7\)); all future inoculations were therefore standardized to between \(10^0\) and \(10^1\) c.f.u. ml\(^{-1}\). \(B.\ vietnamiensis\) G4 colonized pea roots at a significantly higher level than \(E.\ coli\) \((5.4 \times 10^4\) c.f.u. \((\text{root cm})^{-1}\) \()\) and \(S.\ aureus\) \((4.8 \times 10^5\) c.f.u. \((\text{root cm})^{-1}\) \()\); Mann–Whitney test; \(P<0.005\), confirming that these two human-associated species could be considered as non-specialized, less rhizosphere-competent controls. SEM was used to assess if \(B.\ vietnamiensis\) G4 cells that could be cultured at high densities from the roots had adhered to the surface of pea roots, or had become endophytic. \(B.\ vietnamiensis\) G4 cells were observed on the root surface and were commonly visualized clustering around breaks in the root surface or along the margins of plant cells (Fig. 2b–e).

**Screening of Bcc strains for root colonization competence**

A collection of 26 genetically distinct Bcc strains, reflective of environmental and clinical sources, was assembled for rhizosphere colonization analysis (Table 1). All Bcc strains and species examined were capable of root colonization in both plant models (Fig. 3). Root colonization levels achieved by each Bcc strain differed significantly from each other in each model (one-way ANOVA; \(P<0.0001, F=20.21, \text{d.f.}=25\) on \(A.\ thaliana\); \(P<0.0001, F=4.49, \text{d.f.}=24\) on pea). Mean viable counts for individual strains ranged from \(4.2 \times 10^1\) c.f.u. \((\text{root cm})^{-1}\) \((B.\ stabilis\) LMG 14294) to \(1.0 \times 10^6\) c.f.u. \((\text{root cm})^{-1}\) \((B.\ ambifaria\) Ral-3) on the \(A.\ thaliana\) model, and \(7.7 \times 10^4\) c.f.u. \((\text{root cm})^{-1}\) \((B.\ ambifaria\) AU0216) to \(5.8 \times 10^5\) c.f.u. \((\text{root cm})^{-1}\) \((B.\ cenocepacia\ H12424) on the pea model. With the exception of \(B.\ stabilis\) LMG 14294 on \(A.\ thaliana\) and \(B.\ ambifaria\) AU0216 on pea, all Bcc strains colonized at a significantly higher level \((P<0.05)\) than the non-specialized root colonizer controls, \(E.\ coli\) and \(S.\ aureus\). When compared with a known rhizocompetent \(Burkholderia, B.\ phytotrophs\) PsJN (Compant et al., 2008a), 20 Bcc strains colonized \(A.\ thaliana\) roots at statistically similar levels, three Bcc strains showed significantly higher levels \((B.\ ambifaria\ Ral-3, B.\ anthina\) strains LMG 20980 and C1765), and three Bcc strains were significantly lower colonizers \((B.\ stabilis\) LMG 14294, \(B.\ ambifaria\) AU0216 and \(B.\ multivorans\) LMG 13010) (Fig. 3).

When Bcc strains were compared at the species level, overall significant differences in root colonization were detected between Bcc species both on pea (one-way ANOVA; \(P<0.0001, F=5.31, \text{d.f.}=8\)) and on \(A.\ thaliana\) (one-way ANOVA; \(P<0.0001, F=13.75, \text{d.f.}=8\)). There was no concordance in the overall level of colonization achieved by
each Bcc species or the ranking of species for each plant model (see Fig. 3). Significant colonization differences were also detected between strains of the same Bcc species for B. anthina (Mann–Whitney test; P=0.0294, U=0.00), B. cenocepacia (Kruskal–Wallis test; P=0.0204, H=13.13, d.f.=5) and B. multivorans (Mann–Whitney test; P=0.0286, U=0.00) in the pea model (Fig. 3a), and for B. ambifaria (one-way ANOVA; P<0.0001, F=39.22, d.f.=4), B. dolosa (t-test; P=0.0104, t=2.80, d.f.=22), B. multivorans (t-test; P=0.0023, t=3.48, d.f.=21) and B. stabilis (t-test; P<0.0001, t=7.46, d.f.=33) in the A. thaliana model (Fig. 3b).

When all Bcc strains were considered together, there was no overall significant difference in colonization of either plant species by strains from clinical or environmental sources (t-test; P=0.0609, t=2.04, d.f.=14, on A. thaliana; t-test, P=0.3174, t=1.04, d.f.=14, on pea). However, within individual Bcc species, the relative colonization level attained by clinical and environmental strains was different and dependent on the plant model. B. cenocepacia, B. cepacia and B. vietnamiensis clinical and environmental strains did not show significant colonization differences (P>0.05) in either plant model, whereas B. ambifaria environmental strains were significantly (P≤0.05) better root colonizers than their clinical counterparts in both plant models. In comparison, B. multivorans, B. anthina and B. stabilis relative colonization patterns differed between the two plant models. A maximum 77-fold root colonization difference was recorded between environmental and clinical strains of B. stabilis on A. thaliana. Interestingly, genetically highly similar B. cenocepacia strains AU1054 and HI2424, originating from clinical and environmental sources, respectively (LiPuma et al., 2002), achieved high and consistent colonization levels in both plant models (Fig. 3).

In addition to quantitative rhizosphere analysis by viable count enumeration, a qualitative evaluation of colonization was carried out by SEM for the pea root model (Fig. 2). Bacterial cells were observed adhering to the root surface for all Bcc strains analysed (Fig. 2e–i), but it was apparent that colonization did not occur evenly over the root surface, and bacterial cells could be dense in some regions while sparse in others (Fig. 2). Bacterial cells were often observed clustering around plant tissue cracks, tears and cell margins. B. ambifaria AMMD, B. multivorans LMG 20090 and B. cenocepacia LMG 19487 originated from clinical and environmental sources, respectively, and were consistently detected by both methods. B. cenocepacia PC259 and B. cenocepacia LMG 19487 originated from clinical and environmental sources, respectively, and were consistently detected by both methods. B. cenocepacia LMG 19487 originated from clinical and environmental sources, respectively, and were consistently detected by both methods.
13010, and \textit{B. cenocepacia} K56-2 and BC-2 all demonstrated very low numbers of surface visible bacteria, between one and 25 putative bacterial cells over four random root sections (Fig. 2; Table 1), despite all these strains showing viable bacterial counts of greater than $10^5$ c.f.u. per root section (Fig. 3). Bacterial numbers too numerous to count were observed at the root surface for \textit{B. cepacia} ATCC 25416, \textit{B. stabilis} LMG 14294, \textit{B. dolosa} AU0746 and \textit{B. cenocepacia} M54 (SEM score=4; Table 1), yet the mean colonization level for these four strains was not significantly different from the four strains with low root surface colonization scores (Fig. 3).

**Effect of deletion of the third chromosomal replicon on Bcc root colonization**

Agnoli et al. (2012) recently demonstrated that Bcc bacteria could survive complete loss of their third chromosomal replicon (c3) by creation of c3-null mutants. These mutants were viable despite loss of over 1 Mb of DNA, but they had lost key phenotypes associated with virulence in nematode, wax moth larvae and fly models, as well as significant antifungal activity (Agnoli et al., 2012). The \textit{A. thaliana} model was used to evaluate the role of the third Bcc replicon in an active rhizosphere colonization interaction. Chromosome deletion mutants (Δc3) of five Bcc species were available (Table 1) and overall, no significant difference in root colonization was observed between these and their wild-type parents, with the mean root colonization level remaining high at over $1.1 \times 10^6$ c.f.u. (root cm)$^{-1}$ (Fig. 4; one-way ANOVA; $P=0.7391$, $F=3.45$, d.f.=8). However, significant differences were seen for \textit{B. ambifaria} LMG 19182, where the Δc3 mutant had a reduced colonization ($t$-test; $P=0.0002$, $t=3.97$, d.f.=50) compared with its wild-type parent. In contrast, the \textit{B. vietnamiensis} LMG 10929 Δc3 mutant demonstrated an enhanced colonization phenotype ($t$-test; $P=0.0411$, $t=2.10$, d.f.=43).

**DISCUSSION**

We have described the development and utilization of two \textit{in vitro} microcosms that simulate bacterial rhizosphere colonization of pea and \textit{A. thaliana}. Although the pea microcosm was broadly similar to previously described systems (Bevivino et al., 1994; Heungens & Parke, 2000; O’Sullivan et al., 2007), the \textit{A. thaliana} developed herein represents an advance in terms of modelling active root colonization. Previous \textit{A. thaliana} models have been based on seed/seedling immersion into bacterial suspensions or drop/spray inoculation onto roots or leaf surfaces (Conn et al., 2008; Digonnet et al., 2012; Dong et al., 2003; Timmusk & Wagner, 1999). Our method was designed to delimit unequivocally the initial plant–inoculum interaction with the primary root tip. The colonization levels recorded in upper sections of the root therefore resulted from legitimate bacterial colonization, dependent on active bacterial processes such as mobility, adherence and growth, and not by the passive distribution of the inoculum. The \textit{A. thaliana} model was therefore able to successfully discriminate \textit{B. vietnamiensis} STM phenotypes of hypo- and hyper-colonization only previously identified via competition experiments in the pea rhizosphere microcosm (O’Sullivan et al., 2007).

One of the main aims of our study was to determine if rhizocompetence differs between Bcc strains of environmental versus clinical origin. Overall, Bcc strains from both sources demonstrated equivalent rhizocompetence. This observation further highlights the difficulty in identifying phenotypic or taxonomic differences between clinical and environmental Bcc strains. At the species level, significant differences in root colonization were observed between clinical and environmental strains on both plant models for one of the most represented species in our analysis, \textit{B. ambifaria} but not on \textit{B. cenocepacia} (five and six native isolates, respectively, Table 1). \textit{B. cenocepacia} strains showed high root colonization irrespective of their origin, whereas \textit{B. ambifaria} environmental strains were significantly better root colonizers than the clinical isolates. \textit{B. cenocepacia} strains are highly abundant in the rhizosphere (Mendes et al., 2007; Ramette et al., 2005; Zhang & Xie, 2007) and in CF infections (LiPuma, 2010). \textit{B. cenocepacia} has therefore been regarded as the highest risk
species for human infection following hypothetical biotechnological use of Bcc (Chiarini et al., 2006). Our results support this argument by highlighting the fact that B. cenocepacia strains retrieved from lung infections keep an intact potential to return to the natural environment, in particular to the rhizosphere.

In contrast, following this rationale B. ambifaria has low reported prevalence and virulence in CF infections (<3%; LiPuma, 2010) but high abundance in the rhizosphere, suggesting this species may be safer for environmental release. The more limited capability of B. ambifaria as an opportunistic human pathogen has been discussed by others as a reason to relax restrictions on the biotechnological use of this species (Chiarini et al., 2006). The B. ambifaria clinical isolates analysed in this study showed a moderate but significantly lower fitness towards the root than their environmental counterparts. Vial et al. (2010) demonstrated that a clinical isolate of B. ambifaria (HSJ1) spontaneously showed phase variation, mediated by genetic and epigenetic modifications. These resulted in stable B. ambifaria variants with improved rhizocompetence and reduced virulence (Vial et al., 2010). These findings support our data and suggest that the B. ambifaria clinical isolates analysed here derived from environmental ancestors that then evolved via phase variation to infect the CF lung, and in turn partially lost rhizocompetence. Interestingly, despite the known pathogenic potential of Bcc bacteria and the use of plants as models of infection (Agnoli et al., 2012), none of the strains we examined (Table 1) demonstrated detrimental effects on the health of the pea or A. thaliana plants. High numbers of bacteria on the pea root surface were only observed for four of the 14 Bcc strains examined by SEM (Fig. 2; Table 1), yet the root colonization levels for these strains were not significantly different from those with low root colonization scores (Table 1; Fig. 3). These data suggest that certain Bcc strains may be endophytic in the pea model and that this very close internal interaction is not detrimental to the plant. Several plant-beneficial Burkholderia species have been well characterized as endophytes (Suárez-Moreno et al., 2012). Our pea rhizosphere data suggest that multiple Bcc species (Table 1) may also be capable of this lifestyle but further systematic analysis will be required to prove the extent of their endophytic capabilities.

The comparative root colonization data obtained across the Bcc species suggest they are fundamentally environmental bacteria with an intrinsic ability to colonize the plant rhizosphere. The maintained rhizocompetence of Bcc mutants lacking chromosome 3 (Fig. 4) also supports this hypothesis and showed that the majority of rhizosphere fitness determinants were encoded on chromosomes 1 and 2. These are the more ancestral replicons within the Bcc genome, encoding the majority of core phenotypic functions and fewer accessory functions (Agnoli et al., 2012; Drevinek et al., 2008; Holden et al., 2009). The rhizocompetence of four of the five c3-null mutants screened was not diminished in the A. thaliana model (Fig. 4). However, the B. ambifaria AMMD Δc3 mutant colonized at a level approximately 1 log below its wild-type parent (Fig. 4), suggesting that species- or strain-specific differences may occur in terms of certain c3-encoded rhizocompetence traits. The loss of virulence in multiple models of infection and greatly diminished antifungal activity have been observed for these c3-null mutants (Agnoli et al., 2012), and in the case of B. ambifaria AMMD, c3 also seems to play a minor role in rhizocompetence.

Overall, our data on the c3-null mutants suggest Bcc rhizocompetence can be considered a core Bcc attribute, and interestingly, we also showed that loss of this replicon can actually lead to enhanced root colonization in the case of B. vietnamiensis LMG 10929 Δc3 (Fig. 4). Enhanced colonization was also found in the competitive STM experiments that resulted in the identification of B. vietnamiensis G4 hyper-colonization mutants such as 3-E9 (O’Sullivan et al., 2007), which also showed hyper-colonization on our A. thaliana model. Using STM, O’Sullivan et al. (2007) demonstrated that multiple genes were involved in rhizosphere fitness, but at the time of their study, only the draft B. vietnamiensis G4 genome was available. Using the now complete G4 genome sequence, the 67 transposon mutations associated with loss of rhizocompetence map as follows: 41 to c1, 16 to c2, five to plasmid pBV1E01, four to plasmid pBV1E04, one to plasmid pBV1E03 and zero to c3. With greater than 80% of these rhizocompetence genes mapping to chromosomes 1 and 2, and a lack of mutations mapping to c3, our data on the minor role of c3 in Bcc root colonization are supported.

In conclusion, using a specifically developed A. thaliana model of active root colonization and an optimized model of P. sativum rhizosphere competence, we were able to screen the root colonization ability of a panel of Bcc strains systematically. Our results support the notion that the ability to colonize the rhizosphere is an evolutionarily ancient Bcc trait and that, ultimately, all Bcc bacteria may come from the natural environment, making it difficult to establish inherent differences between clinical and environmental Bcc strains. The rhizosphere is a reservoir of Bcc strains which are potential biocontrol agents, potential human pathogens and in certain cases both (Berg et al., 2005). In the case of B. ambifaria, the consistently lower root colonization ability seen in clinical strains suggests a degree of specialization may occur in certain species. The limited effect of deletion of the third chromosomal replicon on rhizocompetence is also worthy of further follow up. As this replicon is primarily associated with Bcc virulence traits (Agnoli et al., 2012), genetic engineering with c3-null mutants as a platform may be a viable strategy from which to derive safe Bcc for biotechnological use.

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