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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.

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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 anthina* 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.

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 phytophirmans* PsJN, 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. cenocepacia* 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. cenocepacia* 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. phytophirmans* PsJN (Compant *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 % (w/v) 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 × 10^8 c.f.u. 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

(Vidal-Quist *et al.*, 2013), with a minimum of 12 seedlings [c.f.u. (cm root)$^{-1}$ or c.f.u. per root section] evaluated for each test organism.

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 10^6–10^7 c.f.u. ml^{-1} 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^{-1} 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)$^{-1}$]. 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 *Photorhabdus 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 super optimal broth medium (SOB) supplemented with 10 mM $MgCl_2$ and 10 mM $MgSO_4$, and incubated for 6 h at 37 °C. The resulting culture was swabbed into ice-cold, sterile 0.5 M sucrose with 10 % glycerol to a final optical density of 0.5 U at 600 nm. Fifty microlitres of the cell suspension was mixed with 5 µl of plasmid DNA (0.5 µg) in a chilled 1 mm electroporation cuvette and subjected to a single electroporation pulse (capacitance 25 µF, voltage 2 kV, resistance 200 Ω; resulting in a time constant of 4.1) using a Gene Pulser apparatus (Bio-Rad). Immediately after the pulse, 0.95 ml of SOB with catabolic repressor medium (SOC), supplemented with 10 mM $MgCl_2$ and 10 mM $MgSO_4$, were added to the cuvette and this was incubated for 1 h at 37 °C without agitation. The suspension was finally plated onto LB containing 50 µg chloramphenicol ml^{-1} and incubated at 37 °C for 2 days. Light emission of transformant colonies was checked using a LUMIstar OPTIMA luminometer (BMG Labtech). A single transformed derivative was designated *B. vietnamiensis* G4lx and its light emission stability determined by monitoring bioluminescence, OD_{600} and viable counts of consecutive subcultures in LB broth, with and without antibiotic supplement.

Bioluminescence imaging. *B. vietnamiensis* G4lx was tested in the *A. thaliana* microcosm as described above. Bacterial light emission was detected on undisturbed and unsealed inoculation plates using an IVIS-200 Imaging System (Xenogen) with the following settings: 20 s exposure, small (Hi-Res) binning, f-stop=1–8, photographic=1. The inoculated plants were kept within the dark chamber for at least 5 min prior to image acquisition to avoid leaf autoluminescence. Captured raw data were processed with Living Image 4.2 software (Caliper).

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 %, w/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_2 (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 $\log_{10}$-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 1. Bcc strains used in this study

Burkholderia species	Strain designation*	Sequence type†	Original source‡	Comments and references pertinent to strain§
B. cepacia (I)	LMG 18821	5	Clin	CF patient respirator; Mahenthiralingam *et al.* (2000)
	ATCC 25416^T (LMG 1222^T)	10	Env	Type strain; isolated from sour skin disease of onions (USA); genome sequenced strain (2509276048); Mahenthiralingam *et al.* (2000). High numbers of bacteria observed on the pea root surface by SEM (score=4)
B. multivorans (II)	LMG 13010^T	397	Clin	CF patient (Belgium), non-epidemic; Mahenthiralingam *et al.* (2000). Low numbers of bacteria observed on the pea root surface by SEM (score=1)
	ATCC 17616 (LMG 17588)	21	Env	Soil (USA), genome sequenced strain (641228482); Mahenthiralingam *et al.* (2000)
B. cenocepacia (III)	K56-2 (LMG 18863)	30	Clin	CF patient (Toronto, Canada), epidemic, MLST lineage III-A, amenable to genetic manipulation; Mahenthiralingam *et al.* (2000). Low numbers of bacteria observed on the pea root surface by SEM (score=1)
	AU1054	122	Clin	CF patient (USA), MLST lineage III-B, genetic clone of strain HI2424, genome sequenced strain (637000046); Mahenthiralingam & Drevinek (2007)
	HI2424	122	Env	Soil (USA), MLST lineage III-B, genetic clone of strain AU1054, genome sequenced strain (639633014); Mahenthiralingam & Drevinek (2007)
	HI2424Δc3	122	Env	c3-null mutant of HI2424; Agnoli *et al.* (2012)
	BCC1283	250	Env	Industrial contamination isolate; Mahenthiralingam *et al.* (2008)
	M36	38	Env	Maize rhizosphere (USA), biocontrol strain 'Type Wisconsin' used in Blue Circle, withdrawn in 2003; Payne *et al.* (2005). Low numbers of bacteria observed on the pea root surface by SEM (score=2)
	BC-2	203	Env	Maize rhizosphere (USA), biocontrol strain, MLST lineage III-B; Payne *et al.* (2005). Low numbers of bacteria observed on the pea root surface by SEM (score=1)
B. stabilis (IV)	LMG 14294^T	50	Clin	CF patient sputum (Belgium), non-epidemic. High numbers of bacteria observed on the pea root surface by SEM (score=4)
	BCC1308	337	Env	Industrial contamination isolate; Mahenthiralingam *et al.* (2008)
B. vietnamiensis (V)	PC259 (LMG 18835)	58	Clin	CF patient (USA), non-epidemic; Mahenthiralingam *et al.* (2000). Low numbers of bacteria observed on the pea root surface by SEM (score=2)
	G4 (ATCC 53617)	60	Env	Industrial waste facility (USA), US patents 4925802 and 55443317, genome sequenced strain (640069307); O'Sullivan *et al.* (2007)
	LMG 10929^T	65	Env	Rice rhizosphere (Vietnam); Mahenthiralingam *et al.* (2000)
	LMG 10929Δc3	65	Env	c3-null mutant of LMG10929; Agnoli *et al.* (2012)
B. dolosa (VI)	AU0746	70	Clin	CF patient (USA), non-epidemic; Coenye *et al.* (2001). High numbers of bacteria observed on the pea root surface by SEM (score=4)
	AU0645 (LMG 18943^T)	72	Clin	CF patient (USA); Coenye *et al.* (2003)
B. ambifaria (VII)	LMG 19467	78	Clin	CF patient (Australia); Coenye *et al.* (2003)
	AU0216	166	Clin	CF patient (USA); Mahenthiralingam *et al.* (2008)
	AMMD^T (LMG 19182^T)	77	Env	Pea rhizosphere (USA), unregistered biocontrol strain, genome sequenced strain (637000047); Coenye *et al.* (2003) and Parke & Gurian-Sherman (2001). Low numbers of bacteria observed on the pea root surface by SEM (score=1)
	LMG 19182Δc3	77	Env	c3-null mutant of LMG19182; Agnoli *et al.* (2012)

Table 1. cont.

Burkholderia species	Strain designation*	Sequence type†	Original source‡	Comments and references pertinent to strain§
	Ral-3	79	Env	Corn rhizosphere, biocontrol strain, application by Agrium (1998) withdrawn in 2000; Parke & Gurian-Sherman (2001). Low numbers of bacteria observed on the pea root surface by SEM (score=2)
	M54 (R-5142)	73	Env	Maize rhizosphere (USA), biocontrol strain 'Type Wisconsin' used in DENY^{TM} revoked in 2004; Payne *et al.* (2005). High numbers of bacteria observed on the pea root surface by SEM (score=4)
B. anthina (VIII)	C1765 (LMG 20983)	85	Clin	CF patient (UK); Coenye *et al.* (2003)
	LMG 20983Δc3	85	Clin	c3-null mutant of LMG20983; Agnoli *et al.* (2012)
	LMG 20980^{T}	86	Env	House plant rhizosphere (Nashville, USA); Coenye *et al.* (2003). Low numbers of bacteria observed on the pea root surface by SEM (score=2)
B. pyrrocinia (XI)	LMG 14191^{T} (ATCC 15958^{T})	41	Env	Soil (Japan); Coenye *et al.* (2003). Low numbers of bacteria observed on the pea root surface by SEM (score=2)
	LMG 14191Δc3	41	Env	c3-null mutant of LMG14191; Agnoli *et al.* (2012)
	BC011 (LMG 21823)	92	Env	Water (USA), biocontrol strain; Coenye *et al.* (2003). Low numbers of bacteria observed on the pea root surface by SEM (score=2)

*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).

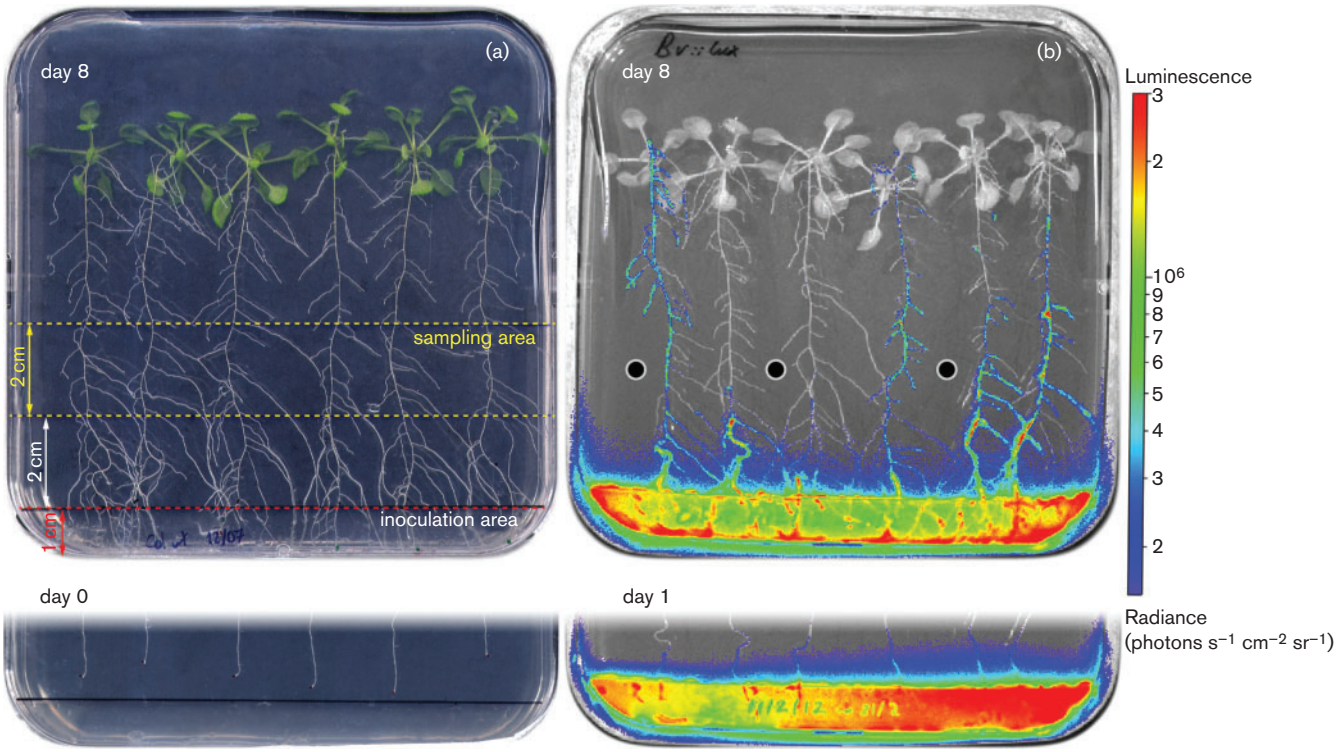

Fig. 1. *A. thaliana* rhizosphere colonization model. (a) *A. thaliana* seedlings were grown for 2 weeks vertically on MS medium until primary root tips were 1.5–2 cm above the lower edge of the plate (see lower section of a day 0 plate). At this point a standardized suspension of *B. vietnamiensis* 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 G4l× 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.

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 ($\pm$SD) $\log_{10}$ c.f.u. per root section$=5.57\pm0.18$].

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

with respect to wild-type G4 (*t*-test; $P=0.0491$, $t=2.06$, 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 10^1 to 10^7 c.f.u. ml^{-1}. After 14 days, no significant difference between the mean colonization level attained, $1.80 \pm 0.25 \times 10^6$ c.f.u. (root cm)$^{-1}$, were observed in relation to inoculation density (Kruskal–Wallis test; $P=0.4994$, $H=5.35$, d.f.=7); all future inoculations were therefore standardized to between 10^6 and 10^7 c.f.u. ml^{-1}. *B. vietnamiensis* G4 colonized pea roots at a significantly higher level than *E. coli* [5.4×10^4 c.f.u. (root cm)$^{-1}$] and *S. aureus* [4.8×10^3 c.f.u. (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).

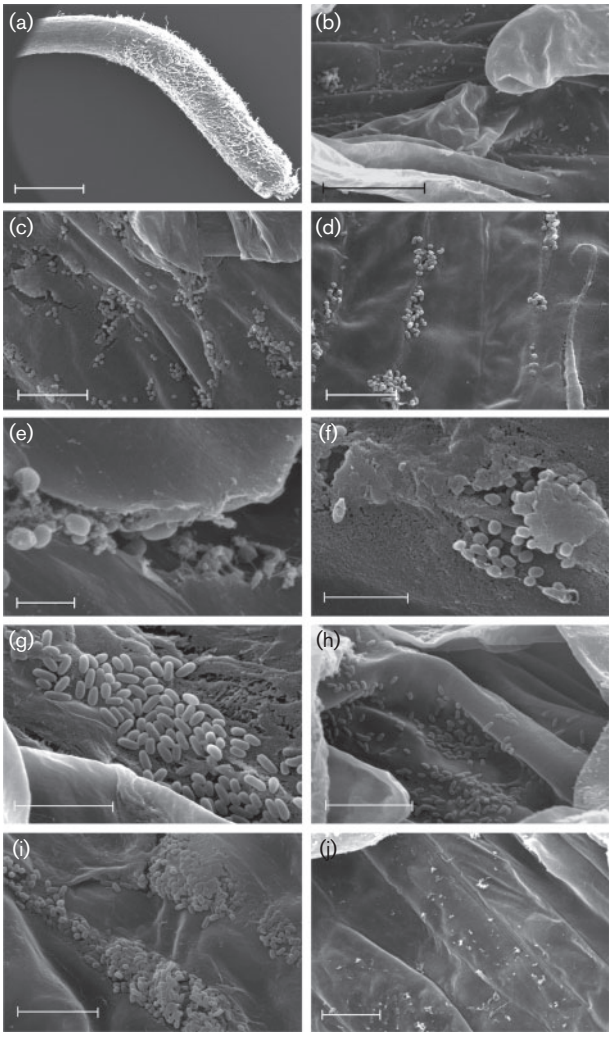

Fig. 2. Scanning electron micrographs showing surface colonization of pea (*P. sativum*) roots by Bcc bacteria. (a–e) Pea root tip and colonization of its surface by *B. vietnamiensis* strain G4 during growth in the microcosm. (f–i) Colonization by the following Bcc species (Table 1): (f) *B. cenocepacia* strain BC-2; (g) *B. cepacia* strain ATCC 25416; (h) *B. cenocepacia* strain M36; (i) *B. ambifaria* strain M54. (j) An uninoculated control pea root section demonstrating the absence of bacteria cells. Bars: (a) 2 mm, (b, j) 20 μm, (c, d, h, i) 10 μm, (e) 2 μm, (f, g) 5 μm.

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: $P<0.0001$, $F=20.21$, d.f.=25 on *A. thaliana*; $P<0.0001$, $F=4.49$, d.f.=24 on pea). Mean viable counts for individual strains ranged from 4.2×10^4 c.f.u. (root cm)$^{-1}$ (*B. stabilis* LMG 14294) to 1.0×10^7 c.f.u. (root cm)$^{-1}$ (*B. ambifaria* Ral-3) on the *A. thaliana* model, and 7.7×10^4 c.f.u. (root cm)$^{-1}$ (*B. ambifaria* AU0216) to 5.8×10^7 c.f.u. (root cm)$^{-1}$ (*B. cenocepacia* HI2424) 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 ($P \leqslant 0.05$) than the non-specialized root colonizer controls, *E. coli* and *S. aureus*. When compared with a known rhizocompetent *Burkholderia*, *B. phytophirmans* 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; $P<0.0001$, $F=5.31$, d.f.=8) and on *A. thaliana* (one-way ANOVA; $P<0.0001$, $F=13.75$, d.f.=8). There was no concordance in the overall level of colonization achieved by

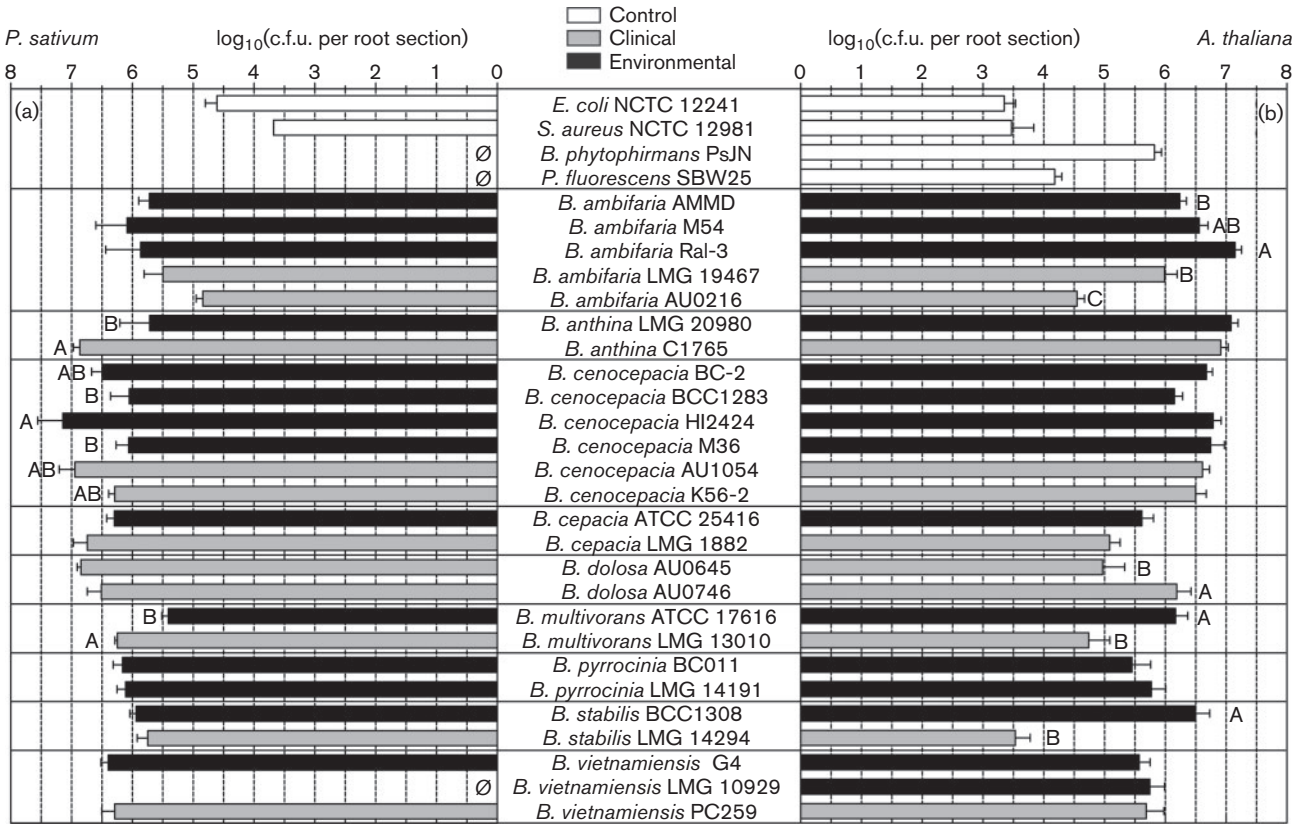

Fig. 3. Root colonization levels of all Bcc strains in the *P. sativum* (a) and *A. thaliana* (b) models. Each bacterial species examined is listed and the following colonization levels are shown: open bars, the non-Bcc control species; grey bars, clinical Bcc strains; filled bars, environmental Bcc strains. The mean $\log_{10}$ c.f.u. per root section and SEM is shown and significant colonization differences ($P \leqslant 0.05$) between strains of the same Bcc species are indicated by the upper-case letters adjacent to the error bars. *B. phytophirmans*, *P. fluorescens* and *B. vietnamiensis* LMG 10929 were not examined in the pea model (Ø).

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 ($P \leqslant 0.05$) 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

13010, and *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 *B. cepacia* ATCC 25416, *B. stabilis* LMG 14294, *B. dolosa* AU0746 and *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 *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 1.1×10^6 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 *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 *B. vietnamiensis* LMG 10929 Δc3 mutant demonstrated an enhanced colonization phenotype (*t*-test; $P=0.0411$, $t=2.10$, d.f.=43).

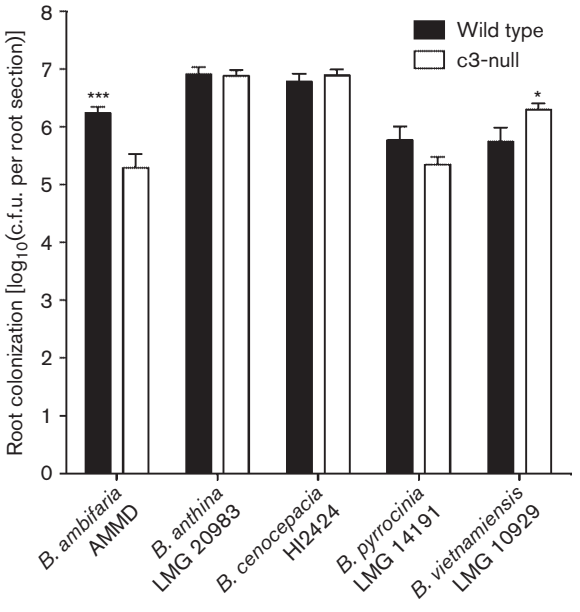

Fig. 4. The effect of third chromosome replicon deletion on Bcc root colonization in the *A. thaliana* model. Root colonization [mean +SEM $\log_{10}$(c.f.u. per root section] in the *A. thaliana* model with wild-type Bcc strains (filled bars) and their respective c3-null mutants (open bars). Asterisks indicate significant colonization differences between the wild-type strain and its c3-null mutant (*$P \leqslant 0.05$; ***$P \leqslant 0.001$).

DISCUSSION

We have described the development and utilization of two *in vitro* microcosms that simulate bacterial rhizosphere colonization of pea and *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 *A. thaliana* developed herein represents an advance in terms of modelling active root colonization. Previous *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 *A. thaliana* model was therefore able to successfully discriminate *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, *B. ambifaria* but not on *B. cenocepacia* (five and six native isolates, respectively, Table 1). *B. cenocepacia* strains showed high root colonization irrespective of their origin, whereas *B. ambifaria* environmental strains were significantly better root colonizers than the clinical isolates. *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). *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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