Genome-wide analysis of DNA repeats in *Burkholderia cenocepacia* J2315 identifies a novel adhesin-like gene unique to epidemic-associated strains of the ET-12 lineage

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Members of the *Burkholderia cepacia* complex (Bcc) are respiratory pathogens in patients with cystic fibrosis (CF). Close repetitive DNA sequences often associate with surface antigens to promote genetic variability in pathogenic bacteria. The genome of *Burkholderia cenocepacia* J2315, a CF isolate belonging to the epidemic lineage Edinburgh–Toronto (ET-12), was analysed for the presence of close repetitive DNA sequences. Among the 422 DNA close repeats, 45 genes potentially involved in virulence were identified and grouped into 12 classes; of these, 13 genes were included in the antigens class. Two trimeric autotransporter adhesins (TAA) among the 13 putative antigens are absent from the other *Burkholderia* genomes and are clustered downstream of the *cci* island that is a marker for transmissible *B. cenocepacia* strains. This cluster contains four adhesins, one outer-membrane protein, one sensor histidine kinase and two transcriptional regulators. By using PCR, we analysed three genes among 47 Bcc isolates to determine whether the cluster was conserved. These three genes were present in the isolates of the ET-12 lineage but absent in all the other members. Furthermore, the *BCAM0224* gene was exclusively detected in this epidemic lineage and may serve as a valuable new addition to the field of Bcc diagnostics. The *BCAM0224* gene encodes a putative TAA that demonstrates adhesive properties to the extracellular matrix protein collagen type I. Quantitative real-time PCR analysis indicated that *BCAM0224* gene expression occurred preferentially for cells grown under high osmolarity, oxygen-limited conditions and oxidative stress. Inactivation of *BCAM0224* in *B. cenocepacia* attenuates the ability of the mutant to promote cell adherence *in vitro* and impairs the overall bacterial virulence against *Galleria mellonella* as a model of infection. Together, our data show that *BCAM0224* from *B. cenocepacia* J2315 represents a new collagen-binding TAA with no bacterial orthologues which has an important role in cellular adhesion and virulence.

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INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) represents a group of bacteria commonly found in soil and aquatic habitats. Over the last 20 years, members of the Bcc have emerged as problematic opportunistic human pathogens in immunocompromised individuals and in patients with cystic fibrosis (CF) (Mahenthiralingam *et al.*, 2005). They have the capacity of spreading between patients and lead

to declining lung function, with necrotizing pneumonia and fatal septicaemia termed ‘cepacia syndrome’ (Mahenthiralingam *et al.*, 2005). The Bcc consists of 17 related species that have been isolated from sputum of CF patients (Vanlaere *et al.*, 2009). The virulence and transmissibility of each species are variable and *B. cenocepacia* and *B. multivorans* are the most dominant species isolated from CF patients (Segonds *et al.*, 1999; Coenye *et al.*, 2004). Based on *recA* gene analysis, *B. cenocepacia* has been subdivided into four subgroups, known as A, B, C and D. Subgroup A contains the Edinburgh–Toronto (ET)-12 epidemic strains, which are associated with ‘cepacia syndrome’ (Vandamme *et al.*, 2003). The genome sequence of *B. cenocepacia* strain J2315, an ET-12 member (Vandamme *et al.*, 2003), has been

Abbreviations: Bcc, *Burkholderia cepacia* complex; BCESM, *B. cepacia* epidemic strain marker; CF, cystic fibrosis; ECM, extracellular matrix; ET, Edinburgh–Toronto; LB, Luria broth TAA, trimeric autotransporter adhesin.

A supplementary table of primer sequences is available with the online version of this paper.

determined (Holden *et al.*, 2009). Although the pathogenesis of Bcc is not well understood, a few virulence factors, such as two quorum-sensing systems (Sokol *et al.*, 2003), iron acquisition via siderophore production (Visser *et al.*, 2004), exopolysaccharide synthesis (Moreira *et al.*, 2003) and a type III secretion system (Tomich *et al.*, 2003), have been identified.

Many pathogenic bacteria, including *Burkholderia*, have a propensity for varying the surface molecules that interact with their hosts (Rocha & Blanchard, 2002; Mahenthiralingam *et al.*, 2005). A genetic mechanism mediating such phenotype variation involves repetitive DNA sequences that are known to promote genetic variability through homologous and illegitimate recombination processes (Achaz *et al.*, 2002; Bichara *et al.*, 2006). Homologous recombination occurs between large repetitive DNA sequences and may involve conversion or reciprocal strand exchange, duplication or deletion, thereby introducing changes in the genome (Rocha *et al.*, 1999). Illegitimate recombination occurs between short closely spaced repetitive DNA sequences with few or no identical nucleotides (Bichara *et al.*, 2006). These repeats are located either within ORFs, such that alteration in the length of the repeat promotes antigenic or phase variation, or within regulatory regions, where sequence change influences transcription (Saunders *et al.*, 2000; Rocha & Blanchard, 2002). This allows the generation of a vast repertoire of abilities by which pathogenic strains can adapt to the different microenvironments of the host and evade host immune responses.

We have searched the *B. cenocepacia* J2315 genome for close repetitive DNA sequences hoping to identify novel genes whose products may be involved in pathogenicity. Particular attention was given to a class of surface-exposed proteins, so-called autotransporter adhesins. These have been shown to play critical roles in processes such as cell-to-cell aggregation, biofilm formation, serum resistance, and adherence to and invasion of host cells (Henderson *et al.*, 2004). Computer-based analysis, combined with a PCR-based assay, permitted us to identify a putative virulence-adhesin cluster that has a distribution restricted to the epidemic ET-12 lineage of *B. cenocepacia*. Furthermore, within this unique cluster, we identified and characterized a novel trimeric autotransporter adhesin (TAA) (BCAM0224) and we suggest a potential role of this protein in the pathogenesis of epidemic isolates of the ET-12 lineage of *B. cenocepacia*.

METHODS

DNA sequences of *Burkholderia* genomes. The sequences of *B. cenocepacia* J2315 and *B. pseudomallei* K96243 were taken from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/B_cenocepacia/ and http://www.sanger.ac.uk/Projects/B_pseudomallei/, respectively). The sequence of *B. mallei* ATCC23344 was taken from the J. Craig Venter Institute (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gbm>). The search for orthologues also req-

uired the use of the following genomes: *B. cenocepacia* PC184, *B. cenocepacia* AU1054, *B. cenocepacia* HI2424, *B. vietnamiensis* G4, *B. dolosa* AU0158, *B. ambifaria* AMMD, *B. pseudomallei* K96243, *B. mallei* ATCC23344, *B. lata* 383, *B. thailandensis* E264 and *B. xenovorans* LB400, available at Genomes Online DataBase v. 3.0 (<http://www.genomesonline.org/>).

Whole genome analysis to identify each class of repeats

Search for tandem repeats. *Burkholderia* genome sequences were processed using the Tandem Repeats Finder (TRF) version 3.21 program (Benson, 1999). The method identifies tandem repeats that are arranged in a contiguous head-to-tail fashion and was carried out using default settings.

Search for generic non-exact repeats. The search for different classes of repeats was computed using RepSeek (Achaz *et al.*, 2007). We searched for repeats with a threshold of $P < 0.001$, which prevents finding spurious repeats and gives a minimum seed length for the repeat in the range of 21–26 nt. This coincides with the minimal region of homology required for homologous recombination in *Escherichia coli* and *Bacillus subtilis* (Cohan, 1994). Repeat outputs were classified into five categories according to the relative distance and orientation between the two copies: distant direct (DD), distant inverted (DI), close direct (CD), overlap direct (OD) and close inverted (CI).

Coding sequence prediction and annotation tools. For all categories of close repeat elements ($n=422$), we identified their location within a gene or an intergenic region by using the Artemis interface (Rutherford *et al.*, 2000). In the cases where no function was predicted, we carried out BLASTP searches and tried to extrapolate their putative function.

Each ORF predicted by Artemis was used to identify homologous sequences with BLASTP. Lipid attachment sites (lipobox) and signal peptide cleavage motifs were searched for using tools accessed at <http://www.mrc-lmb.cam.ac.uk/genomes/dolop/dolop.htm> and <http://www.cbs.dtu.dk/services/SignalP/>, respectively. Domains were assigned by Pfam analysis at <http://www.sanger.ac.uk/Software/Pfam/>. Since we were interested in repeats constraining gene sequence or expression, an intergenic repeat was only considered if located <1000 nt upstream from the start of a gene.

To find orthologues, we compared all proteins available on *Burkholderia* genomes with the *B. cenocepacia* J2315 putative antigens we identified in this work, filtering potential homologues using a threshold E-value in BLASTP of 10^{-15} . Subsequently, we aligned the sequences, using the BLOSUM62 matrix (Henikoff & Henikoff, 1992). Finally, we retained reciprocal best hits with identity >45%.

Bacterial strains and growth conditions. The 47 *Burkholderia* strains used are described in Table 1. Bacteria were cultured in Luria-Bertani (LB) broth, with orbital agitation at 250 r.p.m., at 37 °C. When appropriate, media were supplemented with 150 µg ampicillin ml⁻¹ (for *E. coli* XL1-Blue) or 150 µg trimethoprim ml⁻¹ or 600 µg kanamycin ml⁻¹ (for *B. cenocepacia*). For *B. cenocepacia* K56-2, LB medium was supplemented with 300 mM NaCl and 10 mM H₂O₂.

DNA preparation and PCR screening. Total DNA was purified by using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's protocol. BCAM0218, BCAM0219 and BCAM0224 genes were amplified using the oligonucleotide primers F1 and R1 for the first gene, F2 and R2 for the second and F3 and R3 for the third gene (see Supplementary Table S1, available in Microbiology Online).

PCR amplification was carried out in 50 µl reaction volumes, each containing 100 ng template DNA, 0.2 µM (each) primer,

Table 1. Characteristics of the 47 *Bcc* isolates examined in this studyCF-e, Strain that spread epidemically among patients with CF; *cblA*, the cable pilus gene.

Strain name	Other strain designation	Source, location	Relevant information	Source
<i>Burkholderia cepacia</i> PC783	LMG 1222	Onion, USA	BCESM ⁻ , <i>cblA</i> ⁻	J. J. LiPuma
<i>Burkholderia multivorans</i> HI2229	LMG 17588	Soil, USA	BCESM ⁻ , <i>cblA</i> ⁻	J. J. LiPuma
<i>Burkholderia cenocepacia</i> J2315	LMG 16656	CF-e patient, UK	ET12 lineage BCESM ⁺ , <i>cblA</i> ⁺ , <i>recA</i> -A	G. Döring
K56-2	LMG 18863	CF-e patient, Canada	ET12 lineage BCESM ⁺ , <i>cblA</i> ⁺ , <i>recA</i> -A	J. J. LiPuma
BC7	LMG 18826	CF-e patient, Canada	ET12 lineage BCESM ⁺ , <i>cblA</i> ⁺ , <i>recA</i> -A	D. P. Speert
BC45		CF-e patient, Canada	ET12 lineage BCESM ⁺ , <i>cblA</i> ⁺ , <i>recA</i> -A	D. P. Speert
C5424	LMG 18827	CF-e patient, Canada	ET12 lineage BCESM ⁺ , <i>cblA</i> ⁺ , <i>recA</i> -A	D. P. Speert
AU1054	LMG 24506	CF patient, USA	<i>recA</i> -B PHDC lineage	BCCMTM/LMG
HI2424	LMG 24507	Soil, USA	<i>recA</i> -B PHDC lineage	BCCMTM/LMG
LMG 18829	PC184	CF-e patient, USA	BCESM ⁺ , <i>cblA</i> , <i>recA</i> -B PHDC lineage	G. Döring
MC0-3	LMG 24308	Maize rhizosphere, USA	<i>recA</i> -B	BCCMTM/LMG
AU12250		CF patient, USA		J. J. LiPuma
AU12265		CF patient, USA		J. J. LiPuma
LMG 13013		CF patient, Belgium		G. Döring
LMG 16654	J415	CF patient, UK	BCESM ⁻ , <i>cblA</i>	G. Döring
LMG 16659	C1394	CF-e patient, UK	Manchester epidemic strain BCESM ⁺ , <i>cblA</i> , <i>recA</i> -B	G. Döring
LMG 18828	C6433	CF-e patient, Canada	BCESM ⁺ , <i>cblA</i> , <i>recA</i> -A	G. Döring
LMG 18830	CEP511	CF-e patient, Australia	BCESM ⁺ , <i>cblA</i> , <i>recA</i> -B	G. Döring
R-665		CF patient, Canada		G. Döring
R-1445		CF patient, Canada		G. Döring
R-1448		CF patient, Canada		G. Döring
R-1449		CF patient, Canada		G. Döring
R-1471		CF patient, Canada		G. Döring
R-4194				G. Döring
R-6116		CF patient, Canada		G. Döring
R-9504				G. Döring
R-9932				G. Döring
R-10965		CF patient, Canada		G. Döring
R-13130				G. Döring
R-14086				G. Döring
R-15291				G. Döring
<i>Burkholderia stabilis</i> LMG 14294		CF patient, Belgium	BCESM ⁻ , <i>cblA</i>	D. P. Speert
<i>Burkholderia vietnamiensis</i> PC259	LMG 18835	CF patient, USA	BCESM ⁻ , <i>cblA</i>	J. J. LiPuma
<i>Burkholderia dolosa</i> AU0645	LMG 18943	CF patient, USA		J. J. LiPuma
<i>Burkholderia ambifaria</i> HI2468		–, USA		J. J. LiPuma
<i>Burkholderia anthinia</i> AU1293	LMG 21821	CF patient, USA		J. J. LiPuma
<i>Burkholderia pyrrocinia</i> BC011	LMG 21823	Water, USA		J. J. LiPuma
<i>Burkholderia ubonensis</i> LMG 20358		Surface soil, Thailand		BCCMTM/LMG
<i>Burkholderia latens</i> LMG 24064		CF patient, Italy		BCCMTM/LMG
<i>Burkholderia diffusa</i> LMG 24065		CF patient, USA		BCCMTM/LMG
<i>Burkholderia arboris</i> LMG 14939		CF patient, Belgium		G. Döring
<i>Burkholderia seminalis</i> LMG 24067		CF patient, USA		BCCMTM/LMG
<i>Burkholderia metallica</i> LMG 24068		CF patient, USA		BCCMTM/LMG
<i>Burkholderia contaminans</i> R-10269	LMG 23250	CF patient, Belgium		G. Döring
<i>Burkholderia lata</i> 383	LMG 22485	Forest soil, Trinidad		G. Döring
<i>Burkholderia phymatum</i> STM815		Soil, –		G. Döring
<i>Burkholderia phytofirmans</i> PsJN		Onion roots, –		G. Döring

0.2 mM (each) deoxynucleoside triphosphates, 1.5 mM MgSO₄, 2.5 U Platinum Taq DNA polymerase (Invitrogen) and 1 × PCR amplification buffer. Amplification was carried out as follows: initial

denaturation at 95 °C for 2 min, 25 cycles of 45 s at 95 °C for denaturation, 30 s at 60 °C for primer annealing and 60 s at 68 °C for extension, with a final 7 min extension at 72 °C.

Amplification of the *B. cepacia* epidemic strain marker (BCESM) (*esmR* gene) was also performed by using the BCESM1 and BCESM2 primers (Mahenthiralingam *et al.*, 1997) (see Supplementary Table S1). PCR conditions consisted of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 60 s for 30 amplification cycles.

Quantitative real-time PCRs. *B. cenocepacia* K56-2 was grown in LB media containing NaCl (300 mM) and H₂O₂ (10 mM) (initial OD₆₄₀ 0.2) for 17 h at 37 °C, 60 r.p.m., in 24-well polystyrene microplates under microaerophilic conditions (<5% oxygen). Total RNA was isolated from cells harvested at late-exponential phase of growth using the RNeasy mini kit (Qiagen) following the manufacturer's protocol and RNA was treated with DNase (Qiagen) for 30 min at room temperature. cDNA was synthesized from 150 ng total RNA in 10 µl mixtures containing 2.5 µM random hexamers, 1 × PCR amplification buffer, 500 µM (each) deoxynucleoside triphosphates, 0.4 U RNase inhibitor µl⁻¹, 1.25 U multi-scribe reverse transcriptase µl⁻¹ (Applied Biosystems, Roche). cDNA (25 ng) was used to perform quantitative real-time PCR of *BCAM0224* using the Applied Biosystems 7500 Real-Time PCR system and Power SYBR Green master mix reagents, along with RT1 and RT2 primers (Supplementary Table S1). The melting curves were performed in triplicate with the following parameters: 95 °C for 10 min, followed by 40 PCR cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Relative quantification of *BCAM0224* gene expression was calculated by using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001) for *B. cenocepacia* K56-2 growth in LB supplemented medium compared with LB medium as control sample. The sigma factor gene *sigA* (BCAL0918) was used as normalization gene and amplified using RT3 and RT4 primers (see Supplementary Table S1).

Cloning *BCAM0224* and constructing a *B. cenocepacia* deletion mutant. A 1950 bp fragment of the *BCAM0224* gene from *B. cenocepacia* K56-2 was amplified by PCR using primers F4 and R4 (see Supplementary Table S1), which contain *Bam*HI and *Hind*III restriction sites, respectively. This fragment was cloned into the suicide vector pDrive (Qiagen) to generate pDM1. The trimethoprim resistance cassette was excised from pUC-Tp and cloned into the pDM1 *BCAM0224* *Pst*I restriction site, resulting in pDM2. This plasmid was transformed into *B. cenocepacia* K56-2 by electroporation and transformants were selected by growth on LB agar supplemented with 150 µg trimethoprim ml⁻¹ for 48 h at 37 °C. To distinguish between single- and double-crossover mutants, trimethoprim-resistant colonies were screened by replica plate cloning for kanamycin sensitivity. The candidate insertion mutants were further characterized by PCR using primers F4 and R4, which allowed the *BCAM0224*-deficient *B. cenocepacia* K56-2 to be identified.

Adherence assays. To analyse the ability of the *BCAM0224* protein to interact with extracellular matrix (ECM) proteins, such as collagen type I, fibronectin and laminin, 96-well polystyrene microplates were coated with 10 µg ml⁻¹ of each ECM protein in TBS (137 mM NaCl, 2.7 mM KCl, 25 mM Tris base, pH 7.4) at 4 °C overnight. Cultures of *B. cenocepacia* K56-2 and the mutant strain Δ *BCAM0224* (initial OD₆₄₀ 0.2) were grown for 17 h at 37 °C, 60 r.p.m., in microaerophilic conditions. Cells were harvested, washed with TBS and resuspended in TBS to 10⁶–10⁷ cells ml⁻¹. Equal amounts (200 µl) were added to the ECM-coated wells. After incubation for 2 h at room temperature, non-bound bacteria were removed and adherent bacteria were fixed with 4% (w/v) paraformaldehyde for 20 min and stained with 1% (w/v) crystal violet for 10 min. After washing with TBS, the bound crystal violet was dissolved in 95% ethanol and the absorbance was measured at 595 nm. The experiment was repeated independently three times.

We also examined adhesion of the *E. coli* BL21 strain expressing the gene *BCAM0224*, and the empty expression vector as a control, to collagen type I. *BCAM0224* was amplified by PCR from *B. cenocepacia* K56-2 DNA using primers *BCAM0224F6* and *BCAM0224R6*, containing *Nde*I and *Bam*HI restriction sites, respectively (Supplementary Table S1). The PCR product was digested and ligated into pET-23a vector (which resulted in pDM4), and introduced into *E. coli* BL21 (DE3) by electroporation. To overexpress *BCAM0224*, cells were grown to mid-exponential phase at 37 °C, induced by addition of 1 mM IPTG and incubated for an additional 4 h at 37 °C. *E. coli* BL21 (DE3) transformed with the vector pET-23a was used as a control. *E. coli* adherence to collagen type I was performed as described for *Burkholderia* adherence assays.

***Galleria mellonella* killing assay.** Cultures of *B. cenocepacia* K56-2 and the mutant strain Δ *BCAM0224* (initial OD₆₄₀ 0.2) were grown for 17 h at 37 °C, 60 r.p.m., in microaerophilic conditions. Cells were then harvested and resuspended in a series of 10-fold serial dilutions containing 10⁶–10⁴ bacteria in 10 mM MgSO₄ plus 1.2 mg ampicillin ml⁻¹. A micrometer was adapted to control the injection volume of a micro-syringe. This apparatus was used to inject 3.5 µl aliquots into *G. mellonella* via the hindmost left proleg. Control larvae were injected with 10 mM MgSO₄ plus 1.2 mg ampicillin ml⁻¹. Following injection, larvae were placed in glass Petri dishes and stored in the dark at 37 °C. The survival and appearance was recorded at 24 h intervals until 72 h.

Complementation of the *BCAM0224*-deficient *B. cenocepacia* K56-2. A PCR fragment carrying the complete coding sequence of *BCAM0224* was amplified from *B. cenocepacia* K56-2 total DNA using primers *BCAM0224F5* and *BCAM0224R5*, containing *Nde*I and *Xba*I restriction sites, respectively. This fragment was digested with *Nde*I and *Xba*I and ligated into similarly digested pDA17 under control of the constitutive *dhfr* promoter (D. Aubert and M. A. Valvano, unpublished data), before transformation into *E. coli* DH5 α supplemented with 25 µg tetracycline ml⁻¹. The resulting replicative plasmid, pDM3, was introduced into the *BCAM0224* mutant by electroporation; trans-complementation was assessed by plate adherence assay.

Statistical analysis. Relative comparisons were done between corrected values with ANOVA test for significance. A *P*-value <0.05 was considered statically significant. XLSTAT-Life software (Micro-soft) was used to construct the Kaplan–Meier survival curves for *G. mellonella* infection.

RESULTS

Annotation of DNA close repeats in the *B. cenocepacia* J2315 genome sequence

We first evaluated the number and distribution of the different classes of repeats among the three chromosomes and the plasmid that compose the *B. cenocepacia* J2315 genome (Table 2). The size of the repeats goes from 10 bp to a maximum of 50 068 bp, and overall we found 1056 repeats. Fig. 1 illustrates the distribution of such repeats on the genome. It should be noted that very little difference is observed in the density of repeats for each replicon (Fig. 1).

We focused our interest in the 422 close DNA repeats present in the *B. cenocepacia* J2315 genome. For this, we annotated these repeats and grouped them into four classes (see Methods; Table 2). Among the 422 DNA close repeats, 348 (82%) are located within coding regions and 74 (18%)

Table 2. Number of elements identified in *B. cenocepacia* J2315 for each class of repeats

<i>B. cenocepacia</i> J2315	Length (kb)	Distant		Close				Total
		DD	DI	CD	OD	CL	T	
Chromosome 1	3870	165	165	13	52	2	82	480
Chromosome 2	3271	157	110	54	79	3	50	453
Chromosome 3	876	22	10	18	43	0	14	107
Plasmid	93	3	2	4	4	3	0	16

are in putative promoter regions. As a consequence, illegitimate and homologous recombination among close repetitive DNA will predominantly affect coding sequences.

In order to identify potential virulence factors on the *B. cenocepacia* J2315 genome, we examined the length and number of repeats, as well as the annotation of the 422 DNA close repeats. Among the 422 DNA close repeats, 45 genes potentially involved in virulence were identified and grouped into 12 functional classes (Table 3). Of the 45 genes selected, 28 were located in chromosome 1, 10 in chromosome 2, six in chromosome 3 and one in the plasmid (Table 3). A total of 41 repeat elements are located within coding regions and the remaining four are within putative promoters, where they could influence transcription (Table 3).

Of the 45 genes evaluated, 13 were included into the putative antigens class and are strong candidates for surface-exposed phase variable genes (Table 4). Overall,

this set of genes presents the largest density of repeats in the sorted list of the 422 DNA close repeats and it was selected for further study. Hence, we have made an analysis of the existence of orthologues of the *B. cenocepacia* J2315 antigens subset in other *Burkholderia* genomes. Of the 13 gene products selected, four (BCAL0340, BCAL1524, BCAM2418 and BCAS0236) were found to have length variation in the gene products, due to differences in the numbers of coding tandem repeats. Notably, the putative antigens BCAM0219 and BCAM0223, annotated as TAAs, are absent from the other genomes (Table 4).

BCAM0219 and BCAM0223 belong to a putative adhesin cluster that has a distribution restricted to the ET-12 lineage of *B. cenocepacia*

The putative TAAs BCAM0219 and BCAM0223, may be part of an unstable genomic region. Therefore, we compared the corresponding region of the *B. cenocepacia* J2315 chromosome 2 with the genome sequence of strain *B. cenocepacia* AU1054. The two adhesin genes are part of an approximately 142 kb genomic region which is lacking in the strain *B. cenocepacia* AU1054 (Fig. 2). As shown in Fig. 2(b), BCAM0219 and BCAM0223 are members of a putative adhesin cluster (designated BuceJ2315_pac) located downstream of the previously identified *B. cenocepacia* J2315 *cci* island, which includes the BCESM, an epidemiological marker for virulent *B. cenocepacia* strains (Baldwin *et al.*, 2004). This cluster is approximately 24 kb and is putatively organized with four adhesins

Fig. 1. Distribution of close repeats among the three chromosomes and the plasmid of *B. cenocepacia* J2315. (a) Chromosome 1 (3.9 Mbp), (b) chromosome 2 (3.2 Mbp), (c) chromosome 3 (0.9 Mbp) and (d) plasmid (92.7 kbp). The density of repeats were 0.12 (a) and (c), 0.14 (b), and 0.17 (d). Repeats were assigned to one of six classes: DD, distant direct (■); DI, distant inverse (◇); CD, close direct (□); OD, overlap direct (×); CL, close inverse (△); T, tandem (+). The x-axis represents the position of the first occurrence of the repeat in the genome and the y-axis represents the position of the second occurrence.

(*BCAM0219*, *0223*, *0224* and *0225*), one outer-membrane protein (*BCAM0220*), one sensor histidine kinase (*BCAM0218*) and two regulators (*BCAM0221* and *0222*) (Fig. 2b). The *BuceJ2315_pac* is surrounded by DNA segments with a very low G+C content (Fig. 2b and c). In addition to the *BuceJ2315_pac* cluster, the genomic region also includes the previously reported *B. cenocepacia* pathogenicity island *cci* (Baldwin *et al.*, 2004) among 123 predicted coding sequences (Fig. 2).

A PCR approach to study the distribution of the adhesin locus among Bcc clinical isolates

PCR analysis based on three members of the putative adhesin locus (one sensor histidine kinase, *BCAM0218*, and two TAAs, *BCAM0219* and *BCAM0224*) was performed to verify the presence of the selected genes among a panel of 47 strains representing the 17 species of the Bcc. The majority of clinical isolates examined (29 out of 47) were *B. cenocepacia* strains, since this is the major species isolated from patients

with CF. Two species outside the Bcc were included as controls (Table 1). These three genes were found exclusively in *B. cenocepacia* isolates (Fig. 3), except for *BCAM0219* (also found in *B. arboris* LMG 14939). Of these, the three genes were amplified from the isolates representing the highly transmissible ET-12 lineage, while no products were amplified from the other members of *B. cenocepacia* species, except for gene *BCAM0218* (strains Buce AU12250, Buce LMG 13013 and Buce R-1445) and gene *BCAM0219* (strain Buce R-1449). The *BCAM0224* gene was exclusively detected in *B. cenocepacia* ET-12 lineage (Fig. 3). We also analysed the prevalence of BCESM, a 1.4 kb sequence containing an ORF with homology to transcriptional regulatory genes (*esmR*), which is a previously described marker for transmissible *Burkholderia* strains (Mahenthiralingam *et al.*, 1997). All ET-12 isolates contained both the adhesin genes and BCESM, although other epidemic and non-epidemic *B. cenocepacia* isolates containing the BCESM, but lacking the adhesin genes, were also observed (Fig. 3). Furthermore, the proposed PCR-based assay using

Fig. 2. (a) Part of *B. cenocepacia* J2315_Chromosome 2 and *B. cenocepacia* AU1054_Chromosome 2 visualized with the WebACT tool (<http://www.webact.org/WebACT/home>), which enabled the visualization of TBLASTX (threshold E-value of 0.001 using the BLOSUM62 matrix) comparisons between the sequences. The defined 142 kb genomic region of *B. cenocepacia* J2315 is absent in *B. cenocepacia* AU1054. This region (b) of 123 predicted coding sequences includes the putative adhesin cluster (BuceJ2315_pac), the reported BCESM (*cci* island) described by Baldwin *et al.* (2004), a cluster of genes presumably involved in exopolysaccharide or lipopolysaccharide synthesis, genes encoding membrane proteins, and an invasion protein. *BCAM0219* and *BCAM0223* are circled. (c) The GC content of the region being studied is shown. The line at 67.2% indicates the overall G+C content estimated for the *B. cenocepacia* J2315 chromosome 2.

Table 3. Virulence functions encoded in the *B. cenocepacia* J2315 genome associated with DNA close repeats

Virulence trait	Chromosome/plasmid	Class of repeat/ position (nt)	Location*	Gene product/position (nt)
Bacteriophages	Chr_1	T/105818–105846	G	Hypothetical phage protein/105813–106028 c
	Chr_1	OD/115833–115842	G	Tail fibre assembly protein/115768–116502 c
Type III secretion	Chr_1	T/576538–576560	G	Putative FliI inhibitor involved in flagella biosynthesis/575949–576632 c
Chemotaxis	Chr_1	T/154322–154341	G	Methyl-accepting chemotaxis protein/153107–155065
	Chr_2	CD/761505–762229	G	Methyl-accepting chemotaxis protein/760326–763373
Pilus biogenesis	Chr_1	T/3857610–3857635	G	General secretory pathway protein J (type-4 pili)/3856969–3857640 c
Enzymes	Chr_3	CD/334436–334768	I	Pilus subunit protein/334465–334656
	Chr_1	T/1500582–1500597	G	Hypothetical lipoprotein/1500364–1500843
	Chr_1	OD/2437210–2437545	G	Dihydrolipoamide acetyltransferase E2/2436248–2437891 c
	Chr_1	T/3047958–3047982	G	Putative <i>N</i> -acetylmuramoyl-L-alanine amidase/3047221–3048735
	Chr_1	OD/3865585–3865594	G	Lytic transglycosylase/3865151–3865645
	Chr_2	CD/2384780–2388173	G	Putative haemolysin-related protein/2383411–2392185 c
Multidrug resistance transporters	Chr_1	CD/1171539–1174656	G	Putative membrane protein/1170559–1173867 c
	Chr_1	T/1670177–1670191	G	Putative multidrug resistance protein B/1669180–1670739 c
	Chr_1	T/3049868–3049890	I	Putative permease protein/3048781–3049659 c
	Chr_1	T/3096355–3096373	G	Outer membrane efflux protein/3095788–3097311 c
	Chr_3	T/860050–860068	G	Outer membrane efflux protein/859513–861015 c
Transporters	Chr_1	OD/65748–65763	I	Putative heavy metal resistance membrane ATPase/65909–68044
	Chr_1	T/1850184–1850212	G	Putative iron transport-related membrane protein/1849348–1851438
	Chr_1	T/1977907–1977923	G	Putative iron-transport related protein/1977502–1978209 c
	Chr_1	CD/2321739–2322591	G	Putative ABC transporter/2321263–2323194
	Chr_1	T/3081628–3081656	G	Putative transmembrane ABC transporter/3080773–3081726 c
	Chr_1	T/3191355–3191374	G	Putative proline-rich exported protein/3190341–3192869
	Chr_1	T/3324221–3324244	G	Probable outer-membrane lipoprotein/3323592–3324281 c
	Chr_2	T/2224050–2224074	G	Putative iron transport receptor protein/2223574–2225817
	Chr_2	T/2643585–2643607	G	ABC transporter/2642737–2643696
	Chr_3	CD/358949–360023	G	Autotransporter/357936–370640
	Chr_3	T/636485–636507	G	HlyD family secretion protein/636385–637803
Quorum sensing	Chr_1	TR/1627906–1627920	G	Putative LuxR-family regulatory protein/1627670–1628236 c
Transcriptional factors	Chr_1	OD/3752632–3752862	G	Histone H-1 like protein/3752545–3753195 c
EPS/LPS	Chr_1	T/2028960–2028978	G	Glycosyltransferase/2028519–2029727 c
Putative antigens	Chr_1	T/159960–159985	G	BCAL 0138 lipoprotein/159881–161119
	Chr_1	T/366721–366739	G	BCAL 0340 lipoprotein/366232–366858
	Chr_1	OD/1688274–1688355	G	BCAL 1524 lipoprotein/1688158–1689831 c
	Chr_1	CD/3442677–3443954	G	BCAL 3154 putative glycine-rich surface protein/3442386–3445307 c
	Chr_1	T/3695870–3695899	G	BCAL 3372 lipoprotein/3695865–3696416 c

Table 3. cont.

Virulence trait	Chromosome/plasmid	Class of repeat/ position (nt)	Location*	Gene product/position (nt)
Siderophores	Chr_2	CD/260745–266200	G	BCAM 0219 autotransporter adhesin /259271–268030
	Chr_2	CD/272057–274176	G	BCAM 0223 autotransporter adhesin /270927–275579 c
	Chr_2	OD/769219–769516	G	BCAM 0695 lipoprotein/767884–770160 c
	Chr_2	OD/1784638–1784728	G	BCAM 1598 lipoprotein/1783790–1785088 c
	Chr_2	CD/2723836–2729959	G	BCAM 2418 cell surface membrane protein; haemagglutinin motif/2722300–2730627
	Chr_3	CD/260121–260829	G	BCAS 0236 glycine-rich protein/258387–262877
	Chr_3	OD/280643–280650	I	BCAS 0251 lipoprotein/279266–280423 c
	Plasmid	CD/15829–17872	G	pBCA 011 adhesin/15126–20077 c
	Chr_2	T/2492631–2492645	G	Pyochelin synthetase/2491217–2496699 c

*G or I indicate that the repeat falls within a gene or between two genes, respectively.

the set of primers for *BCAM0224* could be used as a valuable tool to discriminate among *B. cenocepacia* epidemic clones belonging to the ET-12 lineage.

***B. cenocepacia* BCAM0224 is a trimeric autotransporter adhesin required for cell adhesion and virulence**

A BLASTP search revealed that BCAM0224 has high amino acid homology with the prototype member of the trimeric autotransporter family of surface-attached oligomeric proteins, the *Yersinia* adhesin YadA, defined by the domain Pfam 03895 (Hoiczyk *et al.*, 2000). To confirm our prediction, we also used the daTAA program, which is a web-based tool that annotates domains of trimeric auto-

transporter adhesins (<http://toolkit.tuebingen.mpg.de/dataa/>) (Szczesny & Lupas, 2008). BCAM0224 is 953 aa long, with a calculated molecular mass of 90 kDa. Like the other TAA proteins, BCAM0224 has a head–stalk–anchor modular organization (Fig. 4a). Analysis of the BCAM0224 amino acid sequence using InterPro (<http://www.ebi.ac.uk/interpro/>) revealed significant hits for the presence of seven clusters of Hep_Hag (Pfam domain PF05658), six clusters of HIM (Pfam domain PF05662) and two collagen-binding domains (Pfam domain PF01391) (Fig. 4a). Hep_Hag and HIM are short repeat motifs found in many bacterial invasins and haemagglutinins (Valle *et al.*, 2008). Using the daTAA program, we also predict the presence of an extended signal sequence with a predicted cleavage site between amino acids 1 and 43 (Fig. 4a).

Table 4. Proteins of the 11 finished/unfinished *Burkholderia* genomes available that belong to the putative antigens class, listed according to orthology relationships

✓, Orthologue present; ×, orthologue absent; LV, orthologue associated with length variation.

	Chromosome 1					Chromosome 2					Chromosome 3		Plasmid
	BCAL 0138	BCAL 0340	BCAL 1524	BCAL 3154	BCAL 3372	BCAM 0219	BCAM 0223	BCAM 0695	BCAM 1598	BCAM 2418	BCAS 0236	BCAS 0251	pBCA 011
<i>B. cenocepacia</i> PC184*†	✓	LV	LV	✓	✓	×	×	✓	✓	LV	✓	×	×
<i>B. cenocepacia</i> AU1054†	✓	LV	✓	✓	✓	×	×	✓	✓	LV	✓	✓	×
<i>B. cenocepacia</i> HI2424†	✓	LV	LV	✓	✓	×	×	✓	✓	LV	✓	✓	LV
<i>B. vietnamiensis</i> G4†	✓	LV	×	✓	✓	×	×	✓	✓	LV	×	✓	×
<i>B. dolosa</i> AU0158*†	✓	LV	LV	×	✓	×	×	×	✓	LV	✓	✓	×
<i>B. ambifaria</i> AMMD†	✓	LV	LV	×	✓	×	×	✓	✓	LV	✓	✓	×
<i>B. lata</i> 383†	✓	LV	✓	✓	✓	×	×	✓	✓	LV	✓	×	×
<i>B. mallei</i> ATCC 23344	✓	×	LV	×	✓	×	×	✓	✓	×	×	×	×
<i>B. pseudomallei</i> K96243	✓	LV	LV	×	✓	×	×	✓	✓	LV	LV	✓	×
<i>B. thailandensis</i> E264	✓	LV	LV	×	✓	×	×	✓	✓	LV	×	✓	×
<i>B. xenovorans</i> LB400	✓	×	LV	×	✓	×	×	✓	✓	×	×	×	×

*Unfinished genome sequence (July 2009).

†Bcc strains.

Fig. 3. (a) Genetic organization of the putative adhesin cluster (BuceJ2315_pac). Arrows show the direction of gene transcription. Positions of the PCR primers used to amplify *BCAM0218* (F1, R1), *BCAM0219* (F2, R2), *BCAM0224* (F3, R3) and *BCESM* (BCESM1 and -2) genes are indicated. The *B. cepacia* *cci* island is also shown. (b) DNA products amplified by PCR from *Burkholderia* species using *BCAM0218*, *BCAM0219*, *BCAM0224* and *BCESM* primers. The panel of *Burkholderia* species included 47 strains representing the 17 Bcc species. *B. cenocepacia* strains comprised the majority of clinical isolates examined (29 of 47); two species outside the Bcc (*B. phymatum* STM815 and *B. phytofirmans* PsJN) were included as controls. The *BCAM0218*, *BCAM0219*, *BCAM0224* and *BCESM* gene products were 1002, 1553, 1130 and 1418 bp, respectively, as expected. In contrast with previously published data (Mahenthiralingam *et al.*, 2000), *BCESM* was not amplified in strains LMG16659 and LMG18830. Lane M, molecular size marker (1 kb ladder).

Expression of the *BCAM0224* adhesin gene was assessed by real-time PCR in *B. cenocepacia* K56-2. Interestingly, physico-chemical environmental factors similar to those that are encountered in the lung microenvironment of CF patients, such as high osmolarity, oxygen-limited condi-

tions and oxidative stress, enhance (sevenfold induction) its expression level.

In order to evaluate the functional role of *BCAM0224* we constructed a knockout mutant of *B. cenocepacia* K56-2

Fig. 4. (a) Schematic representation of the predicted domain organization of BCAM0224 from *B. cenocepacia* J2315. h, Head; c, connector; s, stalk; a, anchor. (b) Adherence of wild-type *B. cenocepacia* K56-2 (filled bars) and $\Delta BCAM0224$ mutant (open bars) to laminin, fibronectin and collagen type I. The binding capacity to collagen type I was significantly decreased in the mutant (* $P < 0.001$). The results are from three independent experiments; bars indicate SD. (c) Adherence to collagen type I by the wild-type *B. cenocepacia* K56-2 (shaded bars), the $\Delta BCAM0224$ mutant (open bars) and the $\Delta BCAM0224$ -complemented strain (filled bars). (d) Adherence to collagen type I by *E. coli* BL21 (DE3) expressing BCAM0224 (filled bars) and *E. coli* BL21 (DE3)/pET 23-a used as a control (open bars). In (c) and (d), a significant difference is indicated by asterisks (** $P < 0.006$). (e) Kaplan-Meier survival curves for *G. mellonella* infection with wild-type *B. cenocepacia* K56-2 (●) and the $\Delta BCAM0224$ mutant (■) strains and the control (▲) ($P < 0.005$, for comparison of the wild-type and the mutant). Ten larvae were injected with 10^4 c.f.u., and larvae were scored as dead when they ceased moving, changed from their normal pale cream coloration to brown, and failed to respond when gently manipulated with a pipette tip. The results are from three independent experiments.

BCAM0224 ($\Delta BCAM0224$) and examined the adherence capacity of cells to surfaces coated with various mammalian ECM proteins, such as collagen type I, fibronectin and laminin, by *in vitro* binding assays. Compared with the BCAM0224-negative mutant, the wild-type *B. cenocepacia* K56-2 cells adhered strongly to collagen type I matrix (Fig. 4b; $P < 0.001$).

To confirm that the insertion of the trimethoprim cassette into BCAM0224 has no polar effect on the expression of the downstream gene BCAM0223, an *in trans* complementation experiment using BCAM0224 cloned in the replicative plasmid pDA17 was performed. The introduction of pDM3 recombinant plasmid into the BCAM0224 mutant showed a partial restoration of the adhesive phenotype when compared with the phenotype of *B. cenocepacia* K56-2 wild-type (Fig. 4c). This could be a result of the lower expression level of the BCAM0224 protein. We also examined adhesion of the *E. coli* BL21 strain expressing the gene BCAM0224 to collagen type I, using the empty expression vector as a control. A difference was observed between the recombinant and the vector control (Fig. 4c), indicating that BCAM0224

is a collagen-binding TAA. This result is in accordance with the trans-complementation result obtained with the *B. cenocepacia* $\Delta BCAM0224$ mutant.

Finally, in order to test the assumption that the trimeric autotransporter BCAM0224 might somehow be involved in virulence, we compared the ability of the wild-type *B. cenocepacia* K56-2 and the $\Delta BCAM0224$ mutant strains to cause mortality in *G. mellonella* (caterpillars of the greater wax moth) larvae infection assays. As shown in Fig. 4(d), the rate of mortality was dramatically reduced in larvae infected with the $\Delta BCAM0224$ mutant compared with the wild-type K56-2. At 72 h post-infection, all the wild-type-infected larvae ($n = 10$) were killed, whereas 90 % of larvae infected with the mutant had survived ($P < 0.005$) (Fig. 4d).

DISCUSSION

In this study, we carried out a global analysis of repeats in the genome of *B. cenocepacia* J2315, the causative agent of

numerous fatalities in CF patients. We found a large number of repeats in the *B. cenocepacia* J2315 genome (Table 2). This observation suggests that *B. cenocepacia* J2315 has a high genome plasticity that should be important in its capacity to adapt to human and animal hosts as well as to different environments.

Among the 422 DNA close repeats, we selected a total of 45 potential genes assigned into a panel of 12 virulence functional classes (Table 3). Among the 45 genes, 34 have been previously described as virulence factors in *Burkholderia* strains or other bacterial pathogenic genera, such as *Neisseria*, *Mycobacterium*, *Haemophilus* and *Vibrio*. These include genes for phase-variable oligosaccharide structure of lipopolysaccharide (Hughes *et al.*, 1997; Vinion-Dubiel & Goldberg, 2003), siderophore production (Anderson *et al.*, 2004; Visser *et al.*, 2004), multidrug-resistant transporters (Wigfield *et al.*, 2002), cable pili (Sajjan *et al.*, 2003), and intra- and extracellular enzymes, such as haemolysin and lytic transglycosylase (Cloud & Dillard, 2002; Takahashi *et al.*, 2004).

Among the 12 functional virulence classes that we have defined, we focused on putative antigens because the 13 gene products included within this class show the highest over-representation of close repeats and are strong candidates for surface-exposed adhesins and lipoproteins involved in antigenic response. Three interesting findings emerged from our analysis: (i) in general, the putative antigens are under-represented in *B. xenovorans* LB400; (ii) using each of the *B. cenocepacia* J2315 putative antigens as a reference, we found that BCAL0340, BCAL1524 and BCAM2418 have significant differences in the numbers of coding tandem repeats, and for that reason are strong candidates for novel virulence factors; and (iii) even more strikingly, BCAM0219 and BCAM0223, annotated as autotransporter adhesins, are absent from the other genomes (Table 4).

BCAM0219 and BCAM0223 genes are members of a putative adhesin cluster (BuceJ2315_pac) located downstream of the described BCESM (Baldwin *et al.*, 2004) (Fig. 2). BuceJ2315_pac is organized in a modular fashion and contains four autotransporter adhesins genes, one outer-membrane protein, one sensor histidine kinase and two response regulator genes (Fig. 2a). We hypothesized that the periplasmic sensor histidine kinase is responsible for sensing stimuli, and a second component, the two response regulators, regulates two sets of virulence genes, namely the co-transcribed autotransporter adhesin (BCAM0219) and outer-membrane protein (BCAM0220) genes and the three autotransporter adhesins genes (BCAM0223, BCAM0224 and BCAM0225) (Fig. 2). Our analysis revealed that the BuceJ2315_pac is part of a 142 kb genomic region that comprises an upstream and downstream extension of the *cci* island previously discovered by RAPD analysis (Baldwin *et al.*, 2004) and encoding a large number of genes linked to both virulence and metabolism (Fig. 2).

PCR assays using primers targeting three genes of the putative BuceJ2315_pac adhesin locus were designed and used to test a panel of 47 *Burkholderia* strains (Fig. 3). Our findings substantiate that the adhesin genes are located on a chromosomal region unique to some *B. cenocepacia* strains. We also observed that BCAM0224 is an exclusive marker of epidemic *B. cenocepacia* belonging to the ET-12 lineage and we therefore recommend employment of this PCR assay for discrimination of bacteria among Bcc members that belong to this virulent subgroup. These results are compatible with previous studies, which revealed the autotransporter adhesins BCAM0223 and BCAM0225 as unique in the ET-12 lineage (Bernier & Sokol, 2005), but BCAM0223, just like *cblA*, is present in some *B. cenocepacia* strains distinct from ET-12 (Turton *et al.*, 2009).

As a potentially important surface-exposed protein from *B. cenocepacia* ET-12 epidemic strains, we examined BCAM0224 for several features. Bioinformatic analysis of BCAM0224 predicts that this protein is an orthologue of the surface-attached collagen-binding protein YadA from *Yersinia* species, the best characterized member of the TAAs (Tamm *et al.*, 1993; Koretke *et al.*, 2006). Other members of this class, namely NadA from *Neisseria meningitidis* (Comanducci *et al.*, 2002), UspA-1 and -2 from *Moraxella catarrhalis* (Lafontaine *et al.*, 2000) and BadA from *Bartonella henselae* (Riess *et al.*, 2004) are important pathogenicity factors, playing critical roles in processes such as bacterial adherence, biofilm formation, serum resistance and invasion of host cells (Linke *et al.*, 2006). They represent one of the major families of surface-exposed proteins in Gram-negative bacteria and are organized in a modular fashion, i.e. a membrane-anchored C-terminal conserved domain that forms a trimeric α -barrel pore (also called α -domain) and permits, through the type V protein secretion pathway (T5SS), the translocation of a passenger domain (stalk and an N-terminal head) to the bacterial cell surface (Dautin & Bernstein, 2007).

Similar to the results obtained with YadA (Heise & Dersch, 2006), in this study, we found that BCAM0224 promotes bacterial adherence to the ECM protein collagen type I, one of the most abundant components of the ECM. This result supports our *in silico* analysis indicating that BCAM0224 possesses two binding domains responsible for the interaction with collagen (Fig. 4a).

By using quantitative real-time PCR, we determined the expression profile of the BCAM0224 gene. We have observed that BCAM0224 gene expression elicited great changes for cells grown under environmental conditions that mimic the altered microenvironment of the CF lungs. Similar to our work, other studies on CF pathogens exposed to high osmolar conditions have shown a dramatic enhancement of the expression levels of other virulence genes, either from *B. cenocepacia* (Tomich & Mohr, 2004; Bhatt & Weingart, 2008) or *Pseudomonas aeruginosa* (Tan

et al., 1999). Based on these observations, we speculate that expression of *BCAM0224* may play a role in the virulence of *B. cenocepacia* when it infects CF patients.

With a non-polar *BCAM0224* mutant and using adherence assays, we provide evidence supporting that *BCAM0224* has a high affinity to binding collagen type I (Fig. 4b). This result is consistent with experiments in which recombinantly expressed *BCAM0224* in an *E. coli* host was used (Fig. 4c). Using the *G. mellonella* larvae infection model, we demonstrated that the Δ *BCAM0224* mutant strain displays a dramatic reduction in virulence compared with the wild-type strain (Fig. 4d). This behaviour may therefore be associated with the impaired ability of the Δ *BCAM0224* mutant to attach properly to the host cells. These results are compatible with previous studies, which demonstrated that TAAs from other pathogenic bacteria interact with different ECM proteins, enabling them to promote an intimate association with target eukaryotic host cells (Zhang *et al.*, 2004; Heise & Dersch, 2006). Finally, upon sensing this contact, the bacteria translocate an arsenal of virulence factors directly from their cytoplasm into the host cells which cumulatively contribute to pathogenicity.

In conclusion, we identified a novel and unique putative adhesin cluster in the genome of *B. cenocepacia* located within a region that corresponds to an extension of the previously described *cci* island. Moreover, the defined PCR approach for one of the genes within this locus (*BCAM0224*) proved to be a suitable novel specific marker for discrimination of bacteria that belong to the ET-12 lineage of *B. cenocepacia*. Furthermore, we demonstrated that *BCAM0224* from *B. cenocepacia* J2315 represents a new collagen-binding TAA with an important role in cellular adhesion and virulence.

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