Distribution and genomic location of active insertion sequences in the *Burkholderia cepacia* complex

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This study aimed firstly to establish the distribution and copy number within the *Burkholderia cepacia* complex of three insertion sequences (IS402, IS407 and IS1416) that possess the ability to activate transcription and hence influence gene expression. A second aim was to map the genomic insertion sites of one of the active insertion sequences (IS407) to establish putative links between insertion site and downstream gene activation. The resulting data revealed that all three insertion sequences were present in one-third of the 66 isolates tested. The three insertion sequences were prevalent across the nine *B. cepacia* complex species, although IS402 was absent from the 16 *Burkholderia anthina* strains tested and IS407 was absent from all 10 *Burkholderia pyrrocinia* strains. IS407 copies from six strains (two *Burkholderia cenocepacia* strains and one strain each of *Burkholderia multivorans*, *Burkholderia stabilis*, *Burkholderia vietnamiensis* and *B. anthina*) were mapped to the genome using hemi-nested inverse PCR. Insertions were found upstream of genes with wide-ranging functions. This study suggests that the abundance and distribution of these active insertion sequences is likely to affect genomic plasticity, and potentially gene transcription and pathogenicity.

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INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) is composed of a group of highly adaptable bacteria that occupy diverse ecological niches (Coenye & Vandamme, 2003) and cause life-threatening pulmonary infections in patients with cystic fibrosis (CF) and chronic granulomatous disease (Govan *et al.*, 1996; Sirinavin *et al.*, 2004; Nasser *et al.*, 2004). Polyphasic taxonomy has revealed that the Bcc comprises nine closely related species or genomovars (Coenye *et al.*, 2001). While each of these genomovars has been isolated from CF patients, *Burkholderia cenocepacia* (formerly genomovar III) and *Burkholderia multivorans* (formerly

genomovar II) are responsible for approximately 90 % of infections (LiPuma *et al.*, 2001; Mahenthalingam *et al.*, 2005). Originally identified as a cause of soft rot of onions (Burkholder, 1950), and more recently as a cause of ovine mastitis (Berriatua *et al.*, 2001), the group is also characteristically and intrinsically multidrug-resistant (Nzula *et al.*, 2002).

The molecular and genetic basis for the flexibility of these bacteria has yet to be clarified but should be aided by the annotation of the genome of *B. cenocepacia* strain J2315 currently underway at the Sanger Centre (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Recent annotation of the closely related species *Burkholderia pseudomallei* and *Burkholderia mallei* has emphasized the impact of insertion sequences (ISs) and bacteriophages on both the plasticity and adaptability of these organisms (Holden *et al.*, 2004; Nierman *et al.*, 2004). Furthermore, previous studies have shown that ISs are shared between these highly virulent species and *B. cenocepacia* (Mack & Titball, 1998). ISs are

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Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; hINVPCR, hemi-nested inverse PCR; IS, insertion sequence.

small, mobile genomic elements possessing only the genes that allow their transposition from one genomic site to another. In addition to their ability to cause genomic rearrangements such as deletions, duplications and recombination, these elements can also insert into genes and promoter regions and may hence affect gene transcription (Wood *et al.*, 1991; Miché *et al.*, 2001; Mahillon & Chandler, 1998). To date, at least 16 different types of IS have been discovered within the Bcc (Wood *et al.*, 1991; Tyler *et al.*, 1996; Lessie *et al.*, 1996; Miché *et al.*, 2001; Liu *et al.*, 2003), supporting the hypothesis that ISs play a key role in the diversity of these organisms. Of particular interest are active ISs that can upregulate the transcription of genes close to their point of insertion. One of these ISs can affect the transcription of genes involved in adaptation of the Bcc to diverse environmental conditions, thus enabling them to utilize potentially toxic compounds as a carbon source (Hubner & Hendrickson, 1997). In addition, several studies have demonstrated the *in vitro* activation of Bcc genes by ISs (Scordilis *et al.*, 1987; Wood *et al.*, 1991; Miché *et al.*, 2001).

To our knowledge, little work has been done on the potential contribution of active ISs to the evolution of Bcc species as opportunistic pathogens for plants, animals and humans, particularly studies that take account of recent developments in the complex taxonomy of these bacteria. In this study, we examined the distribution and copy number of three active ISs (IS402, IS407 and IS1416) in 66 isolates from clinical and environmental sources; the isolates represented all nine genomovars and included six strains with undetermined genomovar status. In addition, IS407 insertion sites of six strains were mapped to the genome and the impact of their insertion on downstream genes considered.

METHODS

Bacterial strains. Sixty-six strains of both environmental and clinical origin were used in this study. Thirty of these were from the Bcc panel (Mahenthiralingam *et al.*, 2000), 28 were added to represent recently identified genomovars (VI to IX) (Coenye *et al.*, 2003), six strains were found to be of undetermined genomovar status (Peter Vandamme, unpublished data) and two strains were from the Edinburgh Strain Repository. Sixty of these strains were shown to be non-clonal based on PFGE analysis (Butler *et al.*, 1995), four were clones from the ET12 lineage (LMG 16656^T, LMG 18826, LMG 18863 and LMG 18827) and two *B. cepacia* strains from different origins were also clonal (ATCC 25416^T and C2828).

DNA extraction and PCR. Bacteria were grown overnight at 37 °C on Columbia-base agar (Oxoid). DNA was extracted using a Puregene DNA isolation kit (Gentra systems), with a few modifications (Kenna *et al.*, 2003). PCR to test for the presence of IS402, IS407 and IS1416 was conducted using the PCR primers and amplification cycling conditions described by Miché *et al.* (2001). A 25 µl reaction contained the following PCR reagents from Qiagen: 20 ng of DNA, 250 µM (each) deoxynucleoside triphosphate, 1.5 mM MgCl₂, 1 × PCR buffer, 20 pmol each primer (Invitrogen), 1 U *Taq* polymerase and sterile distilled water. Ten microlitres of PCR product was run alongside a 1 kb Plus DNA ladder (Invitrogen) on a 1.2% pre-cast E-gel (Invitrogen). A positive control (the *B. vietnamiensis* type strain LMG 10929^T) that had previously been

found to be positive for all three ISs by Miché *et al.* (2001) was used, and negative controls consisted of PCR reaction mix without DNA.

Southern blotting. Southern blotting was conducted using a digoxigenin (DIG)-labelled probe. Briefly, approximately 2.5–4 µg DNA was digested at 37 °C for 4 h with 10 U *Bgl*II, 10 U *Hind*III or 12 U *Eco*RI (Promega). DNA was separated on a 0.8% 0.5 × TBE agarose gel for 16 h at 1.0 V cm⁻¹ alongside 0.05 µg DIG-labelled DNA molecular mass marker II (Roche Diagnostics). DNA from the agarose gels underwent Southern blotting transfer via a capillary-transfer method onto a positively charged nylon membrane as described by Sambrook *et al.* (1989). The IS407 DIG-labelled probe was created using a PCR DIG probe synthesis kit (Roche Diagnostics) with the primers used by Miché *et al.* (2001) to amplify IS407. The membrane was pre-hybridized with pre-hybridization solution [5 × SSC, 0.1% N-lauroylsarcosine (w/v), 0.02% SDS (w/v), 2% blocking solution (v/v) (Roche Diagnostics)] in a hybridization bag (Roche Diagnostics) for 4 h at 68 °C. This step was followed by 12 h hybridization using 100 ml pre-hybridization buffer and 20 µl DIG-labelled probe. All post-hybridization washes were conducted using materials from Roche Diagnostics, unless otherwise stated. Post-hybridization washes consisted of two 5 min washes at room temperature with 2 × SSC and 0.1% SDS, followed by two 15 min washes at 68 °C with pre-heated 0.1 × SSC and 0.1% SDS. The membrane was then incubated for 30 min in blocking solution, followed by 30 min in blocking solution with anti-DIG AP Fab fragments before two 15 min washes in washing buffer and 3 min in detection buffer. To allow visual detection of the bound probe 20–25 drops of 'CDP* ready-to-use' was added. The membrane was sealed in a hybridization bag and left with Lumi-film chemiluminescent detection film to expose for 2–10 min. Film was processed using an Optimax film processor (Protec Medizintechnik).

Hemi-nested inverse PCR. Hemi-nested inverse PCR (hINVPCR) was carried out, with some modifications, based on a method by Yesilkaya *et al.* (2003). The main steps of the method are illustrated in Fig. 1. To reach as many insertion sites as possible, both sides of the IS were targeted using primers specific for both the 5'- and 3'-ends of the IS. Briefly 2.5–4 µg DNA was digested with 5 U of *Eae*I (New England BioLabs) for 2 h at 37 °C. Digested DNA was heat-inactivated at 65 °C, then self-ligated in a thermal cycler with a heated lid at 16 °C for 16 h. The ligation mixture consisted of 0.5–2 µg DNA in 20 µl 10 × ligation buffer [stock: 50 mM Tris/HCl (pH 7.6), 10 mM dithioerythritol, 500 µg bovine serum albumen ml⁻¹] and 4 U *T4* ligase (Roche Diagnostics) made up to a final volume of 200 µl with sterile distilled water.

Hemi-nested inverse PCR was carried out as follows. A 50 µl primary PCR reaction was set up exactly as described in the previous section but using 1 × Hot StarTaq PCR buffer, 1.25 U HotStarTaq polymerase (Qiagen) and 5 µl circularized DNA. The PCR amplification conditions were as follows: 95 °C for 15 min, followed by 15 cycles of 94 °C for 1 min, 1 min at the appropriate annealing temperature using primers for testing either the right side of IS407 (BC4 and BC6) or the left side (BC1 and BC2) (Table 1) and 72 °C for 2 min and a final extension step of 1 cycle of 72 °C for 3 min. Two microlitres of primary PCR product was used to set up a 50 µl nested PCR reaction. All the other conditions remained the same apart from the primer concentration, which was increased to 60 pmol. The primers used in the nested PCR amplification were BC3 and BC2 (left side of IS407), and BC5 and BC6 (right side of IS407). The PCR amplification conditions were the same as for the primary PCR except that the number of cycles was increased from 15 to 25. Twelve microlitres of the nested PCR product was electrophoresed for 5 h at 1.3 V cm⁻¹ in a 2% MetaPhor agarose gel (Cambrex Biosciences). Agarose gels were examined using a UV transilluminator after staining with ethidium

Fig. 1. (a) Position of PCR and sequencing primers in IS407 (black arrows) in relation to *EaeI* restriction sites (black arrows with dotted lines). BC2, BC4 and BC5, forward PCR and sequencing primers; BC1, BC3 and BC6, reverse PCR and sequencing primers. (b) Hemi-nested inverse PCR. Genomic DNA was restricted with *EaeI*, heat-inactivated and ligated. The resulting fragments consisted partly of IS407 DNA and partly of IS407-flanking sequence. A primary PCR reaction followed by nested PCR aimed to amplify the region from the end of the IS out to the flanking DNA. DNA was electrophoresed on a 2% MetaPhor gel and individual bands separated using a band-stabbing technique (Bjourson & Cooper, 1992). These bands were then purified and the DNA sequenced.

bromide. Bands of interest were separated from one another using a hypodermic needle (BD Microlance 3, Becton Dickinson) as a method of extracting the amplified product from the gel (Bjourson & Cooper, 1992). The DNA attached to the needle was washed into fresh nested

PCR mix and reamplified. Bands were then purified using a QIAquick PCR purification kit (Qiagen) and sequenced. The sequencing programme was as follows: 95 °C for 1 min, and 25 cycles of 95 °C for 30 s, 45 °C for 15 s and 60 °C for 4 min. The sequencing mixture consisted of 4 µl ABI PRISM BigDye terminator v3.1 cycle sequencing mix (Applied Biosystems), 40 pmol either primer BC2 or primer BC6 and 0.35–0.8 µl DNA, depending on the concentration of DNA. The mixture was made up to 20 µl with sterile, distilled water.

DNA sequencing analysis. Vector NTI 8 suite (InforMax) was used for DNA sequence analysis. The raw DNA sequence was checked for the presence of an *EaeI* restriction site and the IS407 sequence. The flanking DNA sequence was BLAST searched against the Sanger centre J2315 genome sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Any significant matches (i.e. those with greater than 89% DNA sequence similarity) were mapped to the genome and 3 kb regions downstream of the insertion site were BLAST searched using the Institute for Genomic Research database (<http://tigrblast.tigr.org/cmr-blast/>) or, if no hits were found here, against the database at the National Centre for Biotechnology Research (<http://www.ncbi.nlm.nih.gov/blast/>) for similarity to known genes. In addition overlapping ORFs were searched for with the Vector NTI software, and the DNA was translated and BLAST searched against the ExPASy database at the Swiss Institute for Bioinformatics (<http://ca.expasy.org/>).

RESULTS AND DISCUSSION

Distribution of IS402, IS407 and IS1416 in the study collection

The first aim of this study was to determine the distribution and copy number of three active ISs (IS402, IS407 and IS1416) across the Bcc including six isolates of undetermined genomovar status. PCR was used to test for the presence of the three ISs in 66 clinical and environmental strains. Thirty-two per cent (21/66) of strains were positive for IS402, 36% (24/66) for IS407 and 35% (23/66) for IS1416. The three ISs were found to be distributed across the nine species of the Bcc, with some notable exceptions (Table 2). IS402 was absent from all 16 strains of *B. anthina* tested, IS407 was absent from all 10 *B. pyrrocinia* strains and IS1416 was absent from all eight strains of *B. multivorans*. IS1416 was more common in strains of *B. pyrrocinia* (8/10, 80%) and *B. anthina* (7/16, 44%) than in strains from other genomovars. To our knowledge this is the first time that the prevalence of IS1416 has been investigated across the whole Bcc. IS1416 was only recently found to be present in *Burkholderia* species other than *Burkholderia glumae*, the

Table 1. Hemi-nested inverse PCR primer sequences and annealing temperatures

Primer	Primer sequence	Annealing temperature
BC1	5'-TGCTGTTCCGTAAAGCGCTT-3'	
BC2	5'-CAAGGACCTCGAGGTGGAGAATGCC-3'	65 °C (BC1 and BC2)
BC3	5'-GAACCTTACTAGACTCCGGC-3'	65 °C (BC3 and BC2)
BC4	5'-CGGGCAAGCCAACGCAGAAT-3'	
BC6	5'-GTGTGACGCCGTTTCGCATAC-3'	60 °C (BC4 and BC6)
BC5	5'-GATGCTCCTGCCGCTTTCCA-3'	60 °C (BC5 and BC6)

Table 2. Prevalence of IS402, IS407 and IS1416 in the Bcc, and IS407 copy number

Abbreviations: CF, cystic fibrosis; CGD, chronic granulomatous disease; UTI, urinary tract infection.

Strain	Source	Presence/absence of IS			IS407 copy number*
		IS402	IS407	IS1416	
<i>B. cepacia</i>					
ATCC 25416 ^T	Onion, USA	—	—	+	—
C2828	CF, UK	—	—	+	—
LMG 17997	UTI, Sweden	+	—	—	—
LMG 18821	CF, Australia	—	—	—	—
ATCC 17759	Soil, Trinidad	—	—	—	—
<i>B. multivorans</i>					
LMG 13010 ^T	CF, Belgium	—	—	—	—
LMG 18825	CF, UK	—	—	—	—
LMG 18824	CGD, USA	—	+	—	6
LMG 18823	Lab., USA	+	+	—	4 (1)
ATCC 17616	Soil, USA	+	+	—	4
LMG 16665	Clinical, UK	—	—	—	—
LMG 16660	CF, UK	—	+	—	7
LMG 18822	CF, Canada	—	—	—	—
<i>B. cenocepacia</i>					
LMG 16654	CF, UK	+	—	—	—
LMG 16656 ^T /J2315	CF, UK	+	+	—	10 (5)
LMG 16659	CF, UK	—	—	+	—
C2836	CF, USA	—	—	—	—
LMG 18826/BC7	CF, Canada	+	+	—	16 (5)
LMG 18863/K56-2	CF, Canada	+	+	—	—
LMG 18827/C5424	CF, Canada	+	+	—	6
LMG 18828	CF, Canada	+	—	—	—
LMG 18829	CF, USA	+	—	+	—
LMG 18830	CF, Australia	+	—	—	—
ATCC 17765	UTI, UK	—	+	+	3
<i>B. stabilis</i>					
LMG 14294 ^T	CF, Belgium	+	+	—	10 (4)
LMG 18870	CF, Canada	+	+	—	5
LMG 18888	Clinical, Belgium	—	—	—	—
LMG 14086	Clinical, UK	—	—	—	—
<i>B. vietnamiensis</i>					
LMG 16232	CF, Sweden	+	+	—	9 (9)
LMG 18835	CF, USA	+	—	—	—
LMG 18836	CGD, Canada	+	+	+	7
LMG 10929 ^T	Rice, Vietnam	+	+	+	3
<i>B. dolosa</i>					
LMG 21820	CF, UK	+	—	—	—
<i>B. ambifaria</i>					
LMG 19182 ^T	Soil, USA	+	—	+	—
<i>B. anthina</i>					
LMG 16670	Rhizosphere, UK	—	+	—	7
J2553	Soil, UK	—	—	—	—
LMG 20980 ^T	Soil, USA	—	—	—	—
J2927	Soil, UK	—	+	+	3
J2928	Soil, UK	—	+	+	3
J2941	Rhizosphere, UK	—	+	+	6
J2943	Rhizosphere, UK	—	—	—	—
J2944	Rhizosphere, UK	—	+	+	3 (1)
J2945	Rhizosphere, UK	—	+	+	3

Table 2. cont.

Strain	Source	Presence/absence of IS			IS407 copy number*
		IS402	IS407	IS1416	
J2946	Rhizosphere, UK	—	+	+	2
J2949	Rhizosphere, UK	—	—	—	—
J2950	Rhizosphere, UK	—	+	+	3
J2951	Rhizosphere, UK	—	—	—	—
J2862	Rhizosphere, UK	—	—	—	—
LMG 20982	Environmental, UK	—	—	—	—
LMG 20983	CF, UK	—	—	—	—
<i>B. pyrocinia</i>					
J2536	Soil, UK	—	—	+	—
J2542	Soil, UK	—	—	+	—
LMG 21824	CF, UK	—	—	+	—
C3909	Clinical, Italy	—	—	+	—
C3918	Clinical, Italy	+	—	+	—
C3928	Clinical, Italy	—	—	+	—
C3930	Clinical, Italy	—	—	—	—
C3993	Clinical, Italy	—	—	+	—
C3995	Clinical, Italy	—	—	+	—
C3997	Clinical, Italy	—	—	—	—
Genomovar status undetermined					
E26	CF, UK	—	+	—	3
E27	CF, UK	—	+	—	3
E571	CF, UK	—	—	—	—
E551	CF, UK	—	—	—	—
J2956	Ovine, Spain	+	—	—	—
LMG 16672	Soil, UK	—	—	—	—

*Numbers in parentheses indicate the number of IS407 copies successfully mapped to the Sanger Centre J2315 sequence for the six strains tested using hemi-nested inverse PCR.

species with which it was first associated (Miché *et al.*, 2001; Hasebe *et al.*, 1998).

Due to the small number of *Burkholderia dolosa* and *Burkholderia ambifaria* strains available, it was not initially possible to draw conclusions about the IS distribution in strains from these species. However, additional strains from the extended Bcc panel (Coenye *et al.*, 2003) were later acquired and tested with the same primers. The three additional *B. dolosa* strains tested were negative for all three ISs, and of the three additional *B. ambifaria* strains tested, only one was positive for the presence of an IS (IS407). The data in Table 2 suggested that IS407 might be uncommon or absent in *B. cepacia* (previously genomovar I). However, when an additional six *B. cepacia* strains were tested, five were positive for IS407. Likewise all eight strains of *B. multivorans* were negative for IS1416, but unlike the previous example, when an additional four *B. multivorans* strains were tested these were all negative also. Overall, our data support previous reports that different *Burkholderia* species possess different active IS elements (Hasebe *et al.*, 1998; Miché *et al.*, 2001).

The only IS to show a different distribution between clinical and environmental strains was IS402, which was present in 41 % (17/41) of clinical isolates, but in only 17 % (4/24) of environmental isolates. However, it should be noted that there was no balance between clinical and environmental isolates in each of the Bcc species. Both IS407 and IS1416 were relatively evenly distributed between clinical and environmental strains, with IS407 present in 46 % (11/24) of environmental strains and 32 % (13/41) of clinical strains, while IS1416 was found in 50 % (12/24) of environmental strains and 27 % (11/41) of clinical strains. The only animal isolate in this study was from a sheep suffering from subclinical mastitis (Berriatua *et al.*, 2001) and this strain was found to have only one of the three IS elements (IS402).

Copy-number variation of IS407

In our study, we decided that IS407 should be examined in more detail to determine copy-number variation amongst the different genomovars. IS407 was chosen because of its well-established mechanism for gene activation, which works on the basis of the outwardly directed σ^{70} -like

promoter within its IR-R (an inverted repeat at the right end of the IS) (Wood *et al.*, 1991). In addition, of the three ISs that were tested by PCR, IS407 was found to be the most common IS in J2315. Initial BLAST analysis of the J2315 sequence from the Sanger centre revealed that there were 13 copies of IS407: 10 copies on chromosome 1, one copy on the plasmid and one each on chromosomes 2 and 3.

Genomic DNA from 23 IS407-positive strains was digested and the IS407 copy number determined using Southern blotting. Three restriction enzymes (*Bgl*II, *Hind*III and *Eco*RI) were used to allow accurate coverage of the genome of the Bcc, which has been shown to vary in size from 5 Mb to just over 9 Mb (Lessie *et al.*, 1996). The copy number ranged from two to 16 with a mean of five, which was in agreement with the range noted by others (Mack & Titball, 1998; Miché *et al.*, 2001). There seemed to be a greater number of IS407 copies in isolates of *B. cenocepacia* than in other species, especially *B. anthina* (a mean of 8.75, compared to 3.75, respectively). A disparity between the clonal ET12 *B. cenocepacia* strains J2315, BC7 and C5424 was found on Southern blotting. The ET12 lineage predominates among CF patients in Canada and the UK (Govan *et al.*, 1993), and, though clonal, reports of morphological differences and variation in antibiotic sensitivities between these strains have been noted elsewhere (Winstanley *et al.*, 2001; Nzula *et al.*, 2002). In this study, BC7 was found to have 16 copies while BLAST analysis found J2315 to have 13 (although this laboratory found only 10) and C5424 had only six (Fig. 2). Likewise, the study by Mack & Titball (1998) found only eight copies for J2315. Perhaps

restrictions with a wider variety of enzymes would be necessary before all copies could be successfully identified. Alternatively, perhaps IS407 copy-number variation does exist between these strains. K56-2 was only found to be positive for IS407 when this study was nearing completion, thus no blotting data exist for this strain.

Interestingly, towards the completion of these studies, Ortega *et al.* (2005) found evidence of further differences between these clonal strains. The authors showed that the interruption, by IS402, of a predicted glycosyltransferase gene in the O antigen LPS biosynthesis gene cluster correlates with the loss of LPS O antigen in J2315. In addition, the presence of an unusual prophage element encoding phage DK4/BCepMu (Summer *et al.*, 2004; Langley *et al.*, 2003) in J2315 but not K56-2 is also associated with possession of an LPS glycosyltransferase gene. It is tempting to speculate that the presence of IS402 within the prophage interrupts glycosyltransferase activity in J2315, leading to loss of O antigen and increased endotoxicity.

IS407 insertion site mapping

The second aim of this study was to map IS407 to the genome to establish putative links between insertion site and downstream gene transcription. IS407 insertion sites from six IS407-positive strains (*B. cenocepacia* LMG 18826, *B. cenocepacia* LMG 16656^T, *B. multivorans* LMG 18823, *B. stabilis* LMG 14294^T, *B. vietnamiensis* LMG 16232 and *B. anthina* J2944) were identified by hINVPCR and mapped to the genome. These results are summarized in Table 3. In

Fig. 2. Southern blot of *B. cepacia* complex DNA digested with *Eco*RI showing the differences in IS407 copy number between the clonal ET12 *B. cenocepacia* strains C5424, BC7 and J2315 (lanes 7, 9 and 10, respectively). Lanes 1–15: LMG 16670, LMG 18836, LMG 16232, ATCC 17765, LMG 18870, LMG 14294, LMG 18827/C5424, DIG-labelled DNA molecular mass marker II, LMG 18826/BC7, LMG 16656^T/J2315, LMG 16660, ATCC 17616, LMG 18823, LMG 18824 and DIG-labelled DNA molecular mass marker II, respectively.

Table 3. Data analysis of IS407 sequencing results

Strain (species)	*Sanger Centre J2315 database matches (position)	Flanking DNA sequence matches in TIGR database (accession number)	†Translated overlapping ORFs in flanking sequence with matches to ExPASy database (accession number)
LMG 18826 (<i>B. cenocepacia</i>)	Plus strand – plasmid (81053)	High number of matches to aldehyde dehydrogenase and carbamoyl transferase proteins (60 % match to <i>B. mallei</i> aldehyde dehydrogenase family) (BMA0347)	17 ORFs; ORF1 (40 bp from IS site) – 53 % match to a probable carbamoyl transferase- <i>Leptospira interrogans</i> (Q8F5P8)
LMG 16656 ^T (<i>B. cenocepacia</i>)			
LMG 14294 ^T (<i>B. stabilis</i>)			
LMG 18826 (<i>B. cenocepacia</i>)	Minus strand – chromosome 1 (6422367)	84 % match to putative outer membrane porin protein of <i>B. mallei</i> (BMA3345)	16 ORFs; ORF1 (50 bp from IS site) – 59 % match to an outer membrane porin protein- <i>Bordetella parapertussis</i> (Q7W3G1); ORF6 (850 bp from IS site) – 52 % match to glutathione reductase- <i>Bradyrhizobium japonicum</i> (Q89NS7)
LMG 18826 (<i>B. cenocepacia</i>)	Plus strand – chromosome 2 (2117361)	~700 bp from IS – 60 % match to putative proline-rich transmembrane protein- <i>Ralstonia solanacearum</i> (RSc3315), 55 % match to putative lipoprotein- <i>B. mallei</i> (BMAA0399)	6 ORFs; ORF 2 (600 bp from IS site) – high number of matches to phage proteins, including a 24 % match to a phage protein of <i>B. cenocepacia</i> BcepBIA (Q6IWQ1)
LMG 18826 (<i>B. cenocepacia</i>)	Plus strand – chromosome 1 (2873471)	83 % match to putative outer membrane porin protein from <i>B. mallei</i> (BMA0743)	5 ORFs; ORF1 (220 bp from IS site) – 32 % match to a probable outer membrane porin lipoprotein transmembrane- <i>Ralstonia solanacearum</i> (Q8Y009); ORF5 (890 bp from IS site) – 74 % match to a probable nitrogen regulatory PII transcriptional regulator protein- <i>R. solanacearum</i> (Q8XWX5); ORF6 (1310 bp from IS site) – 62 % match to a probable NH ₃ -dependent NAD ⁺ synthetase signal peptide protein (nadE)- <i>R. solanacearum</i> (Q8XWX3)
LMG 18826 (<i>B. cenocepacia</i>)	Plus strand – chromosome 1 (450374)	No matches	7 ORFs; no significant matches
LMG 16656 ^T (<i>B. cenocepacia</i>)			
LMG 16656 ^T (<i>B. cenocepacia</i>)	Plus strand – chromosome 1 (2800416)	High number of matches to transcriptional regulators, including 59 % match to a probable transcriptional regulator- <i>R. solanacearum</i> (RSp1146)	10 ORFs; ORF 3 (1771 bp from IS site) – many matches to transcriptional regulators; highest match, 38 % match to LysR transcriptional regulator from <i>Bordetella pertussis</i> (Q7VTR6); ORF 8 (1641 bp from IS site) – 48 % match to putative hydrolase from <i>Streptomyces avermitilis</i> , also high number of matches to other hydrolases including <i>Pseudomonas syringae</i> pv. <i>tomato</i> (Q87XT7)
LMG 16656 ^T (<i>B. cenocepacia</i>)	Plus strand – chromosome 1 (1417058)	High number of matches to transposases, including transposases of <i>Geobacter sulfurreducens</i> and <i>P. syringae</i>	8 ORFs; ORF 7 (2851 bp from the IS site) – 61 % match to a putative transposition helper protein from <i>Salmonella enterica</i> subspecies <i>Enterica</i> serovar <i>cubana</i> (Q8RNK7); ORF 8 (3111 bp from the IS site) – 43 % match to ISPsy4 transposase from <i>P. syringae</i> pv. <i>tomato</i> (Q877U4)

Table 3. cont.

Strain (species)	*Sanger Centre J2315 database matches (position)	Flanking DNA sequence matches in TIGR database (accession number)	†Translated overlapping ORFs in flanking sequence with matches to ExPASy database (accession number)
LMG 16656 ^T (<i>B. cenocepacia</i>)	Plus strand – chromosome 1 (352537)	59 % match to 2-oxoglutarate dehydrogenase E1 component- <i>Bordetella bronchiseptica</i> (BB3669)	9 ORFs; no significant matches
LMG 18823 (<i>B. multivorans</i>)	No matches	‡98 % match to NADH-dependent flavin oxidoreductase- <i>Pseudomonas putida</i> (AE016783)	3 ORFs; ORF 1 (24 bp from IS site) – 100 % match to NADH-dependent flavin oxidoreductase from <i>P. putida</i> (Q88K07)
LMG 14294 ^T (<i>B. stabilis</i>)	Minus strand – chromosome 1 (460752)	61 % match to restriction enzyme type I helicase subunits and hsdR- <i>Nitrosomonas europaea</i>	15 ORFs; no significant matches
LMG 14294 ^T (<i>B. stabilis</i>)	Plus strand – chromosome 3 (817701)	~1 bp after IS site – 100 % identity to 5S rRNA-Bcc (X02629); ~120 bp from IS site – 100 % match to 23S rRNA-Bcc (X16368); ~2.1 kb from IS site – 99 % identity to 16S rRNA-Bcc (L28154)‡	7 ORFs; ORF 3 (937 bp from IS site) 75 % match (with start of sequence only) to cell wall-associated hydrolase from <i>Vibrio vulnificus</i> (Q8CME1); ORF 4 (1963 bp from IS site) – 50 % match to the hypothetical protein from <i>Deinococcus radiodurans</i> (Q9RXQ4)
LMG 14294 ^T (<i>B. stabilis</i>)	Plus strand – chromosome 3 (817699)	58 % match to conserved hypothetical protein- <i>Streptomyces coelicolor</i> (SCO5040)	11 ORFs; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	73 % match to UDP-glucuronic acid epimerase (IspL) from <i>Bradyrhizobium japonicum</i> (r2382)	No ORFs
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	70 % match to DNA helicase- <i>Agrobacterium tumefaciens</i> (AGR_C_92)	3 ORFs; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	66 % match to putative integrase/recombinase protein from <i>R. solanacearum</i> (RSc1554)	5 ORFs; ORF 2 (23 bp from IS site) – 80 % match to putative integrase/recombinase protein from <i>R. solanacearum</i> (Q8XZ52); high matches to other site-specific recombinases, phage integrase family
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	70 % match to lysyl-tRNA synthetase- <i>Bdellovibrio bacteriovorus</i> (Bd0702)	2 ORFs; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	70 % match to arabinofuranosidase (abfB)- <i>S. coelicolor</i> (SCO5932)	2 ORFs; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	64 % match to comED-non-essential protein- <i>Bacillus subtilis</i> (Bsu2556)	5 ORFs; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	67 % match to a hypothetical protein- <i>Xylella fastidiosa</i> (XF0715)	Not enough flanking sequence to look for ORFs
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	61 % match to molybdopterin biosynthesis protein MoeA- <i>Chlorobium tepidum</i> (CT1331)	1 ORF; no significant matches
LMG 16232 (<i>B. vietnamiensis</i>)	No matches	74 % match to a hypothetical protein- <i>R. solanacearum</i> (RSp0512)	2 ORFs; no significant matches
J2944 (<i>B. anthina</i>)	No matches	66 % match to a putative maltotoligosyl trehalose trehalohydrolase protein- <i>R. solanacearum</i> (RSp0512)	No ORFs

*Only DNA sequences that gave ≥ 89 % match with the J2315 sequence at the Sanger Centre were considered to be positive matches. All sequences with lower matches were not classed as matches.

†In cases where the flanking sequence matched that of J2315 flanking sequence ORFs 3 kb downstream were searched for and BLAST searched against the ExPASy database. In cases where this was not possible, existing flanking sequence was checked.

‡Matches found using National Centre for Biotechnology Research database (<http://www.ncbi.nlm.nih.gov/blast/>).

some cases, only a few of the copies from each strain were successfully mapped.

Previous studies have suggested that active ISs generally affect the transcription of genes directly next to their insertion site (Reynolds *et al.*, 1981). IS403, however, was found to have more far-reaching effects at >700 bp downstream from the insertion site (Scordilis *et al.*, 1987; Wood *et al.*, 1991). Although Miché *et al.* (2001) found that certain IS407 insertions were at the 3' end of the genes examined in their study, it was acknowledged that in general these ISs affect genes downstream of their insertion site. Since it is not clear how far from its insertion site IS407 can act, the flanking sequence 3 kb downstream of the IS site was BLAST searched.

In general, flanking sequences could not be matched to one particular gene family but rather to genes with a range of different functions (Table 3). In some cases, matches were found to putative regulatory genes such as transcriptional regulators and methylases, but also to housekeeping genes such as those involved in amino acid synthesis, aromatic compound metabolism and disulphide oxidoreductase activity. Several matches were also found to putative outer membrane porin genes and also to genes encoding transmembrane proteins. In all, there did not seem to be a link to a particular gene family, suggesting that the presence of IS407 affects potential virulence factors but also upregulation of genes crucial to cell survival as noted elsewhere for the β -lactamase genes of the Bcc (Scordilis *et al.*, 1987). Indeed, it seems likely that IS407 inserts at random locations in the genome. Although it is hard to be certain without testing a greater number of strains, there did not seem to be any evidence of a preferential locus for insertion, as has been found for IS6110 in *Mycobacterium tuberculosis* (which, like IS407, is a member of the IS3 family of ISs) (Fang & Forbes, 1997).

Interestingly, from the perspective of flanking sequence matches to J2315, two strains, LMG 18826 (BC7) and LMG 14294^T (*Burkholderia stabilis*) had identical sequence matches to the Sanger centre strain (five of the 16 BC7 IS407 copies that were successfully sequenced were mapped to J2315 and likewise four of the 10 IS407 copies from LMG 14294^T). While it is understandable that both the clonal ET12 strains J2315 and BC7 would share sequence homology, it is interesting that a *B. stabilis* strain would have the same level of similarity to J2315. However, perhaps the flanking sequence for the six IS407 copies that were not successfully sequenced in this study would not exhibit a high level of similarity to J2315, thus giving a deceptive impression of overall genome similarity. It is, however, interesting that strain LMG 16232 (*B. vietnamiensis*), the strain most successfully amplified by hINVPCR (flanking sequence for all nine copies was sequenced), showed no matches to the J2315 genome. Likewise, neither did flanking sequences from strains LMG 18823 (*B. multivorans*) and J2944 (*B. anthina*), although for both these strains only one of each of the IS407 copies was successfully sequenced.

Conclusions

In conclusion, with some interesting exceptions, this study has shown that the three active ISs IS402, IS407 and IS1416 are distributed across the nine species of the Bcc. IS407 copies from six strains were mapped to the genome and were found to have inserted upstream of a wide range of putative housekeeping, informational and hypervariable genes including genes involved in amino acid synthesis, transcriptional regulation and outer-membrane proteins. A fuller analysis of the hemi-nested inverse PCR results will be made easier by the completed annotation of the J2315 genome sequence. A closer look at genomic regions of interest will also help to determine the impact of active ISs on the diversity and variable virulence within the Bcc.

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