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Trimeric autotransporter adhesins in members of the *Burkholderia cepacia* complex: a multifunctional family of proteins implicated in virulence

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Trimeric autotransporter adhesins (TAAs) are multimeric surface proteins exclusively found in bacteria. They are involved in various biological traits of pathogenic Gram-negative bacteria including adherence, biofilm formation, invasion, survival within eukaryotic cells, serum resistance, and cytotoxicity. TAAs have a modular architecture composed by a conserved membrane-anchored C-terminal domain and a variable number of stalk and head domains. In this study, a bioinformatic approach has been used to analyze the distribution and architecture of TAAs among *Burkholderia cepacia* complex (Bcc) genomes. Fifteen genomes were probed revealing a total of 74 encoding sequences. Compared with other bacterial species, the Bcc genomes contain a large number of TAAs (two genes to up to eight genes, such as in *B. cenocepacia*). Phylogenetic analysis showed that the TAAs grouped into at least eight distinct clusters. TAAs with serine-rich repeats are clearly well separated from others, thereby representing a different evolutionary lineage. Comparative gene mapping across Bcc genomes reveals that TAA genes are inserted within conserved synteny blocks. We further focused our analysis on the epidemic strain *B. cenocepacia* J2315 in which seven TAAs were annotated. Among these, three TAA-encoding genes (*BCAM019*, *BCAM0223*, and *BCAM0224*) are organized into a cluster and are candidates for multifunctional virulence factors. Here we review the current insights into the functional role of *BCAM0224* as a model locus.

Keywords: *Burkholderia cepacia* complex, trimeric autotransporter adhesins, virulence factors

INTRODUCTION

Bacteria belonging to the *Burkholderia cepacia* complex (Bcc) have emerged as highly problematic opportunistic human pathogens in immunocompromised individuals and in patients with the genetic disease cystic fibrosis (CF). The virulence of the Bcc members is variable and *B. cenocepacia* and *B. multivorans* are the most common species isolated from the respiratory tract of CF patients. Bcc strains posses a wide range of virulence factors that are critical for colonization and disease. Despite the identification and characterization of some, many details of *Burkholderia* virulence still remains to be clarified (reviewed in Drevinek and Mahenthiralingam, 2010).

To initiate infection in CF patients, Bcc strains must be able to colonize the respiratory epithelium by binding to a diverse group of host cell surface molecules including proteins, glycolipid receptors, and secretory mucins (McClean and Callaghan, 2009). This essential step, although not fully characterized, is mediated by a variety of proteins collectively termed adhesins, which are surface-exposed proteins (Kline et al., 2009). Thus far, only the cable pili-associated adhesin (*cbl*, 22 kDa) of *B. cenocepacia*, has been identified to interacts with cytokeratin 13, a 55-kDa protein which is enriched in CF epithelial cells differentiated into squamous phenotype (Sajjan et al., 2000). However, *cbl* gene is absent in many Bcc isolates (Sajjan et al., 2002), suggesting that other adhesins may play a relevant role in epithelial adhesion and colonization. Among these, the family of the designated trimeric autotransporter adhesins (TAAs) represents a class of proteins found in Gram-negative pathogens that are known to mediate adherence of the bacteria to host tissues and thereby may be relevant for the overall pathogenic potential of Bcc strains.

Trimeric autotransporter adhesins belong to a subtype of an outer membrane family of proteins termed autotransporters, that have been studied and emerging as important virulence factors in a range of pathogenic alpha-, beta-, and gamma-proteobacteria (Linke et al., 2006). Adhesion to extracellular matrix (ECM) proteins and host cells seems to be the major role played by these proteins (Linke et al., 2006). Müller et al. (2011b) have used both static and dynamic adhesion assays, aiming to prove the involvement of three distinct TAAs in bacterial adherence (Müller et al., 2011b). These authors were further able to show that these three TAAs exhibit promiscuous binding to ECM proteins and endothelial cells, albeit with differences in the results obtained in the static and dynamic conditions (Müller et al., 2011b). Despite the importance of TAAs in cell adhesion, these proteins are multifunctional virulence factors involved in several other biological traits of pathogenic Gram-negative bacteria including biofilm formation, cell-to-cell aggregation, protecting the bacterium from host immune responses (serum resistance), and promoting the

invasion of host cells (Heise and Dersch, 2006; Serruto et al., 2009).

Trimeric autotransporter adhesins are multi-domain proteins organized in a modular fashion i.e., an integral membrane-anchored C-terminal domain that forms a trimeric 12-stranded beta-barrel pore and permits, through the type V protein secretion pathway (T5SS), the translocation of a passenger domain (divided in two regions, the stalk and an N terminal head) into the extracellular space (Cotter et al., 2005). Among the various TAAs described, YadA from enteropathogenic *Yersinia* species (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) represent the structural prototype for this family of proteins (Koretke et al., 2006; **Figure 1**). TAAs trimerization is essential for their translocation and function, providing stability and potential for multivalent interactions. Although it is poorly understood and controversial, TAAs biogenesis seems to occur dependently of other protein partners (Lehr et al., 2010). The C-terminal translocator domain is highly conserved among TAAs (generally consists of 70–100 amino acid residues) and therefore used as the defining element of the family (Cotter et al., 2005). In contrast, the passenger domains are fibrous, more or less repetitive, varying in length, and sequence motifs (Linke et al., 2006). Further, these tandemly repeated sequences may undergo contraction or expansion, thereby defining their specific activities (Linke et al., 2006; Sheets and St Geme III, 2011). Often, the passenger domains contain immunogenic Hep_Hag (Pfam PF05658) and HIM (Pfam PF05662) domains that thereby make them good candidates for vaccine development (Tiyawisutsri et al., 2007). Hep_Hag and HIM are short repeat motifs found in bacterial hemagglutinins and invasins. Although it has been shown that TAAs were found only in prokaryotes, Müller et al. (2011a) demonstrated that the expression in yeast of the β-barrel domain of the *Y. enterocolitica yadA* resulted in the synthesis of a trimeric 12-stranded beta-barrel, exclusively targeted to the mitochondrial outer membrane (Müller et al., 2011a).

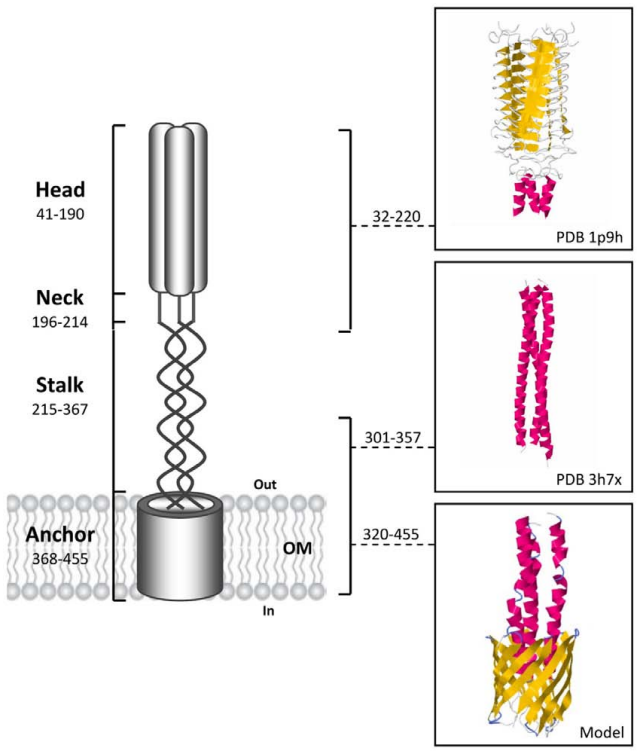

FIGURE 1 | Schematic representation of a full-length trimeric autotransporter adhesin based on the prototypical model of YadA from *Yersinia enterocolitica*, with the domains predicted by the domain annotation of trimeric autotransporter adhesins (daTAA) program. The residues numbers for each domain of the YadA protein are showed. For the head and a part of the stalk the 3D ribbon structures are available and showed with the respective PDB ID. The 3D structure of the YadA anchor has not been solved yet and the monomer model was constructed using the automated Swiss model Workspace (http://www.ebi.ac.uk/newt/; Arnold et al., 2006) with 3EMO as a template. Finally, a predicted 3D model of the trimeric YadA anchor domain (12-stranded beta-barrel) was generated using Cluspro 2.0 software (http://cluspro.bu.edu/home.php; Comeau et al., 2004).

Several TAAs have been characterized in terms of function and structure within a large number of bacterial pathogens, including, among others, YadA from *Y. enterocolitica* (Nummelin et al., 2004), Adhesin A from *Bartonella henselae* (Riess et al., 2004), NadA from *Neisseria meningitidis* (Capecchi et al., 2005), Hia from *Haemophilus influenza* (Meng et al., 2006), an IgD-binding protein from *Moraxella catarrhalis* (Riesbeck et al., 2006), AipA and TaaP from *Proteus mirabilis* (Alamuri et al., 2010), BpaA from *Burkholderia pseudomallei* (Edwards et al., 2010), Sad A from *Salmonella enterica* (Raghunathan et al., 2011), and Cha from *Haemophilus* cryptic genospecies (Sheets and St Geme III, 2011). YadA from *Y. enterocolitica* has been one of the most extensively studied and is found to display a multifaceted activity during host–pathogen interaction (reviewed in Linke et al., 2006).

Herein, as a first approach, we have conducted an *in silico* analysis in completed genomes of Bcc members aiming to identify TAA-encoding sequences. The proteins selected were studied through sequence similarity, phylogeny, and synteny conservation data. Then, we focused our analysis on the epidemic strain *B. cenocepacia* J2315 in which seven TAAs were annotated. Among those, we particularly focused our attention on three clustered TAAs-encoding genes (*BCAM0219*, *BCAM0223*, *BCAM0224*) that are strong candidates for multifunctional pathogenic factors. Finally, we review here recent results arising from the functional analysis of *BCAM0224* as a model locus.

IDENTIFICATION OF TAA-ENCODING SEQUENCES FROM Bcc GENOMES

We used as a starting point the Bcc protein sequences predicted by the domain annotation of trimeric autotransporter adhesins (daTAA) program[1] (Szczesny and Lupas, 2008). These sequences were used to search the 15 Bcc genomes [(9) finished and (6) unfinished] available in *Burkholderia* genome database[2] and integrated microbial genomes (IMG) system[3] searching for other putative TAAs. Next, to confirm the results, we used BLASTP 2.0 against Bcc genomes available at National Center for Biotechnology Information (NCBI) and all proteins identified were verified

[1]http://toolkit.tuebingen.mpg.de/dataa
[2]http://burkholderia.com/
[3]http://img.jgi.doe.gov/

with daTAA program to confirm the presence of the membrane anchor [Pfam YadA domain (PF03895)] and at least another characteristic domain of TAAs. The following Bcc genomes were analyzed: *B. ambifaria* MC40-6, *B. ambifaria* MEX-5, *B. ambifaria* IOP40-10, *B. cenocepacia* AU 1054, *B. cenocepacia* HI2424, *B. cenocepacia* J2315, *B. cenocepacia* MC0-3, *B. cenocepacia* PC184, *B. cepacia* AMMD, *B. dolosa* AUO158, *B. lata* 383, *B. multivorans* ATCC 17616, *B. multivorans* CGD1, *B. ubonensis* Bu, *B. vietnamiensis* G4.

In total, our analysis revealed the existence of 74 putatively TAA-encoding sequences. Compared with other bacterial species, some Bcc genomes contain a large number of TAAs, which probably reflects their large multireplicon genome sizes and may have been acquired by insertions of transposable elements and/or bacteriophages or through horizontal transfer of DNA fragments. Furthermore, the higher number of TAAs found on Bcc genomes also suggests a high genome plasticity that ultimately may be relevant in their capacity to adhere and colonize human hosts as well as other environments. The absolute numbers and the density of TAAs are variable among the Bcc genomes under study. The genome of *B. cenocepacia* MC0-3 contains the highest number (8) and density and *B. vietnamiensis* G4 the lowest number (2) and density. Whether these TAAs have redundant or unique functions is an important question that needs to be answered. In addition, we also calculated the density of TAAs encoding genes relative to non-*Burkholderia* genomes available in the data database. To date, there are only five bacterial genera showing higher TAA gene density than those of Bcc genomes, namely, *Fusobacterium*, *Bartonella*, *Haemophilus*, *Moraxella*, *and Xylella.*

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS OF THE TAAs FROM Bcc GENOMES

To analyze the phylogenetic relationship between the full-length amino acid sequences of the TAAs, we first created a multiple sequence alignment with ClustalW 2.0.12 (Thompson et al., 1994). Despite the conservation in domain architecture, different lengths, and a low overall sequence identity were observed across all TAAs. However, as expected, the C-terminal translocator domains are highly conserved.

The unrooted phylogenetic tree prepared with the 74 TAAs reveals that the sequences have been found to fall into at the least eight clusters with different evolutionary lineages (**Figure 2**). The tree based on the sequences of the C-terminal translocator conserved domain (data not shown) gave essentially the same result as the tree constructed from the entire sequences. The clusters I, V, VII, and VIII, respectively with 12, 12, 14, and 15 members were the tree most representative groups. The clusters I, II, III appear to be related, thereby suggesting that they were derived from a single ancestral. In contrast, clusters V and VI have clearly different lineages that may represent separate evolutionary histories (**Figure 2**). The evolutionary related clusters VII and VIII include only those TAAs with serine-rich repeats; cluster VII is formed with proteins containing only one serine-rich repeat domain whereas cluster VIII grouped the proteins with several extensive serine-rich repeats (**Figures 2** and **3**). As far as we know, these serine-rich repeat proteins have been found only in Gram-positive bacteria where they appear to play a decisive role in colonization; these include, among others, the glycoproteins Fap1 of *Streptococcus parasanguinis*, which mediates bacterial adhesion to saliva-coated hydroxyapatite (Wu et al., 2007), GspB of *S. gordonii* M99, which mediates bacterial binding of human platelets (Bensing et al., 2004) and SrpA of *S. cristatus* which mediates bacterial adhesion in oral biofilms (Handley et al., 2005). A common mechanism involved in these interactions is recognition of surface-associated host sialoglycoconjugates via the hydroxyl groups of S or T residues (O-glycosylation; Zhou and Wu, 2009).

We further analyzed the domain architecture representative of each cluster using the Pfam protein family database (Finn et al., 2010). Each of them contains an identical C-terminal YadA domain and a variable number of the other typical domains found in TAAs, such as the HIM and Hep_Hag domains. In addition, they have variable regions (in size and sequence) that are not conserved between the defined tree clusters (**Figure 3**).

We next analyzed the distribution of the TAAs across the eight clusters defined in the topology of the tree. A general finding was that the TAAs are under-represented in three Bcc species, namely *B. multivorans*, *B. dolosa*, and *B. vietnamiensis* (**Figure 4**). Since *B. multivorans* is one of the most prevalent Bcc species in CF patients, the results showed in **Figure 4** render a clear difficulty to draw a logical distribution of TAAs across the Bcc species understudy. Furthermore, two other interesting findings emerged from our analysis: (i) TAAs included in the clusters I, VII, and VIII are the most representatives within the Bcc species; (ii) although the distribution of TAAs is not species specific, the representatives included in the clusters II and III are almost exclusive found in the genomes of the *B. cenocepacia* strains (**Figure 4**). Further work is needed to characterize the functions and specificities of these proteins, and the role they play in the pathogenesis of Bcc species.

Finally, all of the TAA sequences identified were examined in terms of the chromosomal arrangement and annotation of their neighboring genes. As shown in **Figure 5**, we concluded that each defined tree cluster is likely to represent distinct conserved genetic organizations and ultimately can reflect functional relationships between genes. Interestingly, in the genetic organizations defined for clusters I, II, and VII, our analysis reveals the existence of genes encoding a sensor (histidine kinase or TonB like) and one or more response-regulator proteins in the vicinity of the TAA-encoding gene. This finding suggests a possible two-component signal transduction system, where the periplasmic sensor histidine kinase is responsible for sensing stimuli and a second component regulates the virulence effector, namely the TAA gene (**Figure 5**). The bacterial prototype for this system is the *Bordetella pertussis BvgAS* two-component regulatory system which is involved in the expression of many adhesins and toxins (Jones et al., 2005). It is now important to obtain experimental data in order to validate the hypothesis raised by this *in silico* analysis.

Furthermore, the analysis of synteny between the tree clusters V, VI, VII, and VIII, reveals the existence of a conserved gene encoding an outer membrane protein (OMP) in the vicinity of the TAA-encoding gene (**Figure 5**). It is noteworthy that recent biochemical and structural studies have raised pertinent questions

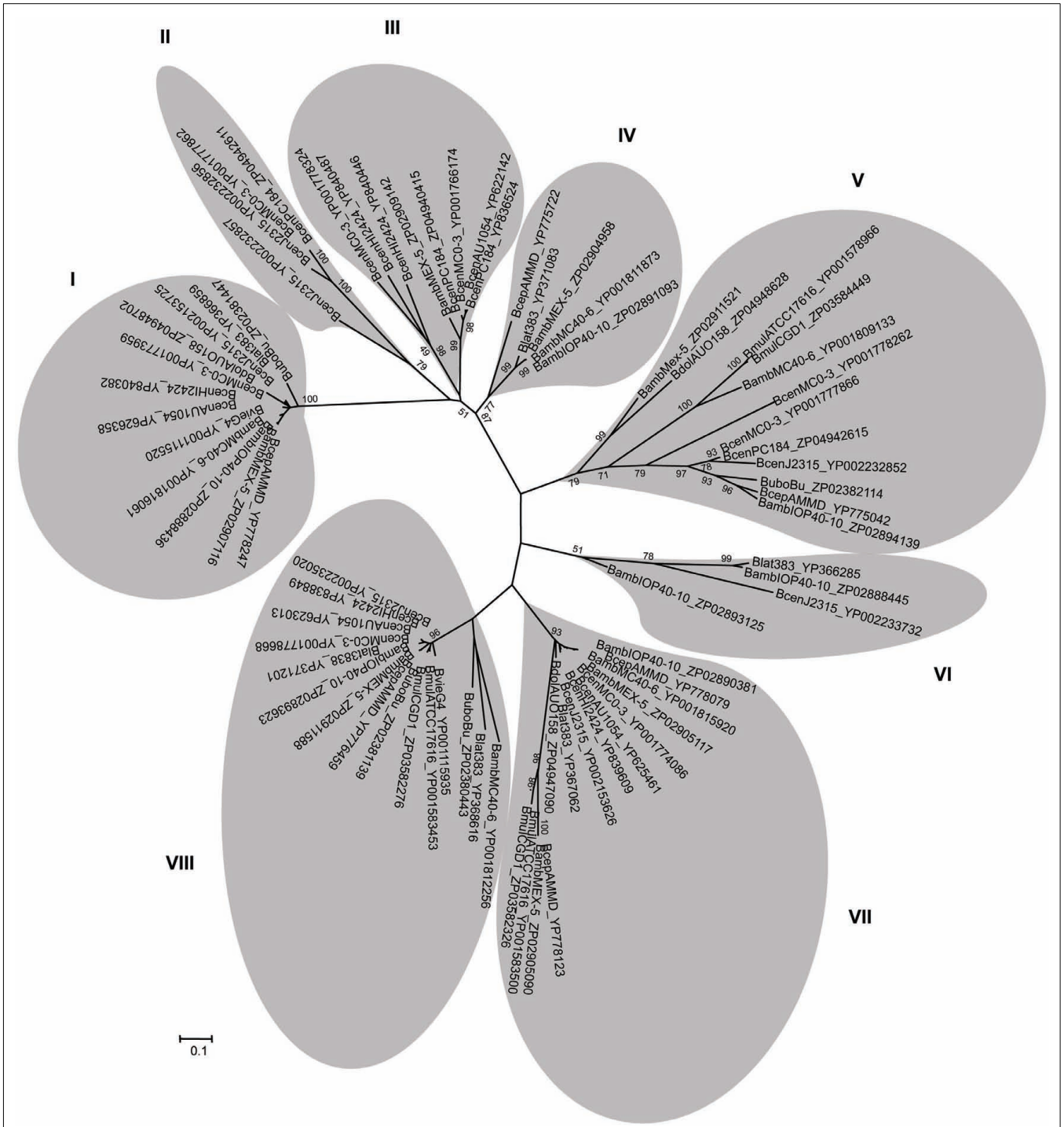

FIGURE 2 | Phylogenetic tree of the 74 TAA sequences, performed with MEGA5 (Tamura et al., 2011), using the neighbor-joining method (Perriere and Gouy, 1996). The phylogenetic tree was constructed based on the result of the global alignment of the 74 sequences performed by ClustalW 2.0.12 (Thompson et al., 1994). A branch length of 0.1 substitution/site is given to phylogenetic distances. The values adjacent to a node indicate the percentage of 1000 bootstrap trees that contain the node. Each sequence is identified using the strain abbreviated name composed by the first letter of the genus, the three first letters of the specie and the strain code, followed by the GenBank accession number.

about the traditional paradigm of TAA biogenesis as self-contained secretion system. In fact, several evidences now support that many passenger domains are transported across the outer membrane by exogenous auxiliary proteins, such as OMPs and periplasmic chaperones (Ieva and Bernstein, 2009; Ruiz-Perez et al., 2009; Wagner et al., 2009).

	Accession nº	Size (aa)	Bcc strain	Domain architecture
Cluster I	YP_778247	1204	*B. cepacia* AMMD	
	ZP_02907116	1204	*B. ambifaria* MEX-5	
	ZP_02888436	1204	*B. ambifaria* IOP40-10	
	YP_001816061	1125	*B. ambifaria* MC40-6	
	YP_001115520	1125	*B. vietnamiensis* G4	
	YP_626358	1197	*B. cenocepacia* AU1054	
	YP_840382	1197	*B. cenocepacia* HI2424	
	YP_001773959	1197	*B. cenocepacia* MC0-3	
	ZP_04948702	812	*B. dolosa* AUO158	
	YP_002153725	1197	*B. cenocepacia* J2315	
	YP_366859	1451	*B. lata* 383	
	ZP_02381447	907	*B. ubonensis* Bu	
Cluster II	ZP_04942611	1510	*B. cenocepacia* PC184	
	YP_001777862	1613	*B. cenocepacia* MC0-3	
	YP_002232856	1550	*B. cenocepacia* J2315	
	YP_002232857	953	*B. cenocepacia* J2315	
Cluster III	YP_836524	597	*B. cenocepacia* HI2424	
	YP_622142	597	*B. cenocepacia* AU1054	
	YP_001766174	597	*B. cenocepacia* MC0-3	
	ZP_04940415	634	*B. cenocepacia* PC184	
	ZP_02909142	422	*B. ambifaria* MEX-5	
	YP_840446	1417	*B. cenocepacia* HI2424	
	YP_840487	967	*B. cenocepacia* HI2424	
	YP_001778324	1072	*B. cenocepacia* MC0-3	
Cluster IV	ZP_02891093	575	*B. ambifaria* IOP40-10	
	YP_001811873	575	*B. ambifaria* MC40-6	
	YP_371083	589	*B. lata* 383	
	YP_775722	536	*B. cepacia* AMMD	
	ZP_02904958	575	*B. ambifaria* MEX-5	
Cluster V	ZP_02911521	480	*B. ambifaria* MEX-5	
	ZP_04948628	263	*B. dolosa* AUO158	
	YP_001578966	650	*B. multivorans* ATCC17616	
	ZP_03584449	613	*B. multivorans* CGD1	
	YP_001809133	737	*B. ambifaria* MC40-6	
	YP_001778262	3718	*B. cenocepacia* MC0-3	
	YP_001777866	3355	*B. cenocepacia* MC0-3	
	ZP_04942615	3355	*B. cenocepacia* PC184	
	YP_002232852	2953	*B. cenocepacia* J2315	
	ZP_02382114	1237	*B. ubonensis* Bu	
	YP_775042	4191	*B. cepacia* AMMD	
	ZP_02894139	1359	*B. ambifaria* IOP40-10	
Cluster VI	YP_366285	1326	*B. lata* 383	
	ZP_02888445	911	*B. ambifaria* IOP40-10	
	YP_002233732	1396	*B. cenocepacia* J2315	
	ZP_02893125	908	*B. ambifaria* IOP40-10	
Cluster VII	ZP_02890381	1484	*B. ambifaria* IOP40-10	
	YP_778079	1353	*B. cepacia* AMMD	
	YP_001815920	1434	*B. ambifaria* MC40-6	
	ZP_02905117	1357	*B. ambifaria* MEX-5	
	YP_001774086	1546	*B. cenocepacia* MC0-3	
	YP_625461	1471	*B. cenocepacia* AU1054	
	YP_839609	1471	*B. cenocepacia* HI2424	
	YP_002153626	1496	*B. cenocepacia* J2315	
	YP_367062	1854	*B. lata* 383	
	ZP_04947090	1378	*B. dolosa* AUO158	
	YP_778123	1117	*B. cepacia* AMMD	
	ZP_02905090	1362	*B. ambifaria* MEX-5	
	YP_001583500	330	*B. multivorans* ATCC17616	
	ZP_03582326	1470	*B. multivorans* CGD1	
Cluster VIII	YP_001812256	898	*B. ambifaria* MC40-6	
	YP_368616	977	*B. lata* 383	
	ZP_02380443	510	*B. ubonensis* Bu	
	YP_001115935	561	*B. vietnamiensis* G4	
	YP_001583453	2505	*B. multivorans* ATCC17616	
	ZP_03582276	2201	*B. multivorans* CGD1	
	ZP_02381139	477	*B. ubonensis* Bu	
	YP_776459	2493	*B. cepacia* AMMD	
	ZP_02911588	511	*B. ambifaria* MEX-5	
	ZP_02893623	511	*B. ambifaria* IOP40-10	
	YP_371201	2866	*B. lata* 383	
	YP_001778668	2814	*B. cenocepacia* MC0-3	
	YP_623013	2728	*B. cenocepacia* AU1054	
	YP_838849	2778	*B. cenocepacia* HI2424	
	YP_002235020	2775	*B. cenocepacia* J2315	

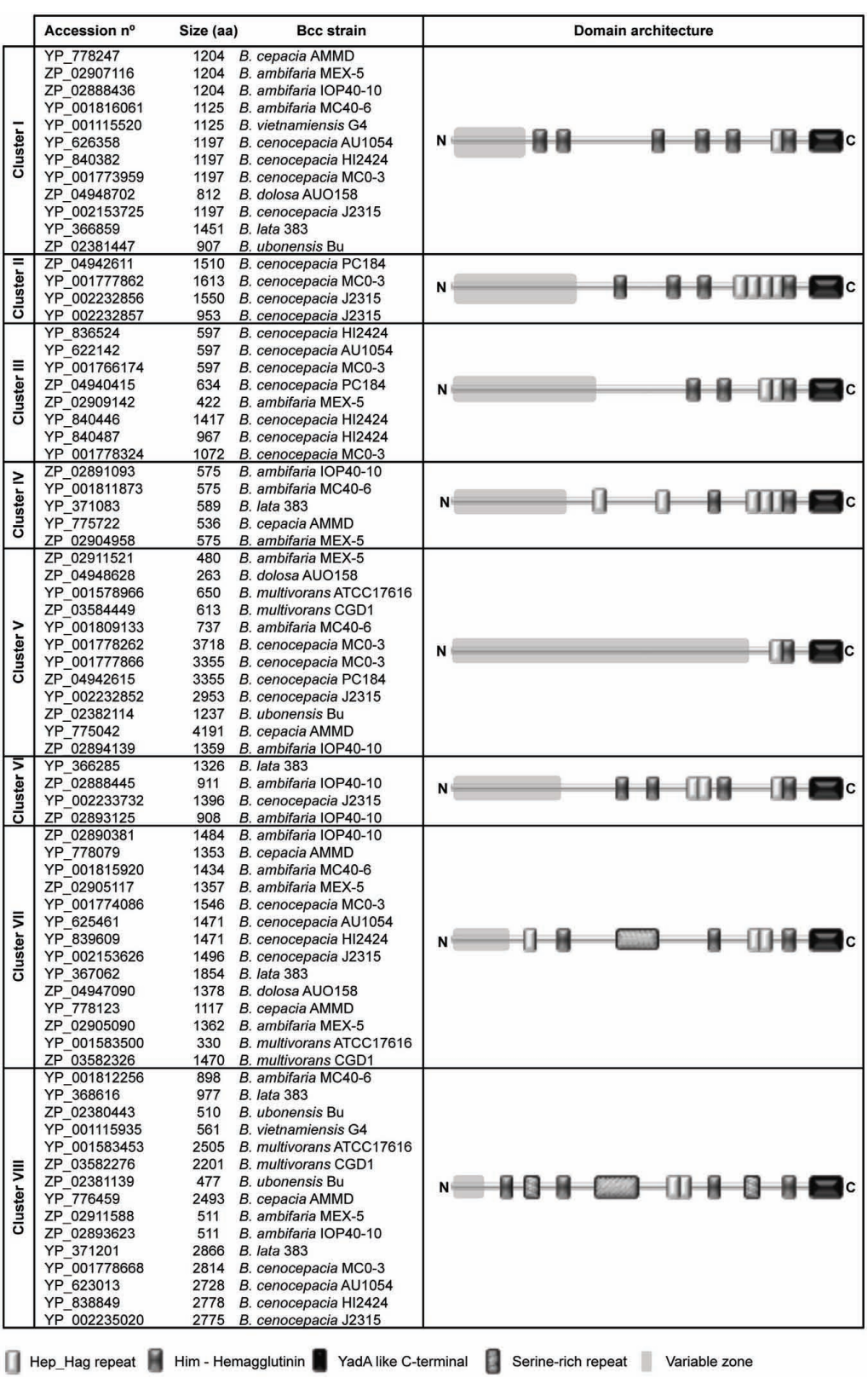

FIGURE 3 | Domain architecture of TAA proteins being conserved throughout the tree clusters defined in Figure 2. The Pfam database (Finn et al., 2010) was used to obtain the details of domain organization. Keys for the Pfam domains are shown in the bottom.

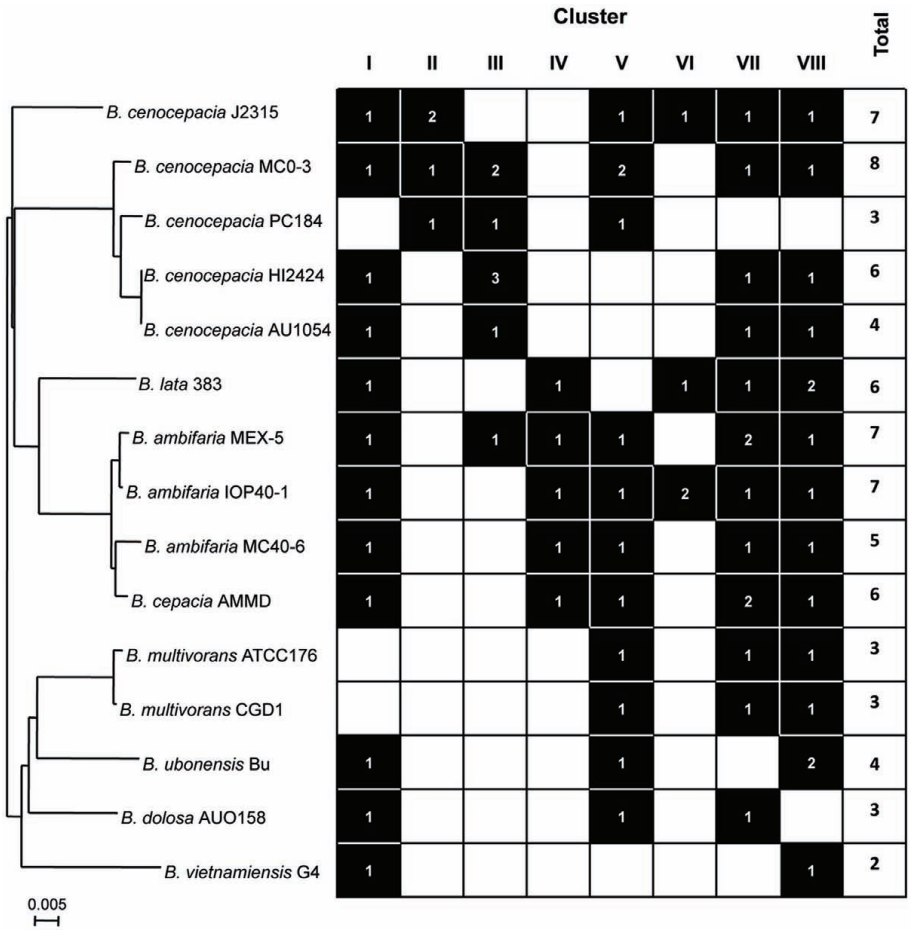

FIGURE 4 | Relationship between Bcc phylogeny (based on *recA* sequence) and the presence/absence of the TAAs across the eight tree clusters defined in Figure 2. Phylogenetic distances were estimated using the neighbor-joining method (Saitou and Nei, 1987), applying a distance matrix and visualized with NJplot (Perriere and Gouy, 1996). A branch length of 0.005 substitution/site is given to phylogenetic distances.

IDENTIFICATION OF NOVEL TAAs IN THE EPIDEMIC CLINICAL ISOLATE *B. cenocepacia* J2315

We further focused our analysis on the epidemic strain *B. cenocepacia* J2315 in which seven TAAs were annotated (**Figure 6A**). Of the seven TAA-encoding genes, five were located on chromosome 2 (*BCAM0219*, *BCAM0223*, *BCAM0224*, *BCAM2418*, *BCAM1115*) and two on chromosome 3 (*BCAS0236*, *BCAS0335*; **Figure 6B**). These observations are consistent with the fact that the chromosomes 2 and 3 contain a large number of virulence genes, whereas chromosome 1, the largest replicon, carries the majority of the core functions (Holden et al., 2009).

As previously stated in **Figure 4**, these seven TAAs are scattered over the phylogenetic tree, as follows: one member in cluster I, V, VI, VII, and VIII and two members in cluster II. Analysis of these TAA proteins reveals the existence of common complex architectures represented by multi-modular and polyfunctional domains, despite only weak sequence conservation (**Figure 6A**). Given the importance of TAAs in the virulence of Gram-negative pathogens, it is likely that these multifunctional proteins may play decisive roles in *B. cenocepacia* virulence.

Of the seven TAAs identified, three (*BCAM0219*, *BCAM0223*, *BCAM0224*) are described by Mil-Homens et al. (2010) and form part of a 24-kb cluster located downstream of the *B. cenocepacia cci* pathogenicity island (Baldwin et al., 2004). This cluster has a unique gene arrangement composed by three TAA-encoding genes (*BCAM0219*, *BCAM0223*, *BCAM0224*), one lipoprotein (*BCAM0220*), two sensor histidine kinases (*BCAM0218*, *BCAM0227*), and three response-regulator genes (*BCAM0221*, *BCAM0222*, and *BCAM0228*; Mil-Homens et al., 2010). Recently, McCarthy et al. (2010) demonstrated the involvement of the *BCAM0227* sensor kinase in the perception of a cell–cell signaling molecule known as the *Burkholderia* diffusible signal factor (BDSF).

Quantitative real-time PCR analysis reveals that these TAAs clustered genes are overexpressed under certain environmental conditions such as, high osmolarity, oxygen limited conditions, and oxidative stress (Mil-Homens et al., 2010). Further, Mil-Homens et al. (2010) have developed a series of PCR-based assays to verify the presence of the selected TAA genes in 47 genomes representing the 17 species of the Bcc (Mil-Homens et al., 2010).

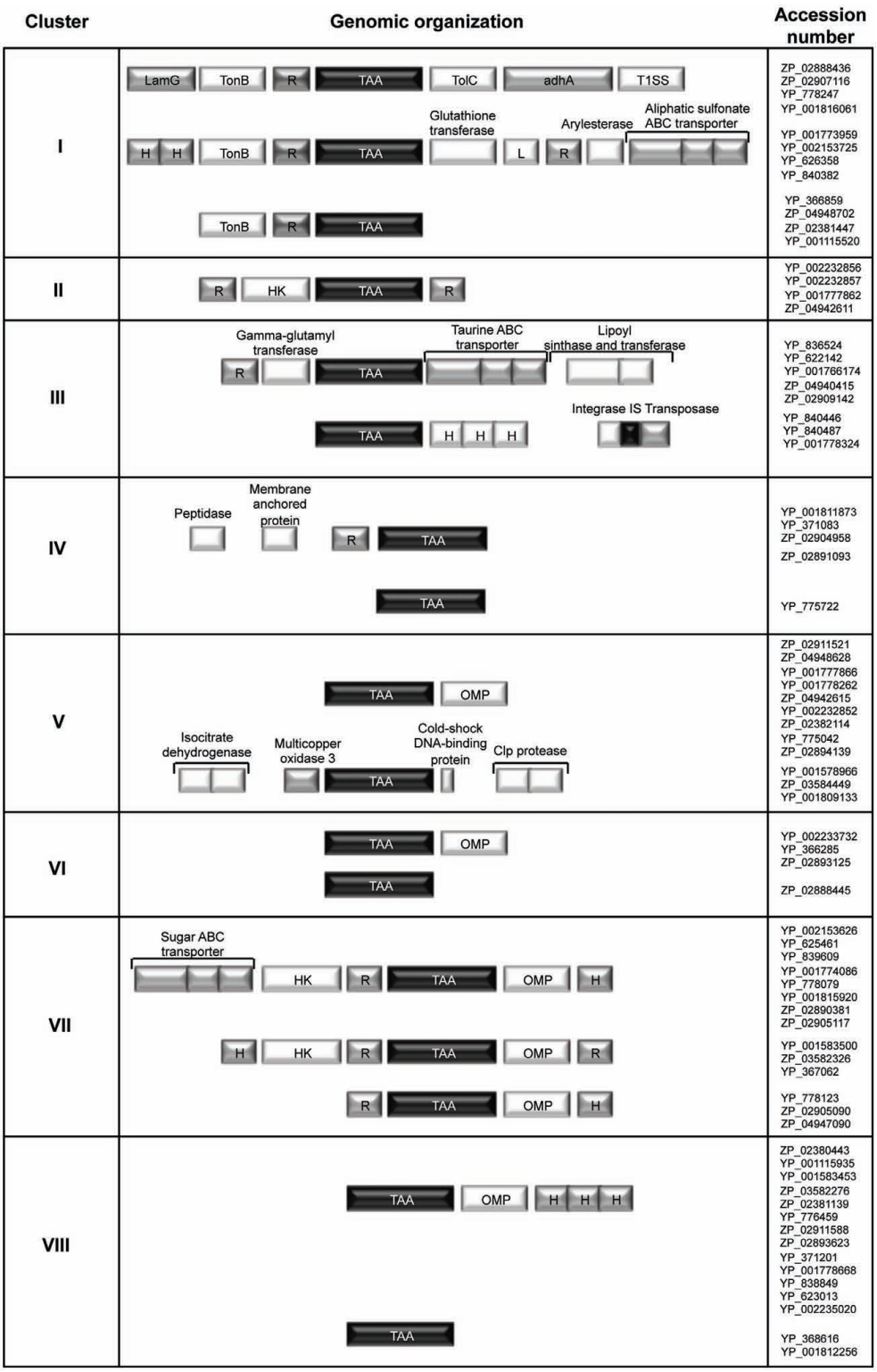

FIGURE 5 | Conserved synteny blocks around the TAA genes in the Bcc genomes. Each syntenic block is representative of each defined cluster of the phylogenetic tree showed in **Figure 2**. The conserved chromosomal arrangement and annotation of their neighboring genes are presented. (TonB, TonB-dependent siderophore receptor; HK, histidine kinase; R, regulator; OMP, outer membrane protein; TAA, trimeric autotransporter adhesin; L, lipoprotein; LamG, laminin G domain of extracellular proteins; TolC, TolC outer membrane channel; adhA, cable pili-associated adhesin; T1SS, type I secretion system; H, hypothetical protein).

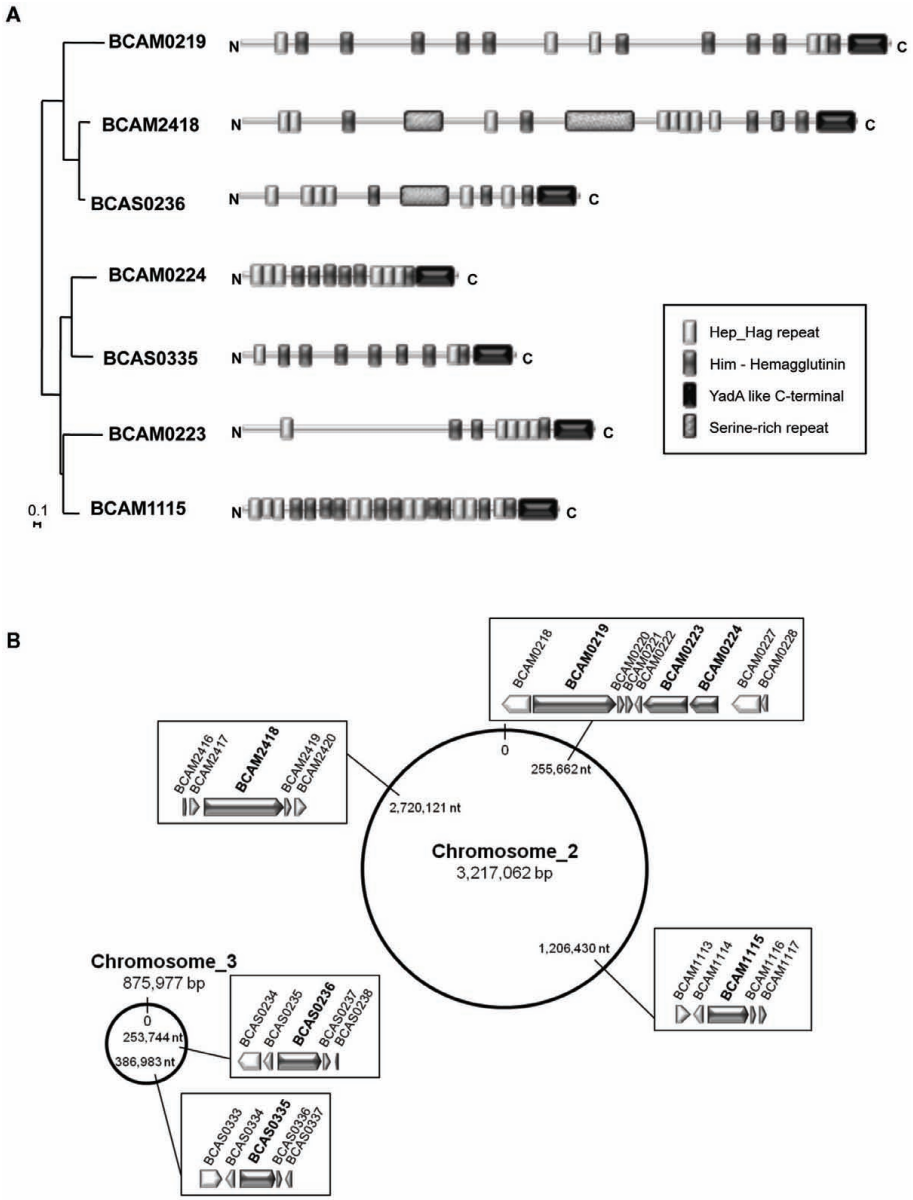

FIGURE 6 | (A) Phylogenetic relationships and domain organization of the seven TAAs from the epidemic strain *B. cenocepacia* J2315. Phylogenetic distances of the C-terminal (70–71 aa) of the seven TAAs from *B. cenocepacia* J2315, were estimated using the neighbor-joining method (Saitou and Nei, 1987), applying a distance matrix and visualized with NJplot (Perriere and Gouy, 1996). A branch length of 0.1 substitution/site is given to phylogenetic distances. Domain organization is defined based on Pfam analysis (Finn et al., 2010). Keys for the Pfam domains are shown in the bottom. **(B)** Chromosomal location of the seven TAA-encoding genes in the genome of the epidemic strain *B. cenocepacia* J2315.

It is noteworthy that a PCR test targeting the TAA-encoding gene *BCAM0224* has been proved to be specific for epidemic *B. cenocepacia* strains belong to the ET-12 lineage (Mil-Homens et al., 2010). Aiming to prove the usefulness of this PCR assay as a genetic marker to discriminate epidemic strains of the ET-12 lineage, Mil-Homens et al. (2010) also assessed the use of the BCESM marker (Mahenthiralingam et al., 1997) across the same panel of Bcc strains. The results obtained have shown that the *BCAM0224* sequence was exclusively detected in members of the ET-12 lineage whereas the BCESM sequence was found in ET-12 isolates as well as in some other epidemic and non-epidemic *B. cenocepacia* isolates (Mil-Homens et al., 2010). Thereby, we consider that this novel PCR-based assay may serve as a valuable tool to aid in Bcc strain identification.

The deduced protein encoded by *BCAM0224* is composed of 953 amino acids, with a calculated molecular mass of 85 kDa and a pI of 4.02 (Mil-Homens et al., 2010). Analysis of the amino acid composition revealed that the protein contained 7.1, 3.8, 41.6, and 58.4% acidic, basic, polar, and hydrophobic residues, respectively. The presence of an extended signal sequence with a

predicted cleavage site between amino acids 1 and 43 was identified. The deduced amino acid sequence encoded by *BCAM0224* showed a head–stalk–anchor modular structure composed by seven clusters of Hep_Hag (Pfam domain PF05658), six clusters of HIM (Pfam domain PF05662), and two collagen-binding domains (Pfam domain PF01391; **Figure 6A**; Mil-Homens et al., 2010).

In order to investigate the contribution of BCAM0224 for virulence, Mil-Homens et al. (2010) have constructed a knockout mutant and tested its ability to adhere to ECM components and to kill the larvae *Galleria mellonella*, used as a model host to study *Burkholderia* pathogenesis (Mil-Homens et al., 2010). Overall, the TAA BCAM0224 protein showed adhesive properties to collagen type I, one of the most abundant components of the ECM. Furthermore, the same authors also examined adhesion of the *Escherichia coli* BL21 cells expressing the gene of interest. A significant difference was found between the recombinant and the vector control, confirming that BCAM0224 has collagen-binding properties. Finally, Mil-Homens et al. (2010) used the insect *Galleria mellonella* as a model of infection to analyze whether BCAM0224 is a virulence determinant in the pathogenesis of *B. cenocepacia*. At 72 h post-infection, compared to the wild-type *B. cenocepacia* K56-2, the BCAM0224-mutant exhibited attenuated (10%) killing ability in comparison to the wild-type (Mil-Homens et al., 2010). Collectively, these results strongly suggest that BCAM0224 is important for adhesion and virulence of *B. cenocepacia* cells.

CONCLUDING REMARKS

Over the last years, important advances have been made in the study of TAAs, as novel virulence factors produced by Gram-negative bacteria, where their main function is to act as adhesins. To initiate infection Bcc species must be able to colonize the respiratory epithelium by binding to specific host macromolecules. This process has only begun to be studied and remains to be fully characterized. With these aspects in mind, here we present an *in silico* approach to identify TAA-encoding sequences in Bcc pathogenic strains. As a result, 74 TAA-encoding genes potential implicated in functional aspects associated to Bcc pathogenicity, such as cell adhesion, were predicted and classified by phylogenetic analysis. Among the candidates, we review experimental data supporting that the BCAM0224 from *B. cenocepacia* J2315 represents a collagen-binding TAA with an important role in cellular adhesion and virulence. Overall, the TAA proteins identified in this study are promising targets for future experimental analysis and could represent a valuable resource for unveiling mechanisms underlying the pathogenesis of Bcc bacteria.

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