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Research Paper

Trimeric autotransporter adhesins in *Acinetobacter baumannii*, coincidental evolution at work

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ABSTRACT

Trimeric autotransporter (TAA), also known as type Vc secretion system, is expressed by many strains of *Acinetobacter baumannii*, an opportunistic pathogen, which is responsible for nosocomial infections worldwide. TAAs, are modular homotrimeric virulence factors, containing a signal peptide, complex stalk, and conserved membrane anchoring domain. The evolutionary mechanisms underlying the evolvement of these adhesins are not clear. Here, we showed that TAA genes were laterally acquired and underwent gene duplication and recombination.

The heterogeneity of TAA nucleotide sequences, GC content, codon usage, and the probability of recombination and duplication events were assessed by MEGA7. Given the heterogeneity of sequences, we used all-against-all BLAST for clustering the TAAs. The pattern of distribution of TAAs are highly scattered; GC content and codon usage for these genes are variable. Multiple events of lateral gene transfer from the early history of Acinetobacter and the occurrence of gene duplication, gene loss, and recombination after acquiring the alien genes may explain the scattered pattern of distribution of TAAs.

Additionally, this gene is not present in many clinical isolates of *A. baumannii*, thus is not a single virulence factor attributing to the infection. The advantage of harboring such genes might be adopting to different environments by developing the biofilm communities. We suggested that TAA genes were laterally acquired in the environmental context and incidentally provided some benefits at the infection site. Thus, coincidental evolution theory may be better suited for describing the evolution of TAA genes in *A. baumannii* genomes.

1. Introduction

Acinetobacter species are vastly distributed in the environment and recently have been emerged as an opportunistic human pathogen. *Acinetobacter baumannii* is the most frequent cause of infections in the *Acinetobacter* genus. It is well separated from the other species based on the genome-wide analysis (Chan et al., 2012). This species is a notorious opportunistic pathogen (Howard et al., 2012) accountable for nosocomial infections all over the world. The pathogen is able to infect multiple anatomic sites (Harding et al., 2018) such as respiratory tract, urinary tract, skin, and soft tissue (Jahangiri et al., 2018; McConnell et al., 2013).

The evolution of *A. baumannii* is dictated by an open pan-genome, which is prone to the acquisition of alien virulence factors. This property could lead to the transformation of an innocuous commensal organism to a serious threat for human health (Peleg et al., 2008). The plasticity in the genome of Acinetobacters (Harding et al., 2018; Imperi et al., 2011) has significantly facilitated the adaptive evolution (Ghalambor et al., 2007; Ogier et al., 2010) and persistence in harsh environments.

Expansion of highly problematic clonal lineages of *A. baumannii* is mainly owing to the antimicrobial resistance trait of the organism (Diancourt et al., 2010). Whereas, the importance of many other putative virulence factors shared by the majority of strains is not clear (Imperi et al., 2011). Virulence factors are known as pathogen determinants whose loss specifically weakens the virulence but not the

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viability. Secretion systems and adhesins are classic examples of virulence factors (Brogden et al., 2006).

A. baumannii is primed to anchor the tissue of the host (Murray et al., 2017). Thus, the key factor at the primary site of infection is the attachment of the bacteria to the cellular components. Similar to other pathogenic organisms, this initial event of infection is mostly invoked by the surface components of *A. baumannii*. In this regard, trimeric autotransporter adhesins (TAAs) of Gram-negative bacteria have recently garnered a lot of attention. These adhesins are endowed with properties such as domain complexity, binding properties (Łyskowski et al., 2011), agglutination (Bentancor et al., 2012b), auto transportation (Linke et al., 2006), and holding repetitive modules within their sequences (Meng et al., 2008). TAA representing the type Vc secretion system (T5cSS) (Dautin and Bernstein, 2007; Navarro-Garcia et al., 2004), are homotrimers, containing an N-terminal signal peptide, a stalk or passenger domain, and a membrane-anchored domain responsible for projecting the stalk through a pore of 12 beta strands from the outer membrane to the external milieu; hence it is nominated as autotransporter (Bassler et al., 2015).

T5cSS has also been shown to be expressed by *Acinetobacter* species. The first member of T5cSS in Acinetobacter species, $Ata_{A.\ baumannii}$, has been introduced in clinical isolates of *A. baumannii*. It has been shown that $Ata_{A.\ baumannii}$ plays a role in the infection onset and promoting biofilm formation (Bentancor et al., 2012a). $Ata_{A.\ baumannii}$ was also found to be implemented in adherence of bacterial cells to surface components of human epithelial cells (Weidensdorfer et al., 2016), therefore is considered as a virulence factor. Another member of TAAs, namely AtaA, has been introduced in the environmental species, *Acinetobacter* Sp. Tol5 (Ishikawa et al., 2012). AtaA was reported to be required in high adhesiveness of the bacterium to abiotic surfaces (interested readers are referred to (Koiwai et al., 2016) who has discussed several aspects of AtaA molecular biology, which indicated great similarity to $Ata_{A.\ baumannii}$).

TAAs have been vastly characterized in several species. Multiple functions, which are contributed to the multiple domains with intrinsic structures are ascribed to these adhesins. Majority of the available data have been unraveled in the light of molecular infection biology, in which the observed symptoms of infection are interpreted by physical or deterministic interactions of pathogens with their hosts (Diard and Hardt, 2017). These approaches would partly describe the virulence factors. Then, the evolutionary studies are required, as a complement, for better understanding the conservancy, evolutionary relationships, and their role in the pathogenesis of *A. baumannii*.

Although many clinical and environmental species express homologs of TAAs, their expression pattern appears to be highly heterogeneous (Weber et al., 2017). Given these circumstances, getting a better grasp of the existing relationship between harboring a TAA and *Acinetobacter* infections would be drastically important for arming against this multi-drug resistance pathogen. Moreover, developing an evolutionary theory could be important to determine the functional properties of the target protein, the rational design of new vaccines, and provide opportunities for designing new anti-virulence drugs (Rasko and Sperandio, 2010). In the present study, we showed that the sequences of TAAs are heterogeneous among multiple species. Hence, we chose to use an all-against-all BLAST approach for clustering the TAA sequences. Clustering the sequences of TAAs allowed us to explore some aspects of the TAAs evolution. We employed an integrated in silico approach to investigate the correlation between the existence of TAA to *A. baumannii*-caused infections. We proposed that TAA encoding genes acquired through lateral gene transfer (also known as horizontal gene transfer), afterward, affected by gene duplication, recombination, and GC-biased gene conversion. We also argued that coincidental evolution theory (Brown et al., 2012) may account for the evolution of this virulence factor among Acinetobacters.

2. Methods

2.1. Sequence data sources

The UniProt Knowledge Base (Consortium, 2017), Interpro (Finn et al., 2017), and Pfam (Finn et al., 2016) databases were surveyed to extract the currently available data on TAA sequences and domains.

The sequence IDs of TAAs in Acinetobacters were extracted from Interpro (Finn et al., 2017) database. All nucleotide and amino acid sequences were retrieved by employing the ID mapping function of the UniProt server. Related coding sequences (CDS) were downloaded from the PATRIC bioinformatics resource center (Wattam et al., 2016). All amino acid sequences were obtained from the UniProt database.

All sequences were archived in MEGA7 (Kumar et al., 2016) for the further edition, conducting evolutionary analyses, generating alignments, reconstructing the phylogenetic tree, and defining the duplication events.

The genomes and phylogenetic tree of Moraxellacea family were obtained from the PATRIC bioinformatics resource center (Wattam et al., 2016).

The isolation sources of *A. baumannii* strains were extracted from the BioSample database of NCBI.

2.2. Evolutionary analysis

The homogeneity of substitution patterns calculating all pair-wise sequence comparisons was tested in MEGA7 (Kumar et al., 2016). For this analysis, the library of nucleotide sequences was trimmed in such a way that for each strain, just one TAA sequence was selected. This trimmed library of nucleotides of TAA encoding genes was used to calculate the disparity index (Kumar and Gadagkar, 2001). Corresponding *P*-values were estimated with the use of 1000 Monte Carlo based replicates as implemented in MEGA7 (Kumar et al., 2016).

2.3. Duplication events

The neighbor-joining tree (Saitou and Nei, 1987) was constructed on all TAA encoding genes downloaded from the PATRIC database. The gene duplications were identified by searching for all branching points in the topology with at least one species that was present in both subtrees of the branching point. An unrooted gene tree was used for the analysis such that the search for duplication events was performed by finding the placement of the root on a branch or branches that produced the minimum number of duplication events. Duplication events were inferred by duplication finder, implemented in MEGA7 (Kumar et al., 2016).

2.4. Recombination events

The recombination detection methods are sensitive to multiple sequence alignment lengths. Therefore, we selected a set of TAA encoding genes of similar sizes (approximately 8100 base pairs).

The aligned selected sequences were curated by CleanStopCodons of HyPhy package (Pond and Muse, 2005) for removing gaps and stop codons. The trimmed alignment was analyzed by GARD algorithm (Kosakovsky Pond et al., 2006) as provided by Datamonkey (Weaver et al., 2018) at www.datamonky.org. The analysis was also repeated employing PHI statistic test (Bruen and Bruen, 2005) as implemented in SplitsTree ver. 4 (Huson, 1998).

2.5. GC content and codon usage analysis

Percentage of the GC contents of bacterial genomes was obtained from ftp://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt.

Codon usage tables of bacteria and codon usage of each CDS were viewed in codon usage database at https://www.kazusa.or.jp/codon/.

The GC contents of two sets of fifteen TAA sequences from Acinetobacters and Burkholderia species were determined at Ensembl genome browser system (Kersey et al., 2017) at http://bacteria.ensembl.org. The two sets of core genes also were subjected to similar analysis as the control; aliphatic sulfonates import ATP-binding protein SsuB (Higgins et al., 2017) as a control for Acinetobacter, and fimbrial gene clusters (Sim et al., 2008) for Burkholderial species.

The best model for analysis of codons of TAA encoding genes was predicted to be General Time Reversible model (Nei and Kumar, 2000) of nucleotide substitution, by MEGA7 software.

Estimates of the numbers of inferred synonymous (s) and nonsynonymous (n) substitutions along with the numbers of sites that are estimated to be synonymous (S) and nonsynonymous (N) were produced using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model (Muse and Gaut, 1994) of codon substitution and General Time Reversible model (Nei and Kumar, 2000) of nucleotide substitution. For each codon, for estimating ML values, a tree topology was automatically computed. The test statistic dN - dS was used for detecting the codons that have undergone positive selection (dS is the number of synonymous substitutions per site (s/S) and dN is the number of nonsynonymous substitutions per site (n/N)). Maximum Likelihood computations of dN and dS were conducted using HyPhy software package (Pond and Muse, 2005). Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated.

2.6. tBLASTn searches

A target database of genomes, including 222 genomes of Moraxellaceae family was made locally and searches against genomes to find special targets were conducted by PerfectBLAST (Santiago-Sotelo and Ramirez-Prado, 2012) using tBLASTn algorithm (Gertz et al., 2006).

The same analysis was conducted for all available Acinetobacter bacteriophages and plasmids, separately. For all searches, the consensus sequence of the membrane anchoring domain was queried.

2.7. Clustering the protein sequences

A clustering analysis was performed on the sets of sequences, based on all-against-all BLAST+ similarities by CLANS (Frickey and Lupas, 2004) at https://toolkit.tuebingen.mpg.de/ (Alva et al., 2016). A variant of the Fruchterman and Reingold graph layout algorithm was used by CLANS to generate graphs providing a graphical representation of pairwise sequence similarities. A search against orthologue groups was conducted at www.orthodb.org (Zdobnov et al., 2016) to find out the distribution of TAAs, or other target proteins among Moraxellaceae family and the levels of evolutionary rates (Zdobnov et al., 2016).

2.8. Exploring genomic context of ata

Gene synteny analysis was explored via SyntTax server (Oberto, 2013) at http://archaea.u-psud.fr across available Moraxellaceae chromosomes using 10% normalized blast and the best blast hit matches (Shpaer et al., 1996). In order to define the role of neighbor protein coding regions, all gene products were extracted from the synteny file and subjected to gene ontology analysis. Gene ontology searches were conducted at http://cello.life.nctu.edu.tw by Cello2GO software (Yu et al., 2014).

A search through String database (Szklarczyk et al., 2014) was done for finding co-expression and co-occurrence profiles of genes across several genomes. An order-level pre-built tree was constructed in PATRIC database by an automated pipeline that begins with amino acid sequence files for each genome (Fig. 3). To understand the distribution of TAAs in this tree, a non-redundant library of trimeric autotransporters was produced through tBLASTn search against a locally built database of the related genomes. The membrane anchoring region of $Ata_{A.\ baumannii}$ (Uniprot Acc. No. A3M3H0) was queried for tBLASTn search because this sequence is currently the only reviewed member of the family in the databases.

3. Results

3.1. Sequence extraction

The Interpro and PATRICK databases were well suited for our analysis (in terms of annotations and clustering) in order to extract the true members of TAAs. The Interpro database was searched for extracting the TAA protein IDs and viewing the domain architectures. Contemporary, there are 6717 protein sequences which match for YadA-like C-terminal (IPR005594). Among, 5650 proteins belong to Proteobacteria, 551 sequences belong to Moraxellaceae family, 374 sequences belong to Acinetobacters, and 74 sequences belong to *Acinetobacter baumannii/calcoaceticus* complex. Similar domain architectures were found in several genomes specially Burkholderials.

3.2. Homogeneity of TAA encoding genes

In order to examine whether the pattern of nucleotide substitution is constant in TTA encoding genes of Acinetobacters, the disparity index tests of substitution pattern of homogeneity were performed for each pair of TAA sequences. In the disparity index tests, all (1st + 2nd + 3rd) codon positions were used. The pattern was analyzed separately for nucleotide sequences of *A. baumannii* and other species excluding *A. baumannii* (henceforth, we call them non-baumannii Acinetobacters). The probability of rejecting the null hypothesis of homogeneity was rejected for 62.04% of sequence pairs in non-baumannii Acinetobacters, while for *A. baumannii* TAAs this value was 61.43% (and 64.17% for all sequence pairs). This implies that the substitution pattern may not be constant among TAA encoding genes in *Acinetobacter* species, suggesting that TAA in Acinetobacters have evolved via different evolutionary processes. In Addition, no clear dichotomy was observed between pathogenic and environmental species.

3.3. Recombination and duplication events

The whole library of TAA genes from Acinetobacter species was analyzed for defining the recombination and duplication events. The genetic algorithm for recombination detection (GARD) did not find any sign of recombination. Since the repetitive proteins such as TAAs are prone to recombination, the existence of such a phenomenon is potentially expected. Thus, we did not confine our analysis to GARD; we also attempted to trace the recombination by the PHI test, which is shown to be more sensitive than other methods (Bruen and Bruen, 2005). The PHI test found statistically significant evidence for recombination (P = 2.97e-4).

Duplication events were searched through a phylogenetic tree that was constructed by TAA encoding genes of Acinetobacter species. The method predicted at least 29 duplication events within the dataset (Fig. 1).

3.4. Codon usage and GC contents analysis

To determine if the TAA genes are alien in Acinetobacter genomes or not, the codon usage and GC contents of a set of TAA genes were analyzed. The test statistics, conducted in MEGA7, indicated an overabundance of nonsynonymous substitutions in the codon usage of TAA encoding genes suggesting that these genes underwent a positive selection. In addition, comparison of the codon usage of each TAA encoding genes with the whole coding sequences of Acinetobacter showed relatively different patterns. These differences are evident in the heat map (Fig. 2).

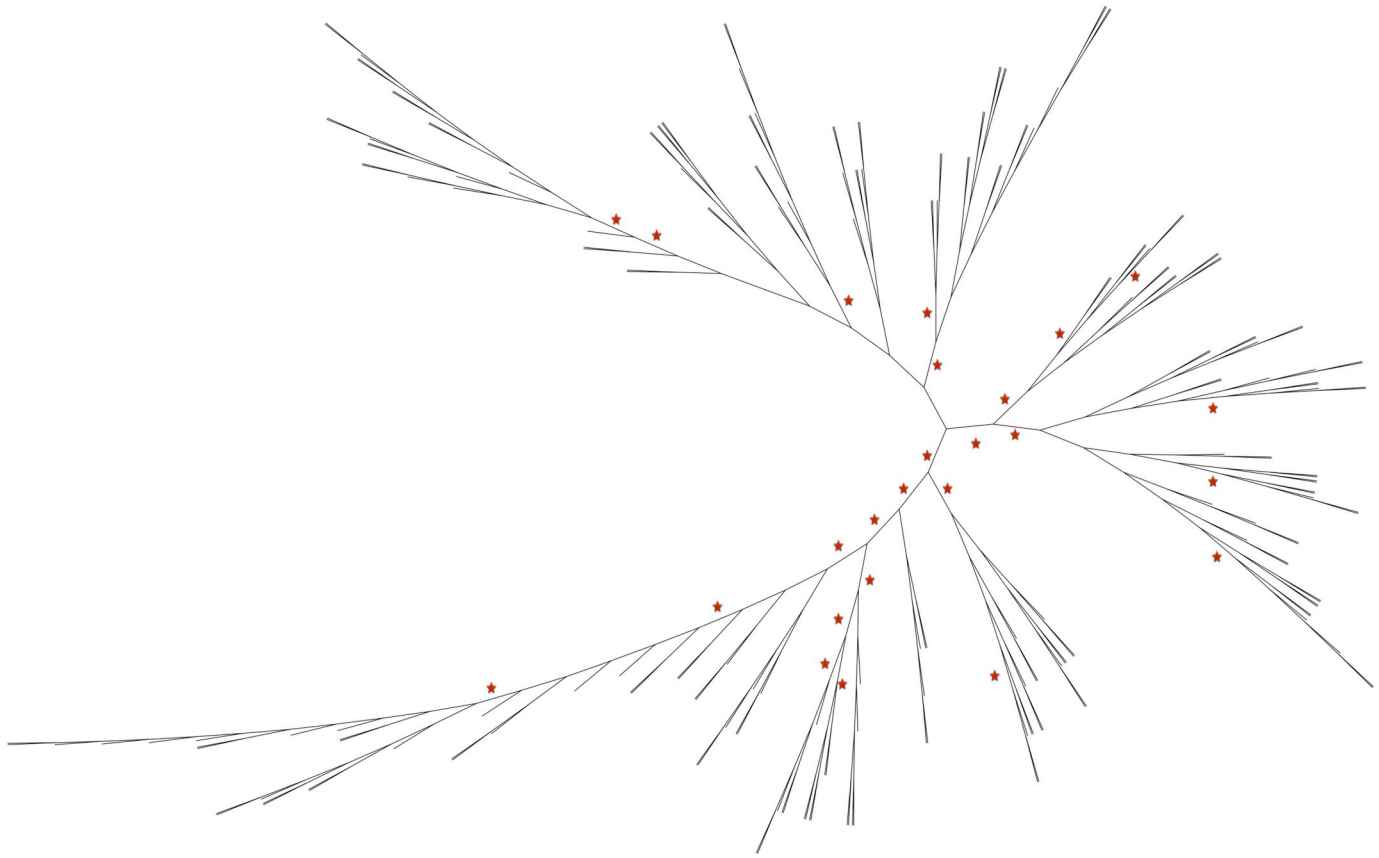

Fig. 1. Duplication events. The phylogenetic tree constructed on TAA encoding genes of Acinetobacters is presented; the red marked branches shows the duplication events. Leave labels are omitted for simplicity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A nucleotide composition analysis of TAAs showed that most TAAs in Acinetobacters had a higher GC content than the average GC content of Acinetobacters (40%). Average differences between the GC content of TAAs of Acinetobacters and the average GC content of Acinetobacter genome (Bias) were calculated for 50 randomly selected TAAs. This value was estimated as 8.12 (it can be compared to ssuB genes set (a confirmed core gene of Acinetobacters), which was estimated as 3.29).

3.5. tBLASTn searches

In order to find the presence of TAA genes in *A. baumannii* strains or plasmids or Acinetobacter related phage genomes, a tBLASTn search was conducted against the libraries of mentioned genes. TAAs were appeared to be present in more than two thousand species of *A. baumannii*. There was no homologous sequence in the genomes of Acinetobacter bacteriophages. Among all sequenced plasmids, TAA was found in one *A. baumannii* plasmid (pA1296_1, complete sequence, hypothetical protein BS103_17785 (plasmid)).

3.6. Conservancy throughout TAA sequences

In order to find the conserved blocks across TAA amino acids sequences the alignment approach was used. The alignment of 74 *A. baumannii* specific TAA sequences of various strains revealed their different lengths, low overall sequence identity, and various conserved blocks. These facts could be construed as multiple recombination events. The most conserved block, as expected, was the membrane anchoring region.

As a conserved protein family, the Serralysin-like metalloprotease, C-terminal (IPR011049) was also observed (based on Interpro database reviews); this domain is related to the beta-roll head domains of TAAs, however, none of the consensus patterns related to metal binding motives of this domain family was observed in TAA amino acid sequences.

3.7. Clustering analysis of TAAs

We attempted to clustering the TAA protein sequences to better viewing the properties of these adhesins. A neighbor-joining tree was constructed on 74 TAAs of *A. baumannii*. The sequences were categorized into 5 distinct groups (Fig. 3). Some species harbored more than one TAA sequence, implying gene duplication events. In all cases, paralog sequences were scattered among the five groups (Supplementary data 1). Multiple duplication events might be led to the occurrence of different paralogs.

Phylogenetic reconstructions were not robust and the bootstrap values were usually low. Using models that do not account for this heterogeneity between and within species may be inadequate for these data. Hence, we chose to use the CLANS analysis.

Two separate libraries were built by 5650 TAA sequences of Proteobacteria (library 1) and 551 TAAs of Moraxellaceae family (it is a sub-collection of TAA sequences of Proteobacteria, library 2).

Clustering classification was done to represent the groups of more similar sequences within our two libraries, separately (BLOSUM62 matrix, E = 10–20 for library 1 BLOSUM62 matrix, E = 10–45 for library 2). The CLANS clustering analysis of library 1 showed the accumulation of significant BLAST hits related to AtaA. baumannii in one central clade (Fig. 4b) (including 1424 members; Supplementary data 1) and more scattered sequences outside the focused clades. The clade containing TAAs of Acinetobacters, included 514 TAA sequences from Gammaproteobacteria (excluding Moraxellaceae) and also filled by 490

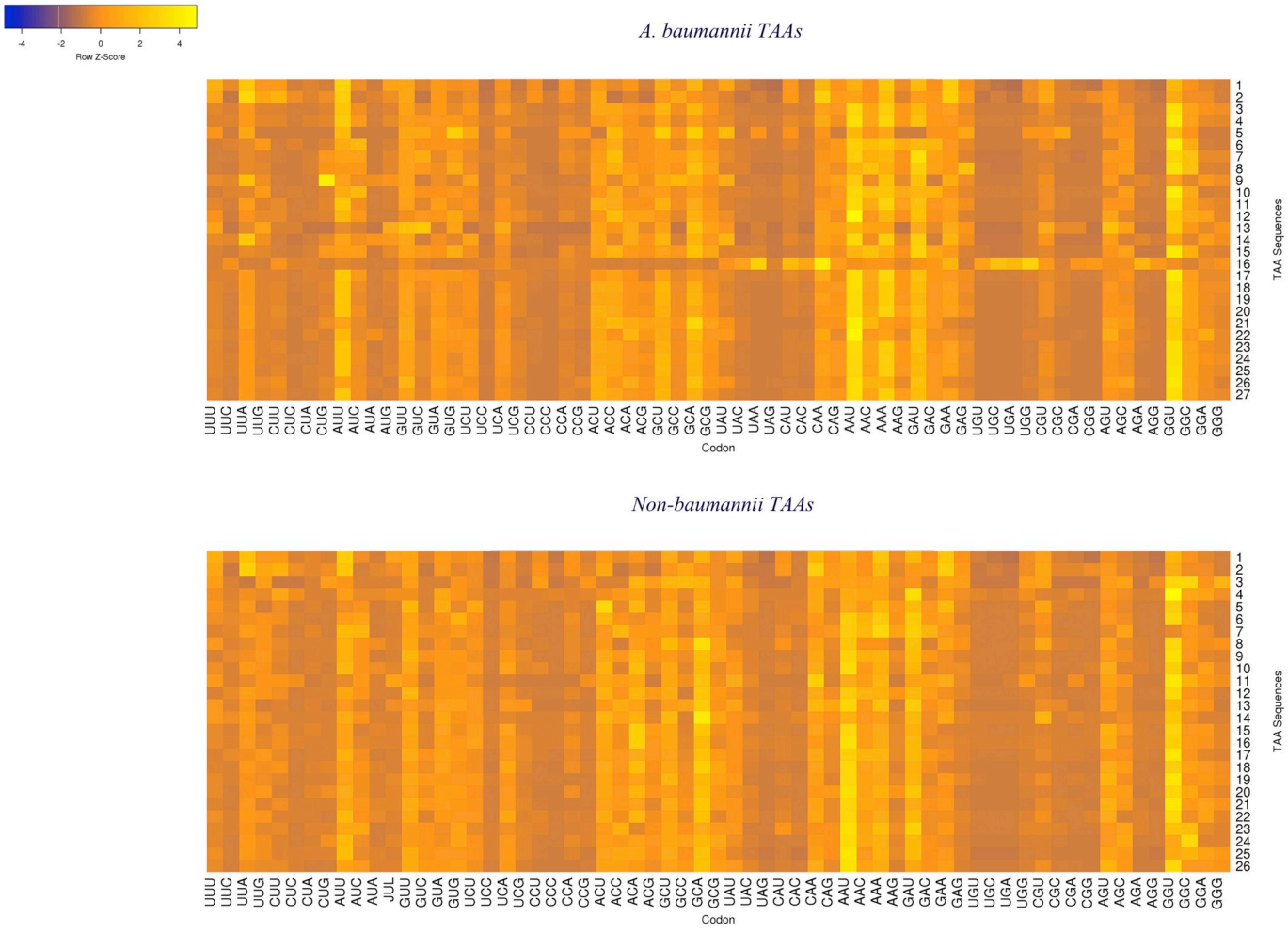

Fig. 2. Heat map of codon usage. The percentage of codon usage for each codon is presented as a heat map. The map on the top is related to TAAs of *Acinetobacter baumannii*, and the bottom is related to non-baumannii Acinetobacters (see the text). First and second lines are codon usage of the whole coding sequences of Acinetobacters and a core gene as a control, respectively.

sequences from Betaproteobacteria (mostly Burkholderia species (415 sequences)), suggesting a significant similarity between the TAA sequences of Acinetobacters and Betaproteobacteria. Through clustering analysis, six clades were identified in library 2 (Fig. 4a). Clade one included 313 sequences; the longest sequence in this clade (and also within the whole library) belonged to Moraxella bovoculi (Acc. No. A0A0U2BMI1, 7042 aa). This clade additionally contained AtaA. baumannii reference sequence, sequences from Psycrobacter, and the members of Moraxella genus (Supplementary data 1). Clade two included Acinetobacters and Psychrobacters; Clade three and four were genus specific, relating to Acinetobacter species (mostly environmental species). Clade five and six were also genus specific, relating to Moraxella and Psychrobacter genera, respectively. The average of isoelectric pH, as well as the average sequence length and cluster density, discriminated clade one from the other clades.

When the consensus sequence of membrane barrel of TAA of Acinetobacters was queried at Orthodb database, two groups of 28 and 30 genes were observed at Acinetobacter level. The density of TAA encoding genes was different among Acinetobacter genomes, due to the gene duplication incidents. The highest density of TAAs belonged to Acinetobacter Sp. CIP 70.18 (6 sequences), and most of the chromosomes contained a single copy of TAA encoding gene. Based on the data bank of orthologs (Orthodb), the evolutionary rate of TAAs at Moraxella level was calculated as 1.60 and 1.43 at the Acinetobacter level, revealing the higher sequence diversity of TAAs in Moraxella than Acinetobacter genus. The OmpA-like domain containing protein, which follows the TAA genes in many genomes (see below), showed the evolutionary rate of 1.37. These similar evolutionary rates revealed the evolutionary rate co-variation of these two genes.

3.8. Gene arrangement in the context of TAAs in Acinetobacters

When the consensus sequence of membrane anchoring region of TAAs of Acinetobacters (51aa) queried against 208 chromosomes of Moraxellacea at SyntTax server, higher scores of gene synteny were related to *A. baumannii* and *A. nosocomialis* (76 species with scores of > 85); most of these species were clinical isolates (66 strains), mainly isolated from respiratory tract source (29 strains). 51 chromosomes showed lower scores (< 80), and 40 chromosomes did not show any synteny. The existence of environmental samples with the same gene order is worth mentioning (Supplementary Data 2 and 3). Moreover, the same synteny was not observed across the genomes of Burkholderiacea members and Acinetobacters (supplementary data 4). This might be deemed as evidence for rolling out sharing of the same common ancestry for these two genera.

Regardless of synteny scores, TAAs in all chromosomes with the same gene arrangement were followed by OmpA-like domain containing protein, which suggests a functional correlation with these two genes. Additionally, searches through genome databases at String database server revealed the co-occurrence of TAAs with OmpA-like domain containing proteins as well as co-expression of these two genes (data not shown). Two-component histidine kinase systems were also

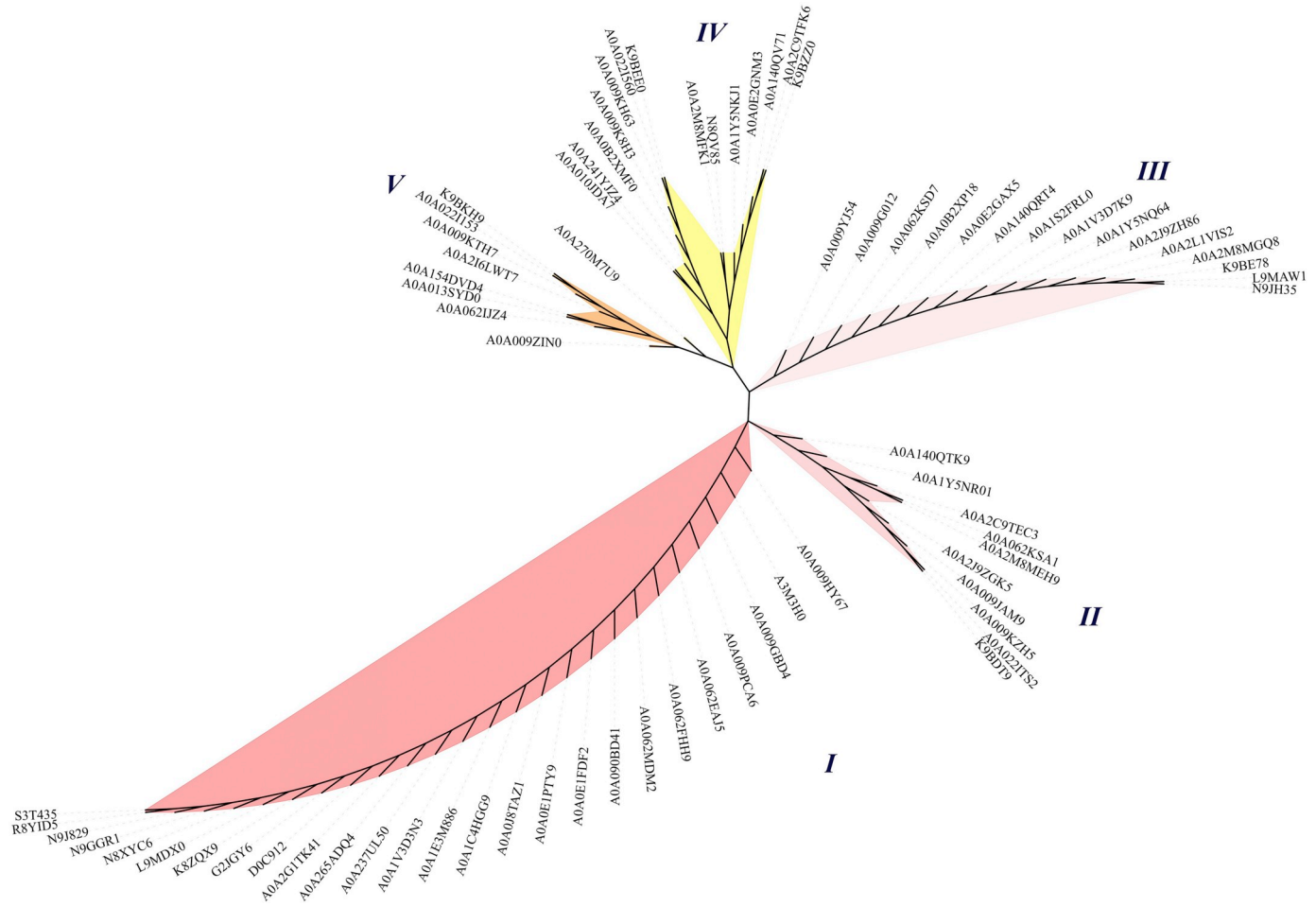

Fig. 3. The evolutionary relationship of 74 *A. baumannii* specific TAAs.
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.93 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 74 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 62 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

observed upstream of TAA encoding gene. These genes and TAA encoding gene are not in the same direction.

Viewing TAA orthologs across Acinetobacter genomes showed that all the multi-copies of TAAs in one species were not followed by OmpA-like domain containing protein. For example, *A. bouvetii* DSM 14964, harbors a short TAA (Locus tag: F941_01139, Acc. No. N9DK69) and three OmpA-Like protein viz. F941_01140 (N9DQV3), F941_02268 (N9C8Q2), and F941_02717 (N9DGY7). In contrast, *A. baumannii* UH8107, contains two TAAs (P680_0786, P680_2657), and one OmpA-like domain containing protein (P680_4078 SmpA/OmlA family protein).

3.9. The genomic context in the vicinity of TAAs in Acinetobacters

To draw a conclusion on the function of TAA harboring region of chromosomes, the neighbor protein-encoding genes were extracted from the synteny file. The extracted genes were subjected to a gene ontology analysis. 653 out of 1311 gene identifiers were successfully mapped to 402 UniProtKB IDs. Out of the remaining IDs, 645 gene identifiers were successfully mapped to the UniParc sequence archive. Gene ontology analysis resulted in 36 molecular functions (Fig. 5a) and 47 biological processes (Fig. 5b). The majority of proteins were cytoplasmic (49%) (Fig. 5c) and GO terms were enriched by binding, metabolic activity, and stress response. This implies that TAAs in the genomes of the same synteny are located in a metabolic island.

3.10. TAA harboring clinical species of Acinetobacter mostly caused localized infections

Fig. S1 shows that TAAs are distributed over the leaves of different and somehow distant clades. Totally, 136 leaves contain TAA across Moraxellaceae family, among which 113 are related to Acinetobacters (Supporting data 1, sheet 4). In order to find out any correlation between the existence of TAA and the infections caused by Acinetobacters, the isolation sources of species were taken into account (Supporting data 1, sheet 5). Most TAA harboring species were appeared to cause localized infections (e.g. wound infection). Urine source (6), body fluid (3), or bloodstream (10 species) sources were rare.

4. Discussion

The accurate description of selection, fortifying, and maintaining the virulence factors are arguable (Brown et al., 2012). Virulence factors presumably are the products of natural selection because they can be mechanistically multifaceted. Given the heterogeneous pattern of substitution and the scattered pattern of distribution of TAAs across the analyzed genomes, arriving at a robust conclusion about the

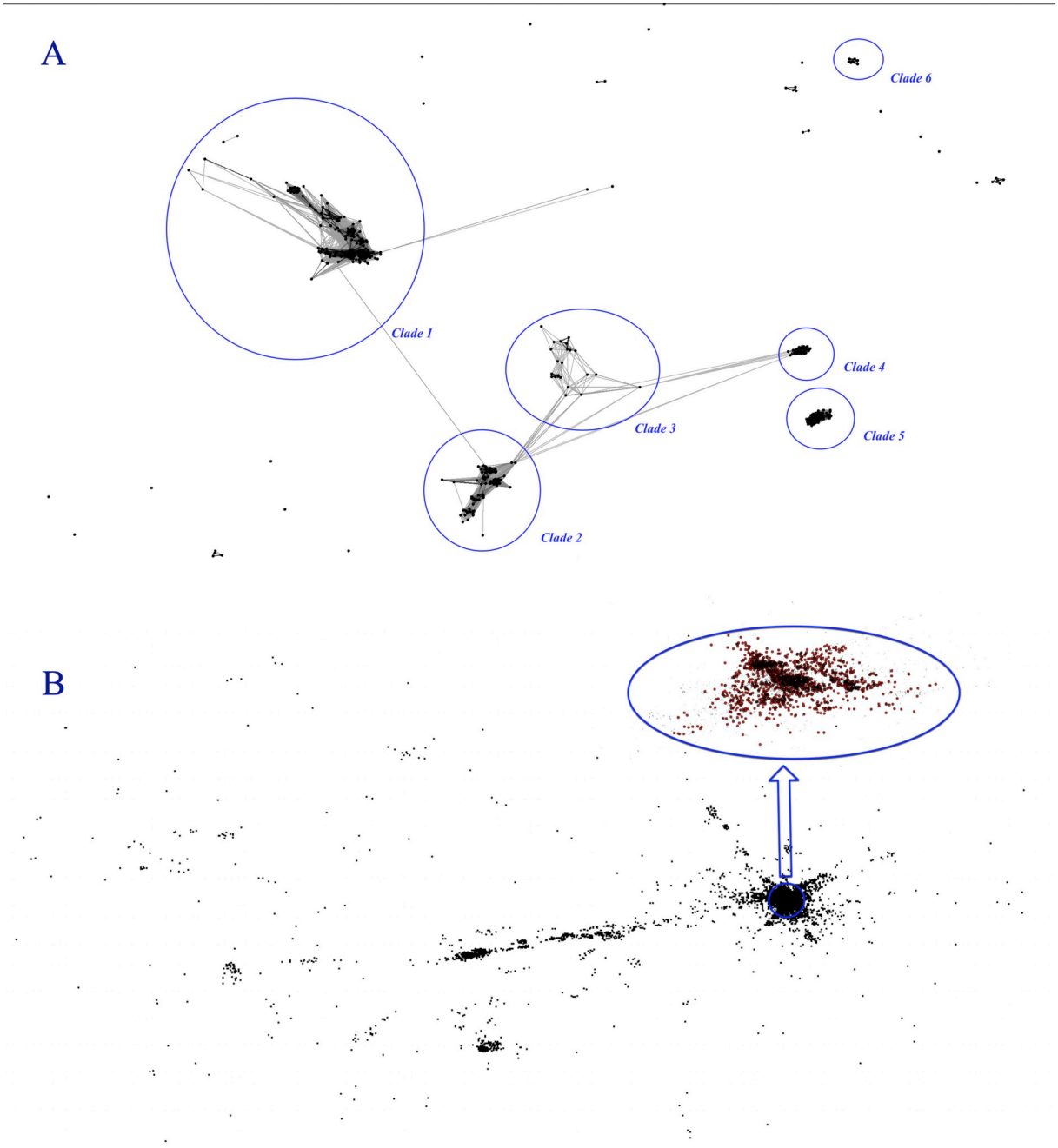

Fig. 4. Graphical projection of the clusters, obtained through the CLANS analysis. A- Six well-separated clades were identified by all-against-all BLAST of TAA protein sequences of Moarxellaceae family. Each dot in the graph represents a protein sequence. BLAST high scoring segment pairs (HSPs) are shown as edges connecting dots reflecting attractive forces proportional to the negative logarithm of the HSP *P*-value, proportional to the intensity of the gray shading for connections. Different groups of TAAs are shown as clouds of assigned blue-colored circles. B- Clustering analysis of all TAAs of Proteobacteria. The focused clade containing TAAs of Acinetobacters is separated from other clades. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evolutionary history of TAAs in Acinetobacters becomes highly controversial. This controversy has been addressed by other authors for other genera (Fialho and Mil-Homens, 2011; Pina et al., 2009) and has been explained by substitution saturation and technical limitations related to the repetitive nature of TAAs, complexity, and diversity of their domains. The differences in the number of homologs in the genomes of Moraxellacea family (owing to a dissimilar rate of gene duplication and probable gene loss), make this conclusion even more difficult. However, lateral gene transfer (LGT) would be a proper interpretation for our observations. LGT is an important evolutionary mechanism common in prokaryotes (Andersson et al., 2003). LGT affords the potential for adaptation to new environments and sometimes promotes the virulence of pathogenic bacteria (Stubenrauch et al., 2017). This genetic phenomenon was proposed by the phylogenetic analysis of TAAs in *Burkholderia cepacia* complex (Fialho and Mil-Homens, 2011) and *Haemophilus parasuis* (Pina et al., 2009). The latter two reports have also highlighted the other mechanisms in TAAs evolution, including gene duplication and gene loss.

Aside from gene acquisition and gene loss, allelic diversity (as a common trait of TAAs) can be observed in the TAA encoding genes in several Acinetobacter species (Bentancor et al., 2012a). This allelic diversity results in the presence of different TAAs of various length and

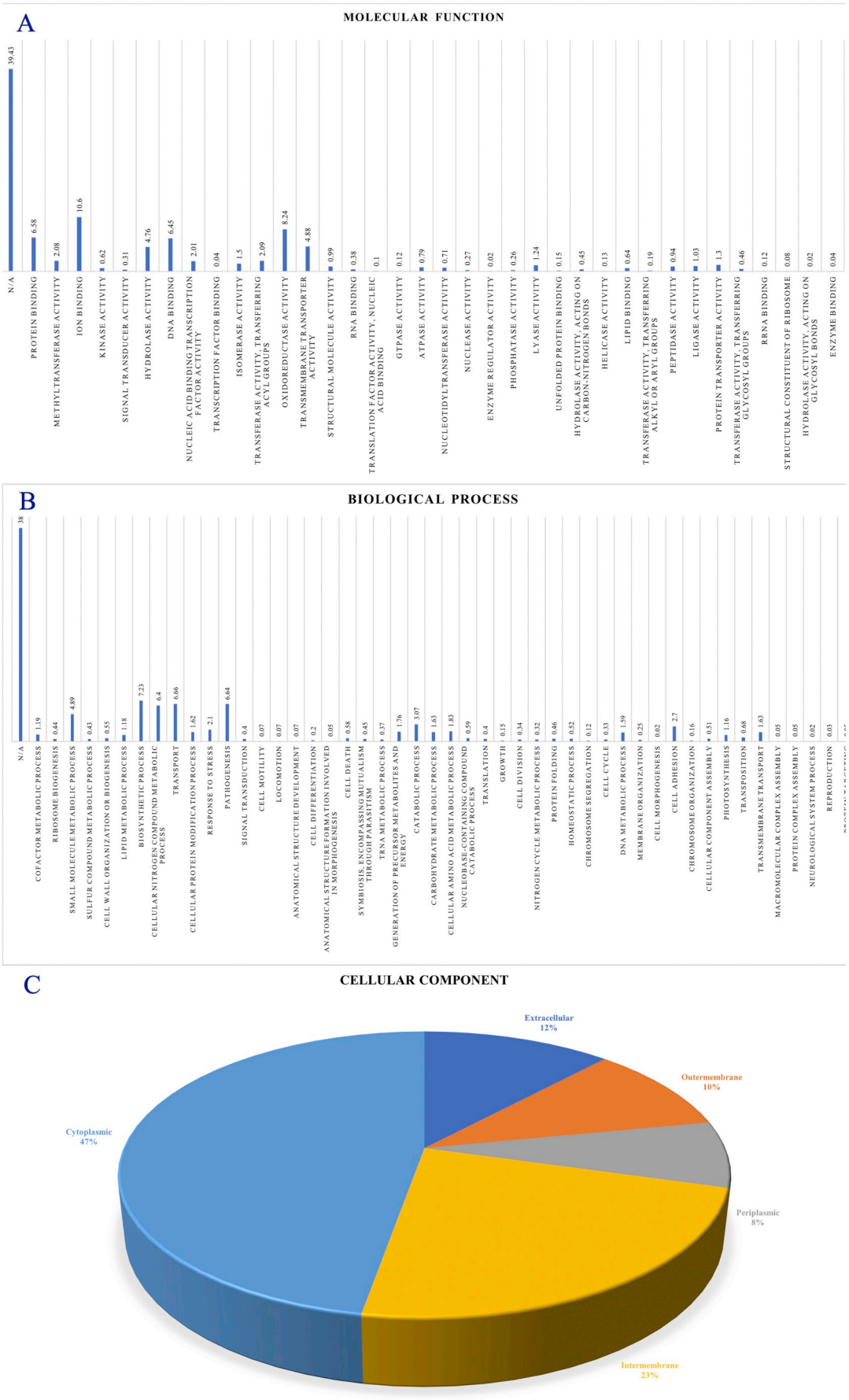

Fig. 5. GO terms analysis of the genes on the location of Ata. Bar plots show the gene ontology components of syntenic genes including molecular function (A) and biological process (B), C. Circular plot shows the subcellular localization of homologous gene products extracted from synteny genome map.

domain architectures and even differences in the sequence of repeated modules in a single protein. We found recombination occasions in a subset of TAA sequences, which would be conceived for this diversity. The repetitive nature of TAAs facilitates the recombination procedures (Persi et al., 2016). It has also been shown that such genes with evidence of recombination, on average, represent considerably higher GC-content than other genes (Lassalle et al., 2015).

The variation observed in the GC content of TAA encoding genes potentially suggests that TAAs are alien genetic materials. Our observations are in agreement with Eijkelkamp et al. (2013) findings. Viewing the GC content of $ata_{A.\ baumannii}$ (strain ATCC 17978, locus tag: A1S_1032), Eijkelkamp et al. (2013) proposed that $ata_{A.\ baumannii}$ is horizontally acquired; they also found that $ata_{A.\ baumannii}$ is under tight regulatory control of histone-like nucleoid structuring protein (locus tag: A1S_0268) (Eijkelkamp et al., 2013). Histone-like nucleoid structuring proteins act as a regulator for the horizontally acquired genetic materials (Lang et al., 2007).

Heterogeneity in the GC content of TAAs in Acinetobacters could be explained by GC- biased gene conversion. Given the fact that the acquired genes tend to be AT-rich (Daubin et al., 2003), the GC- biased gene conversion could be responsible for their GC-reach content. It has a major impact on the evolution of base composition in bacteria. The phenomenon favors G/C nucleotides at polymorphic sites in the conversion of intermediates of recombination (Lassalle et al., 2015). On the other hand, the accumulation of non-synonymous substitutions might be the indicator of positive selection. Herein, we assume that since many elements other than TAAs are involved in the attachment of Acinetobacters to different surfaces (such as OmpA (Gaddy et al., 2009), biofilm-associated protein (Rahbar et al., 2010), and many other factors (Longo et al., 2014)), TAAs do not represent a unique selective advantage for bacterial cells. Therefore, positive selection may not be considerably associated with the evolvement of TAAs in Acinetobacters. By contrast, GC- biased gene conversion, which can imitate some aspects of positive selection (Ratnakumar et al., 2010), may affect the probability of fixation of these presumably laterally acquired alleles.

It is well established that gene transfer between subclasses of the Proteobacteria is a common phenomenon (Coombs and Barkay, 2004), especially those that populated the same habitats (Kloesges et al., 2010). Burkholderia and Acinetobacter share very similar characteristics regarding the pattern of TAAs dissemination and density of TAA encoding genes (Fialho and Mil-Homens, 2011). *A. baumannii* and Burkholderial species are vastly distributed in the environment (Eberl and Vandamme, 2016; Stoyanova et al., 2007) and known to cause nosocomial infections (Gade et al., 2016). Although rare, the coinfection of *B. cepacia* and *A. baumannii*, has been also reported (Gade et al., 2016). Our CLANS clustering analysis showed that the Acinetobacter TAAs, despite variations in length and domain complexity, were completely related as the sequences were collected in a single focused clade. This clade was also enriched by the sequences from the Beta subclass of Proteobacteria. A dominant family in the clade was Burkholderiaceae. These pieces of evidence led us to the conclusion that TAA transfer mainly has occurred between Beta- and Gamma- subclasses of Proteobacteria through LGT. The most likely participant for this gene transfer could be the Burkholderiacea family members. The existence of very similar TAA architectures in the two genomes further strengthens the hypothesis of LGT between the two aforesaid genera.

The exact period of gene transfer through evolutionary time is debatable. The same gene arrangement in the vicinity of TAA encoding genes in the members of Moraxellaceae family suggested a deep common ancestor for these genes. The deep ancestry for TAA is further supported by the distribution of proteins with similar architecture in more distant organisms and co-occurrence of the same genes in other prokaryotic chromosomes. Indeed, it might be hypothesized that LGT has occurred far in the past. Since the intra-species transfer of the chromosomal genes through LGT was reported for *A. baumannii* (Krahn et al., 2016), it could be deduced that after the primary gene acquisition, further expansion of TAAs among Acinetobacters have occurred by gene transfer among family members. Our tBLASTn searches showed that transduction would be ruled out due to the absence of any trace for TAA in bacteriophage genomes. However, finding a plasmid containing TAA gene have revealed the potential for plasmid-based gene transformation.

TAA is absent in many clinical isolates of *A. baumannii*. The absence of TAA encoding genes in some genomes could be rationalized by gene loss events or lack of any TAA genes acquisition by these species. Thus, direct attribution of infection to the existence of TAA in the genome of the pathogens is a challenging issue. For instance, no TAA-like protein exists in hyper-virulent *A. baumannii* LAC-4 (Harris et al., 2013), which is responsible for bloodstream infections and shows a high level of serum resistance. It seems that TAA is not the most important single factor contributing to the virulence, unlike *Y. enterocolitica*, which YadA knock-out strain is not virulent in a mouse infection model (Pepe et al., 1995). Contrary to *Y. enterocolitica,* TAA encoding gene is present in the genome of *Y. pestis*, but is not expressed due to a frameshift deletion; interestingly, introducing this gene to the *Y. pestis* significantly abolished the virulence of the pathogen due to the inhibition of pathogen spreading after fleabite (Mühlenkamp et al., 2015). The *A. baumannii* harboring Ata may presumably be trapped in the infection location, while TAA negative species would be spread via body fluids more efficiently. This may clarify the paucity of TAA positive strains in the body fluid samples.

Some shreds of evidence showed that virulence may evolve for optimal transmission (Alizon et al., 2009). However, this theory did not account for all pathogens, especially the opportunistic ones (Brown et al., 2012). It seems that although TAAs are known as virulence factors, they are not critical for transmission and within-host growth. A comprehensive and simple definition of opportunistic pathogens has been proposed by Brown et al. (2012) as “non-obligate and/or non-specialist parasites of a focal host” (Brown et al., 2012). This demarcation explains that opportunistic pathogens can differentiate two sections: the location where they cause disease and anywhere else that they do not. In the case of *A. baumannii* and TAAs, coincidental evolution may be better fitted as an evolutionary theory for explaining the evolvement of these virulence factors. The direct evolution states that evolutionary selection should favor clones acquiring a new virulence factor, because it may promote the optimization of host exploitation in favor of maximizing their reproductive success. On the other hand, the canonical focus of coincidental evolution model is on the virulence factors that have been evolved in an environmental context different from where they promote infection (Levin and Eden, 1990). The coincidental evolution can be perceived when pathogens can occur in two different niches (e.g., soil and host). Then, the virulence factor that has been selected in a special ecological niche may exert a phenotype of interest in the other sites.

The main advantage of harboring TAA gene might be the ability to adapt to the new environments through developing biofilm communities. The role of TAAs in establishing a biofilm community has been previously assessed in several species (Adler et al., 2013; Łyskowski et al., 2011; Raghunathan et al., 2011; Valle et al., 2008). The same function has also been ascribed for TAAs of Acinetobacters (Bentancor et al., 2012a; Ishikawa et al., 2012).

Biofilms are communities of aggregated cells (Flemming et al., 2016); these dynamic systems are the most abundant and successful form of bacterial life. In fact, planktonic form in bacterial lifestyle is infrequent (Hall-Stoodley and Stoodley, 2005). Biofilm formation is a key element during the lifestyle of Acinetobacters both in natural environment (Espinal et al., 2012) and health care settings (Lindsay and Von Holy, 2006), the sites that they do not cause infection. In addition, it was confirmed that biofilm formation is an important factor in the infection onset of *A. baumannii* (McConnell et al., 2013) within the host, the place that the opportunistic pathogen instigates the infection. This virulence factor might have assimilated in an ecological niche separate

from the infection site, but incidentally provide some benefit for growth in the infection place (i.e., within the host).

Whole transcriptome analysis of *A. baumannii* has revealed a significant (p-value < .001) increase in the expression pattern of TAA in biofilm-associated cells versus both exponentially growing (4.41 folds) and stationary-phase cells (2.63 folds) (Rumbo-Feal et al., 2013). In contrast, the TAA encoding gene showed an unchanged expression pattern after the infection (in serum versus in vitro) (Murray et al., 2017). It can be concluded that biofilm may be considered as a prestage to prepare the pathogen for effective initiation of the infection. This assumption might be tied to the fact that a large number of pathogens would disperse from a biofilm (Purevdorj-Gage and Stoodley, 2004), in the present case, the pathogens that have undergone a phenotypic shift.

An OmpA-like domain containing protein was observed in several genomes in the vicinity of TAA genes. In addition, co-expression of these two proteins (bit score: 0.467 at String database) is corroborated to the existence of coherence between TAA and this protein. Ishikawa (2016) suggested the TpgA name (TAA- and peptidoglycan-associated protein A) for this neighbor protein (Ishikawa et al., 2016). They showed that TpgA and AtaA form a stable complex through which a single molecule of TpgA interacts with each subunit of the trimeric AtaA; therefore, they suggested a polycistronic pattern for the expression of AtaA.

The aforementioned features highlighted a constraint on the expression of TAAs and TpgA (as an accessory protein) in Acinetobacters to ultimately develop a new phenotype. Since TAAs and TpgA in Acinetobacters share similar evolutionary rate and co-expression pattern, they may exhibit evolutionary rate covariation (Clark et al., 2012). Although lateral gene transfer of TpgA along with TAA could be an appealing hypothesis, the observed dispersion for each of these genes highlights the possibility of genetic events such as gene duplication (we found at least 29 sign of gene duplication events in our dataset), which make them apart in some genomes. Indeed, such factor that required complex machinery to result in a final phenotype is necessary to be followed by acquiring all accessories (Stubenrauch et al., 2017). The two-component histidine kinase system (West and Stock, 2001) was also observed upstream of TAA encoding gene. These genes and TAA encoding genes are not in the same direction. The system is neither under regulatory control of H-NS (Eijkelkamp et al., 2013), nor have increased expression pattern in biofilm associated cells (Rumbo-Feal et al., 2013). It is appeared that they may not involve in the same operon. However, sharing similar response to stressful conditions is not completely ruled out.

TAAs are an exquisite example of beauty in the natural adaptation of bacteria to their environment and promoting different lifestyles. Acinetobacters are ancient bacteria (apparently as ancient as Enterobacteriaceae (Touchon et al., 2014)), healthcare environments are the new habitat of Acinetobacters; living well and vigorously in such a harsh new environments demands novel phenotypes to be developed in response to such stressful conditions. The plastic genome of Acinetobacters (Harding et al., 2018; Imperi et al., 2011) warranties the persistence of bacterium by acquiring new traits through various mechanisms. In the case of *A. baumannii,* the outcome is completely against human health. In this respect, acquiring antibiotic resistance genes is not the exclusive concern; many virulence factors could be obtained by the pathogen, emphasizing an emergent requisite for developing new strategies to face this pathogen.

In conclusion, it should be noted that the scattering pattern of distribution of trimeric autotransporters across Acinetobacter genus, heterogeneity of nucleotide substitutions, variation in codon usage and GC contents, allelic diversity, and existence of TAA containing plasmids suggest multiple events of LGT from the initial history of Acinetobacter and occurrence of gene duplication, gene loss, and recombination after acquiring alien genes owing to the plasticity of Acinetobacter genome. The coincidental evolution theory may explain some aspects of the evolution of trimeric autotransporters in Acinetobacters. Thus, for any evolutionary analysis of TAA, at least in Acinetobacter species, the coincidental evolution theory should be taken into account.

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