Pan-resistome insights into the multidrug resistance of *Acinetobacter baumannii*

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Abstract

BACKGROUND

Acinetobacter baumannii is an important Gram-negative opportunistic pathogen, which is responsible for many nosocomial infections. This etiologic agent has acquired, over the years, multiple mechanisms of resistance to a wide range of antimicrobials and the ability to survive in different environments. In this context, our study aims to elucidate the resistome from the *A. baumannii* strains based on phylogenetic, phylogenomic, and comparative genomics analyses. *In silico* analysis of the complete genomes of *A. baumannii* strains was carried out to identify genes involved in resistance mechanisms and the phylogenetic relationships and grouping of the strains based on the sequence type.

RESULTS

The strains belonging to the same sequence type (ST) were phylogenomically closer than from distinct STs, and the most prevalent ST was ST 2. The presence of genomic islands containing most of the resistance gene repertoire indicated high genomic plasticity, which probably enabled the acquisition of resistance genes and the formation of a robust resistome. *A. baumannii* displayed an open pan-genome and revealed a still constant genetic permutation among their strains, similarly to the genera *Escherichia*, *Klebsiella*, and *Pseudomonas*.

CONCLUSIONS

Our study is the first one to evaluate the resistome of the species *A. baumannii* using comparative multi-omics methods. Furthermore, the resistance genes suggest a specific profile within the species throughout its evolutionary history. Moreover, the current study performed the screening and characterization of the main genes present in the resistome, which can be used in applied research for the development of new therapeutic methods to control this important bacterial pathogen.

1 Background

Acinetobacter baumannii is a Gram-negative bacteria, which is an aerobic, non-fermenting, catalase-positive coccobacillus with cosmopolitan distribution [1, 2]. Most of the clinical cases involving this bacterial species are related to one or more of the following pathological conditions: severe pneumonia, meningitis, bacteremia, and erysipelas [1, 3–5]. Although members of the genus *Acinetobacter* are ubiquitous, rarely isolated in the environment outside hospitals, even during outbreaks [6].

A. baumannii has several intrinsic resistance mechanisms, such as (I) the presence of β-lactamases responsible for the degradation of β-lactam drugs; (II) the presence of multiple drug efflux pumps that prevent the increase in the concentration of antimicrobials in the cytoplasm; (III) changes in the molecular

pattern of proteins associated with plasma membrane; (IV) ribosomal methylation that hinders the action of antimicrobials related to the regulation of protein translation processes, such as tigecyclines and quinolones, and; (V) the presence of enzymes capable of degrading multiple antimicrobials [7–9].

The recommended treatment usually prescribed for infections with *A. baumannii* is based on β-lactam antibiotics, such as cephalosporins and carbapenems [10]. This class of antibiotics interferes with peptidoglycan biosynthesis and avoids the formation of the cell wall [11, 12]. Nonetheless, over time, because of its high adaptation skills, strains capable of resisting the high concentrations of these antimicrobials have been detected [2, 8]. Such cases of resistance can be classified into three categories: (i) Extensively Drug Resistant (XDR) is when it is resistant to more than three classes of antimicrobials; (ii) Multidrug Resistant (MDR), when it is resistant to almost all the antimicrobials except for two antimicrobials; or (iii) Pandrug Resistant (PDR), when it is resistant to all known antimicrobials [9, 13, 14].

Some new treatments have exhibited promising results avoiding the various mechanisms of resistance of *A. baumannii*[15, 16]. Tigecycline, the only representative of the glycylcyclines class, is a drug that acts by inhibiting the protein translation process by binding the 30S ribosomal portion, and it may be used in combination with other medicinal products [10]. Nevertheless, there is still some concern about the use of tigecycline due to the ability of *A. baumannii* to develop resistance to certain antimicrobials by their prolonged exposure to low concentrations of tigecycline [16]. Furthermore, microorganisms can acquire resistance mechanisms by exchanging genetic material with other bacteria in the environment [17].

Because of all these factors associated with the ability of *A. baumannii* survival to adverse conditions (grow under a wide thermal range and the environment with low concentrations of nutrients) and, the resistance exhibited by *A.baumannii* generates numerous obstacles for the hospital treatment team, making it difficult to treat patients [1, 4, 6, 9, 18]. Some resistance mechanisms of protein origin have been previously evidenced, such as changes in the DNA-gyrase complex and an increase in the expression of the *ampC* that confers carbapenem resistance to *A. baumannii*[8]. This study explores the genes occurring on the resistome of 206 complete genomes of strains of *A. baumannii* that are related to the resistance of this species, by using multi-omic methodologies for the comparative genomics, phylogenomics, and pan-resistome of this species.

2 Results

2.1 Genomic analysis and geographic distribution

Acinetobacter baumannii is a genetically diverse bacterial species, and there is a variety of typing methods to identify genetic differences among the strains that could be associated with pathogenicity, epidemiological origin, dissemination, and evolutionary patterns [19]. Sequence type and phylogenetic analysis allow to identify genotype groups with a phylogenetic relationship and explore the diversity among the strains [20]. Similarity nucleotides and MLST analysis with geographical data can reveal a better knowledge of the epidemiological context and population structure among the strains around the

world [21, 22]. The analysis of genomic similarity based on sequence alignment and geographic distribution, it is possible to infer bacterial clonality, considering that strains of bacterial species isolated from the same region tend to have the same genic repertoire. Even though events of gene drift and vertical gene transfer cannot be ruled out, genetic characteristics are generally conserved when dealing with isolated bacteria in the same site or nearby sites.

Numerous epidemiological studies of *A. baumannii* associate with the presence of ST by local origin, as seen in the occurrence of ST 848 (CC 208) (Oxford scheme) carrying resistance gene to carbapenems in India [23], and likewise the frequent presence of ST15, ST25, ST79, and ST1 in South America [24, 25]. A recent phylogeographical analysis of the Italian isolates belongs to an only clonal group ST78 (Pasteur scheme) [19].

The 206 *A. baumannii* complete NCBI genomes sequences were analyzed (see Additional file 1). The genomes have a size varying from 3.48 Mb - 4.43 Mb with a genomic GC content of 39.05%. Considering that nearby isolated bacterial genomes tend to maintain the same genetic characteristics, the study of the geographical distribution of *A. baumannii* is an essential method for evaluating the conservation of the species in the global context.

A total of five relevant clusters with high similarity ($\gtrsim$ 98.5%) belonging mainly to specific STs (1, 2, 10, 79 and 437) were retrieved (see Additional file 2). This finding corroborates the conservation of genomes belonging to the same ST. Consequently, strains related to the same ST were expected to be isolated at locations to justify the high genomic similarity. Nevertheless, the geographic distribution of the strains according to the ST proved to be misplaced. Considering that different STs were isolated on distinct continents, possible factors that could justify this misplacing are microbial ubiquity and globalization (Figure 1). There is a higher number of deposited genomes belonging to ST 2 (50% of the used dataset), as well as a more significant number of strains isolated from the Asian continent (51.2% of the used dataset). These data do not corroborate the epidemiological information on the distribution of outbreaks caused by the bacterium *A. baumannii*[9, 20, 23]. Thus, this leads to the conclusion that there is a more significant number of sequencing performed on the Asian and North American continents since epidemiological outbreaks have been reported in several developing countries over time (Argentina, Brazil, and South Africa). Furthermore, outbreaks of infections by this pathogen have also been reported in the European continent; however, the number of isolates from that continent is still much lower.

2.2 Phylogeny and phylogenomics

Phylogenetically, all the *A. baumannii* strains were grouped in the same clade within the *Acinetobacter* genus, confirming the monophyly of this species (Figure 2). This result was expected because not only is observed in different microbial species but also is consistent with reports from the literature on phylogenetic analysis, indicating that the use of housekeeping genes to infer evolutionary history is a good qualifier of phylogenetic distance and epidemiology [26].

This result also points out that the *A. baumannii* strains represented in blue are highly conserved within the species (Figure 2). Moreover, this result revealed that strains of other species (represented mainly by the colors red, green, and yellow) did not group with *A. baumannii* strains, because they have different metabolic and phenotypic characteristics. A relevant fact is the distant phylogenetic relationship of *A. radioresistens* strains (represented in pink), which formed a basal monophyletic clade; therefore, it is a species of interest for comparative analysis.

Three strains (FDAARGOS_494, FDAARGOS_493, and FDAARGOS_560), previously identified as *Acinetobacter sp.*, were grouped together and inside the *A baumannii* clade, strongly suggesting that they are, in fact, of this same species. This taxonomic re-classification has already occurred in other cases of bacterial species [27–29]. More phylogenomic studies, including tetranucleotide analyses, Average Nucleotide Identity (ANI), and the presence and absence of species-specific genes evaluation, are needed to confirm this hypothesis and assure taxonomic reclassification based on genomic data and theoretical background [27, 30].

The *A. baumannii* strains were grouped according to their respective STs in the phylogenomic tree, using the core genome sequence (Figure 3). Nonetheless, in the phylogenomics analyses, the strains ST 2 (represented in green) formed paraphyletic clades, and, thus, these strains cannot be considered in the same group. The strains represented in gray do not have a defined ST, but they all grouped in the same clade, indicating the high similarity among them (see Additional file 2).

2.3 Genomic plasticity

During the analysis of genomic plasticity, a large gap in the *A. baumannii* strains can be observed when visually compared. Even strains belonging to the same ST are not identical, although they are genomic and phylogenomic closer and share the same clade. This result suggests that the strains of this species are not very clonal and tend to have a high rate of gene permutation since there are many gaps between genomes (Figure 4).

Comparative genomic analyses of the 206 *A. baumannii* genomes, using de strain AYE as a reference, showed the presence of fourteen genomic islands (Figure 4). Among these 14 genomic islands, four were Pathogenicity islands, two were Metabolic islands, one was a Symbiotic island, and seven were Resistance islands. Furthermore, one full-sized resistance island (RI7 or AbaR1) was identified within the AYE strain. This genomic region has a length of 96878 nucleotides and contains the highest amount of resistance genes found in this species. There are 25 resistance genes within this island divided into efflux pumps and proteins with enzymatic activity.

The islands RI2 (80220 bp) and RI7 (96878 bp) are conserved within the species, which are more present within strains belonging to ST 1. Outside of this cluster, however, both islands were not completely found. A similar result is observed in smaller islands, such as RI1 (20317 bp), RI3 (6077 bp), RI4 (12534 bp), RI5 (14763 bp) and RI6 (10374 bp), indicating that they are unstable regions within the genome.

There is a great number of genomic islands for the species *A. baumannii*, which reveals its high genomic plasticity. Although we identified a reduced number of type sequences and phylogenetically close strains, analyzing the complete genomes, one can see how all the strains are different in their gene content. This could be due to the horizontal acquisition of mobile genetic elements or gene duplication events.

2.4 Analysis of the pan-genome for understanding this species

There is an intensive effort to know the total repertoire of the species *A. baumannii*. As a result, according to the power-law regression model, the pan-genome of *A. baumannii* remains open (γ = 0.46), which by each newly added genome, the number of new genes will increase the genetic repertoire of the species. This result was obtained using the formula $n = a.x^{\gamma}$, where: *n* is the estimated size of the pan-genome for a given number of genomes; *x* is the number of genomes used; and γ are fitting parameters [31]. As a rule, when $0<\gamma<1$, the pan-genome is considered open. Figure 5 shows the development of the pan-genome. The fact that it was not possible to reach the plateau of the total number of genes concerning the total of genomes corroborates the assumption that the pan-genome of the species remains open. This fact also corroborates the high genomic plasticity already reported for this species, especially considering that this bacterium has an exceptional ability to obtain new gene content through transposable elements [19, 32].

The pan-genome analysis revealed a total of 27682 genes, which 1373 genes are shared for all strains (complete genome sequences of *A. baumannii*), and 10683 were strain-specific genes. The accessory genome, except for single genes, is made up of 15626 genes. For these results, the > 95% threshold was considered for the prediction of orthologous genes.

The presence and absence of genes associated with the phylogenomic analysis of the strains under study, the diversity of presence patterns regarding different strains, and the accessory genome can be visualized in Figure 5. Therefore, each gene presence pattern accompanies the genomic nearness of the core genome. The distribution of accessory genomes and unique genes show high variability and the clustering of strains with shared gene content. Genomic proximity pattern is also observed in genomic similarity analysis and is connected to the grouping of strains by sequence type (Additional file 2 and Figure 3). Thus, strains belonging to the same ST tend to maintain similar profiles of the accessory genome distribution.

The different patterns of the presence of genes of the SDF strain can be observed in a detailed analysis. This strain is already known to be susceptible to antimicrobials and is the only representative of the sequence type 17. Its pattern of accessory genes differs from all the others and has about 803 unique genes, which contrasts with the pattern of the super-resistant AYE strain, which contains about 62 unique genes. This fact, combined with the distant phylogenomic position of the strain, shows how different the susceptible strain is from the others.

A more accurate analysis of the total pan-genome indicates the number of genes related to specific bacterial metabolic pathways. Such analysis is based on the KEGG database. It demonstrates a high number of core genes related to metabolic pathways intrinsic to microbial existence, such as energy metabolism (8.68 %) and molecular translation (5.87 %) (Figure 7). The accessory genes are related to amino acid metabolism (14.78 %), carbohydrate metabolism (15.55 %), and xenobiotics biodegradation and metabolism (5.93 %). Most of the genes related to drug resistance are part of the accessory genome (2.45 %) when compared to their percentage represented in the core-genome (1.76 %). Similarly, genes related to infectious diseases are represented in the core genome (0.80 %), accessory genome (2.07 %) and strain-specific genes (1.44 %).

As for genes related to adaptation to the environment, there is a very low gene repertoire associated with this process in the general pan-genome, with less than 0.5 % of the total repertoire linked to such a pathway in any subdivision of the pangenome.

2.5 Pan-resistome characterization of *Acinetobacter baumannii*

Considering a similarity criterion greater than 70 % and an E-value < $5e^{-6}$, all the studied strains present a pan-resistome of 171 genes, and within that, a core resistome constituted by five genes is shown in Table 1 [11].

In these analyses, the strains that presented *ade*-type bombs were expected to have the complete gene repertoire to be functional. Nevertheless, this pattern was observed exclusively for the *ade*IJK efflux pump, as all the genomes presented the genes *adeI*, *adeJ*, and *adeK*. The same pattern, however, was not observed for the other genes of the same family (Figure 8 and Additional file 3). Similarly, to the genes capable of constituting the *ade*FGH pump, the presence only for the *ade*F and *ade*G genes were detected in all the strains. The gene *ade*H (outer membrane factor protein in the *ade*FGH multidrug efflux complex) was not found in three strains (XDR-BJ83, ORAB01 and DS002), which, in theory, makes the activity of the pump unfeasible. Our study also identified an interesting protein present in all strains: *ampC* enzyme. This is responsible for generating resistance to beta-lactams, more specifically to cephalosporin, and is thought to cause hydrolysis of the drug [33, 34].

Analyzing the accessory portion of the resistome, an interesting distribution profile of specific genes was retrieved. The OXA-66 gene, responsible for coding variant 99 of beta-lactamase with action against penam and cephalosporin, for example, is present in 78 strains, which is equivalent to approximately 48% of the dataset. Among these, 93 belong to the ST 2. This fact makes this gene almost exclusive to strains belonging to ST 2. Regarding the other ST, only six strains have the OXA-66 gene, and they do not belong to ST 2, which are: BAL062 - ST unknown; SAA14 - ST 187; XH857 - ST 215; XH906 - ST 922; 7847 - ST unknown; TP1 - ST 570.

A similar pattern was observed with the ADC-76 gene, responsible for encoding a beta-lactamase that causes cephalosporin inactivation and was present in strains belonging exclusively to ST 23, 10, 85, 464, 575 and 639. The same is true for the OXA-68 gene, identified only in strains belonging to STs 23 and 10, but not present in all the strains. The same for the OXA-180 gene detected only in strains of STs 267. The gene responsible for encoding OXA-69 is almost exclusive to strains belonging to ST 1, 20, 81 and 195.

Other different patterns of gene distribution can be seen in Additional file 3. Nonetheless, there is no significant pattern of visible distribution related to the geographic location of the isolates, except in some cases. The OXA-67 gene is exclusive to isolates (strains EC and EH) from the Czech Republic while ADC-81 and OXA-92 genes are entire to the A388 strain.

As the distribution related to the number of antibiotics is linked to each subpartition of the pan-resistome, the antibiotic with the highest amount of resistance mechanisms linked to it is cephalosporin with about 103 resistance proteins within the formed pan-resistome (Figure 9). In contrast, antimicrobials (sulfonamide, sulfone, cephamycin and pleuromutilin) have low amounts of resistance mechanisms related to the predicted resistome of *A. baumannii*.

In accordance with the distribution of the types of resistance mechanisms found, 131 cause the enzymatic inactivation of the antibiotic (Figure 10). This total is equivalent to 76.6% of the predicted pan-resistome. Also, almost all the core resistome-related proteins are efflux pumps (8 proteins).

The genomics islands of resistance identified some genes, such as *adeS*, *adeR*, *adeA*, *adeB* and *adeC* (within resistance island 2). Moreover, on resistance island 7 (or AbaR1 island), the following antimicrobial resistance-related genes and products were detected: *sul1*, *qacH*, AAC(3)-Ia, APH(3')-Ia, *catI*, tet(A), dfrA10, ANT(3")-IIa, OXA-10, cmlA5, arr-2, ANT(2")-Ia VEB-1, AAC(6')-Ian, tet(G), *floR*, dfrA1, APH(6)-Id, APH(3")-Ib.

3 Discussion

3.1 Similarity analysis, geographic distribution, and phylogenomic reconstruction

Recently, a more significant number of sequences of the *A. baumannii* genome provided resources for studying genomic epidemiology. Out of the 206 genomes analyzed, we identified uniquely 47 STs, of which the ST 1, 2, 18, and 79 were distributed in significant prevalence in North America, Europe, and the East Asia continent, but also a diverse set STs were indistinctly spread across the globe. The presence of few STs could be because most of the genome sequencing projects come from a single outbreak or several strains representing the same geographic location, and, in some cases, a unique ST was reported by geographic information.

According to Jeannot et al. (2014), there is a higher prevalence of strains belonging to ST 2 across the globe, but mainly in the European continent. The previous work also pointed out the polyclonality of *A.*

baumannii strains within the French nation, considering the existence of different STs randomly isolated throughout the country [20].

Based in the analysis of sequence similarity by ST around the global distribution of *A. baumannii* strains, we reported several genogroups (STs) with genomic similarity less than 98 % identity, in the same geographic region (Figure 1 and Additional file 2), which is opposite to the initial hypothesis that isolates from the same region exhibit a high genomic similarity [26]. We also observed that, for strains with the same ST, the obtained result revealed a discrepancy (Figure 1 and Additional file 2). Strains from different ST in the same geographic region and the same STs isolated on different continents may maintain a high similarity (> 99 %). In contrast, diverse genomic strains, with less than 98% identity, are shared in nearby locations.

In previous studies, Kazmierczak and et al. (2016) reported a heterogeneous global distribution between strains of *Pseudomonas aeruginosa* and *Enterobacter* spp. based on genetic variants of lactamases. In our study, geographically close isolates may or may not have the same variant. Consequently, phylogenetic proximity is not mandatory for members of the same geographically close species [35].

The phylogenomic tree of 206 genomes displays concordance with the ST distribution, which is expected since both analyses depend on the vertical evolutionary relationship among strains. The tree phylogeny and distribution of ST; however, show a relationship with the geographical origin. This would indicate that the dissemination of *A. baumannii* does not present a population structure. Nevertheless, it is not definitive, because a higher and representative number of strains is required to evaluate population structure.

It is biologically interesting to understand the evolution and speciation of *A. baumannii* when compared to the other species of this genus. Using such analysis, it is possible to infer the origin of specific mechanisms expressed in this species, as well as to identify the closest and the most distant members within the genus, to standardize comparative analyses better. Phylogenetic analysis based on a few housekeeping genes does not represent the complete evolutionary history or the final diversity between strains or members of the same genus. Nonetheless, a study based on phylogenomics shows a refined ancestry and variety that could be caused by changes in the niche or geographic location of bacterial populations [36].

In comparison, a previous study pointed out that, for a limited number of genes, phylogenetic inference using concatenated genes is better at portraying genetic diversity and distance between different species than the use of consensus trees derived from individual genetic analyses [37]. Therefore, through the proposed method, one can evaluate a significant distancing of *A. baumannii* strains from the other species belonging to this genus, which indicates that the use of concatenated rpoB and 16S rRNA genes is an excellent option for inference of phylogenetic distance of strains belonging to specific genera. A previous study obtained a similar result when performing phylogenetic inference using the complete genomes of 136 strains of *Acinetobacter* within the genus [38].

3.2 Genomic plasticity in *Acinetobacter baumannii*

Previous work has reported genomic plasticity among persistent *A. baumannii* strains in Italy [19], Argentina [39], and Australia [40]. Historically, it is a species capable of receiving and donating genes to other microorganisms in the environment, a mechanism mediated by recombination events [38].

The analysis of *A. baumannii* genomes revealed the presence of 14 essential elements of resistance within genomic islands that are acquired through horizontal transfer of genomic recombination events [41]. The largest genomic island found (RI7) has a length of 96878 nucleotides and presents in its content a total of 25 resistance genes characterized by an identity of more significant than 70% against the database. This same island is partially shared by strains of different ST 1 and is more similar within strains belonging to ST2 than ST1. Previously, this island was described as AbaR1 resistance island, and considered to be one of the leading genomic elements responsible for the high resistance of the species members due to its size and quantity of elements. Currently, its mobile elements are known to originate from bacteria of the genera *Pseudomonas, Salmonella,* and *Escherichia*[1]. Based on several studies, more than ten islands of resistance have already been identified in genomes of *A. baumannii*[42]. In the study of the AYE strain, seven islands of resistance were detected.

Similar events of gene displacement and the presence of specific factors related to pathogenicity within genomic islands are also reported in other species with high intrinsic resistance to antimicrobials. The presence of mobile elements containing virulence or resistance factors allows a better adaptation and proliferation on *A. baumannii*. These are usually included in some phylogenetic groups, which have a greater global distribution [32]. Therefore, monophyletic clades have stability in gene content, which may explain its low clonal incidence compared to other clones, which are characterized by high genomic plasticity [19]. In the literature, such mechanisms are revealed of transfer and translocation in species such as *Pseudomonas aeruginosa* [43] and *Klebsiella pneumoniae*[44], microorganisms that, as *A. baumannii*, are also considered models for understanding resistome, virulence, and pathogenicity. This fact indicates that resistance islands are persistent on the distribution of nosocomial bacteria due to selective pressure, and they are spread and fixed in bacteria to generate adaptive fitness.

3.3 Functional characterization through pan-genome analysis

Currently, pan-genome analyses, which allow observing the total genetic repertoire of a species, are incredibly relevant for determining similarity, functional characterization, and analysis of exclusive characteristics of certain strains of a microbial species. Such a report aims to assess the number of genes shared by all representatives of a taxon, as well as the genes shared by more than one but not all strains belonging to the group, known as accessory genomes [45]. Presently, pan-genome analyses are relevant for determining genetic variability, similarity, essential genes, functional characterization, and prediction of exclusive genes by phenotypic groups to characterize species and strains. In addition to

being able also to view the discrepancies between genomes that are not perceived by conventional analyses [46]. Nowadays, there are reports of pan-genome analysis of several pathogens, such as *Streptococcus agalactiae*[47], *Legionella pneumophila*[48], *Corynebacterium pseudotuberculosis* [49], *Pasteurella multocida* [50], *Pseudomonas aeruginosa* [51] and *Treponema pallidum* [52].

In the pan-genome analysis of *A. baumannii,* a core genome containing 1373 genes was identified. Biologically, using the Kyoto Encyclopedia of Genes and Genomes database, the core genome contains all the essential genes for the survival of the bacteria in a favorable environment. Therefore, it includes pathways related to metabolism and cell division, genetic processes, and energy production [45, 52]. Among the genes related to the core-genome, only five are related to resistance, and these genes represent in the core resistome.

On the other hand, the accessory genome has genes related to microbial adaptation mechanisms, such as antimicrobial resistance factors, symbiosis, adaptation to the environment, and virulence, which may or may not be acquired via horizontal gene transfer [53, 54]. In our study, the accessory genome revealed a total of 26309 genes that may be related to adaptation to the host and are more represented in pathways of carbohydrate and amino acid metabolism, xenobiotic metabolism, and drug resistance. Considering the pathogenic cycle of the species, xenobiotic biosynthesis and degradation pathways are essential facilitators of bacterial adaptation, mostly when related to microbial antibiosis associated to the adaptation to the host [55, 56], which, in theory, provides a more prolonged microbial survival. The prevalence of carbohydrate, amino acid, and xenobiotic metabolism pathways comes in part from the pathogen's evolutionary history [57, 58].

3.4 Resistome of *Acinetobacter baumannii*

In the pan-resistome analysis, it was possible to ascertain the numerical presence of a variant of beta-lactamases such as the OXA genes. Previous analyses have already pointed out that this gene is widespread among the distinct geographic locations of *A. baumannii* strains, reporting that the coding gene for OXA-143 is exclusive for Brazilian strains [59]. Besides, the same study points out that the enzyme OXA-58 is very prevalent across the globe but has a higher incidence in strains from southeastern Europe [59]. Nevertheless, using the methodology employed, the OXA-143 gene was not detected, but a similar pattern was observed for the beta-lactamase SAT-1, which is exclusive to the Brazilian strain MRSN15313. As for the OXA-58 gene, its presence was inferred for isolated strains in Italy, India, Greece, Ghana, China, and two Mexican strains, indicating and corroborating its higher prevalence in the East.

As for the efflux pumps presented, one of the most important and studied is the *ade*ABC pump, which belongs to the RND family (resistance-nodulation-division) [9]. The same *ade*IJK pump family is adequately represented in the core resistome. Previous studies indicate that efflux pumps are excellent targets for drugs, considering that their inhibition greatly amplifies the action of antimicrobials that under normal conditions would be eliminated by the cell [7]. Recent studies report that inhibition of the *ade*B and *ade*J portions lead to a significant reduction in microbial resistance [60]. Both are present inside the

cell and anchor the pump to the membrane. As for proteins with enzymatic action, the one that stands out most is *ampC* beta-lactamase. It has been described with high prevalence in *A. baumannii*, which is considered one of the main responsible for the resistance to beta-lactams [33].

Interestingly, the preferred treatment for susceptible strains of *A. baumannii* is based on carbapenems [61]; however, evaluating the pan-resistome, many mechanisms of resistance to this class have been reported, which may suggest that its presence in the genome does not indicate expression, mainly when relating to the presence of resistance mechanisms in the genome of the SDF strain. In the case of resistant strains, tigecycline treatment has been used with varying success [10, 61].

4 Conclusion

There is a wide variety of genes in the total repertoire of the species studied. Unfortunately, there is no visible clustering for the host and geographic location; however, the grouping of the strains based on ST reveals a coherent pattern, corresponding to the core genome similarity. The repertoire of the resistome was characterized in terms of the presence and similarity of genes in the total pangenome. It demonstrated enormous plasticity when evaluating the distribution of factors throughout the groups and the analyzed phylogeny. The pan-resistome also pointed out the presence of the *ade*IJK efflux pump and ampC enzyme in all the strains of this species, as well as the heterogeneous distribution of resistance factors across the globe. Another interesting fact to be inferred is the higher amount of resistance factors to cephalosporins, aminoglycosides, and tetracycline in the studied genomes. Therefore, there is a contraindication to the use of these drugs in *A. baumannii.* These facts point mainly to the discrepancy of strains belonging to different ST within the species *A. baumannii* and its high capacity to remodel the gene repertoire to adapt to the environment or host, and, hence, can remain as an important pathogen for years. Therefore, the data collected are pertinent to evaluate better the high resistance of the species in a hospital environment and, consequently, can be used for a targeted prescription of antibiotics based on phenotyping related to a genetic presence profile. As a perspective, it is possible to use the data obtained in this work to carry out studies for new drug candidates based on the core genome and taking advantage of the assembled pan-resistome to anticipate possible escape mechanisms of *A. baumannii*.

5 Methods

5.1 Genomes database, annotation, and data retrieval

All the complete *A. baumannii* genomes and their plasmids were obtained through the National Center for Biotechnology Information (NCBI)/GenBank -RefSeq [62]. An *in house* Python3 script was developed for the extraction of chromosome sequences from strains with plasmid sequences, using as a criterion the extraction of the largest contig present in the fasta file. Both files, those containing only the chromosome and those containing chromosome and plasmid, were annotated using the same parameters in the PROKKA pipeline version 1.13.7 [63], with an additional setting: the prediction of RNAs is using RNAmmer software version 1.2 [64].

5.2 Multilocus sequence typing and phylogeny

The similarity analysis was performed using all the complete genomes as input to the software FastANI [65], using default parameters. The sequence type was predicted using the MLST 2.18.0 software, based on the PubMLST platform [66]. The scheme used to be *abaumannii_2* determined and made available by the Pasteur Institute, based on seven sequenced housekeeping alleles: *cpn60* (Chaperonin family protein), *fusA* (Elongation factor G), *gltA* (Citrate synthase), *pyrG* (CTP synthase), *recA* (Protein RecA), *rplB* (50S ribosomal protein L2), and *rpoB* (a beta subunit of RNA polymerase) [67].

A customized Python3 script was used for the extraction of nucleotide sequences of 16S rRNA and rpoB genes, ranked by BLAST similarity (> 98 %) against *A. baumannii* AYE reference sequences. Subsequently, the extracted sequences were concatenated into a single file. The phylogeny was performed using the maximum likelihood method using the *rpoB* and 16S rRNA sequences. The alignment was performed using MAFFT software version 7.31.0 with default parameters [68], and the phylogenetic tree was inferred with the MEGA7 software [69] using the maximum likelihood method with statistical support of 10,000 bootstrap iterations to amplify the reliability of the formed clades. The generated tree figure was optimized using FigTree 1.4.4 software [70].

5.3 Resistance genes profile

The Comprehensive Antibiotic Resistance Database (CARD) [11] was used for the comparison of the local alignments and the determination of the presence of genes related to microbial resistance. For this purpose, the predicted proteome product of the automatic annotation of *A. baumannii* (206 strains) was used.

A customized Python 3.6 script was used to automate BLAST alignments [71] of proteomes against the CARD database. Only the results whose identity and coverage was equal to or greater than 70% and the E-value below $5e^{-06}$, respectively, were used. It was also used to generate the binary matrix of presence and absence genes, considering the files of the previous mining of the multiple alignments [72]. The final result was to generate the clustermap.

5.4 Genomic islands analysis

Genomic Islands Prediction Software (GIPSy) [41] was used to perform the prediction of genomic islands. In this analysis, the AYE strain was selected for the reference genome due to both its history of resistance on the European continent and the high presence of resistance genes. As a subject, the genome of *A. baumannii* SDF was selected, which is a strain previously described as susceptible [1, 41, 73, 74]. Subsequently, the BLAST Ring Image Generator (BRIG) software was used to visualize the genomic islands present in the genomes [75].

5.5 Pan-genome and pan-resistome analyses

The significant pangenomics analyses were performed using the software Roary [76], Bacterial Pan-Genome Analysis Tool (BPGA) [31], and Parallel tools [77]. In BPGA, there is a functional analysis tool, based on similarity, with references in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [78] and Clusters of Orthologous Groups (COG) [79] databases. The determination of the pan-genome was carried out through the BPGA analysis, using the power-law regression model [31].

The extracted core genome, resulting from the previous analysis using Roary, was aligned with MAFFT [68] for subsequent phylogenomic inference using FastTree software with maximum likelihood methodology.

Abbreviations

NCBI
National Center for Biotechnology Information
ANI
Average Nucleotide Identity
RI
Resistance island
KEGG
Kyoto Encyclopedia of Genes and Genomes
BLAST
Basic Local Alignment Search Tool
MAFFT
Multiple Alignment using Fast Fourier Transform
MEGA
Molecular Evolutionary Genetics Analysis
CARD
Comprehensive Antibiotic Resistance Database
GIPSy
Genomic Islands Prediction Software
COG
Clusters of Orthologous Groups
BPGA
Bacterial Pan-Genome Analysis Tool

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is deposited in the NCBI database. The genome of the strains used in these analyses is available in Additional file 1.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

DLNR, FMR, RH, RGS, FA performed comparative genomics analyzes. All authors contributed to the writing of the manuscript. FMR, DCC, DB, PG, SCS, RR, AGN were involved in the data interpretation and critically revised the manuscript. FMR, VA and FA supervised and conducted this work.

All authors have read and approved the manuscript.

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Tables

Table 1. Description of the genes present in the core resistome of the studied strains containing mechanisms of action and antibiotics associated with these mechanisms [11].

Gene	Definition	Mechanism	Antibiotic
*ade*K	Outer membrane factor protein in the *ade*IJK multidrug efflux complex	Antibiotic efflux	Phenicol, rifamycin, penem, diaminopyrimidine, tetracycline, carbapenem, macrolide, lincosamide, floroquinolone, cephalosporin
*ade*J	An RND efflux protein that acts as the inner membrane transporter of the *ade*IJK efflux complex	Antibiotic efflux	Diaminopyrimidine, phenicol, tetracycline, rifamycin, carbapenem, penem, fluoroquinolone, macrolide, cephalosporin, lincosamide
*ade*I	The membrane fusion protein of the *ade*IJK multidrug efflux complex	Antibiotic efflux	Phenicol, rifamycin, penem, diaminopyrimidine, tetracycline, carbapenem, macrolide, lincosamide, floroquinolone, cephalosporin
*ade*F	The membrane fusion protein of the multidrug efflux complex *ade*FGH	Antibiotic efflux	Tetracycline, fluoroquinolone
*ade*G	The inner membrane transporter of the *ade*FGH multidrug efflux complex.	Antibiotic efflux	Tetracycline, fluoroquinolone
*ade*L	A regulator of *ade*FGH in *A. baumannii*. *ade*L mutations are associated with *ade*FGH overexpression and multidrug resistance.	Antibiotic efflux	Tetracycline, fluoroquinolone
ampC	AmpC type beta-lactamases are commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria.	Antibiotic inactivation	Cephalosporins
*ade*N	*ade*N is a repressor of *ade*IJK, a RND-type efflux pump in *A. baumannii*. Its inactivation increases expression of adeJ.	Antibiotic efflux	Carbapenem, diaminopyrimidine, rifamycin, penem, tetracycline antibiotic, phenicol, lincosamide, fluoroquinolone, cephalosporin, macrolide
*abe*M	*abe*M is a multidrug efflux pump found in *A.* baumannii.	Antibiotic efflux	Acridine dye, fluoroquinolone antibiotic, triclosan

Figures

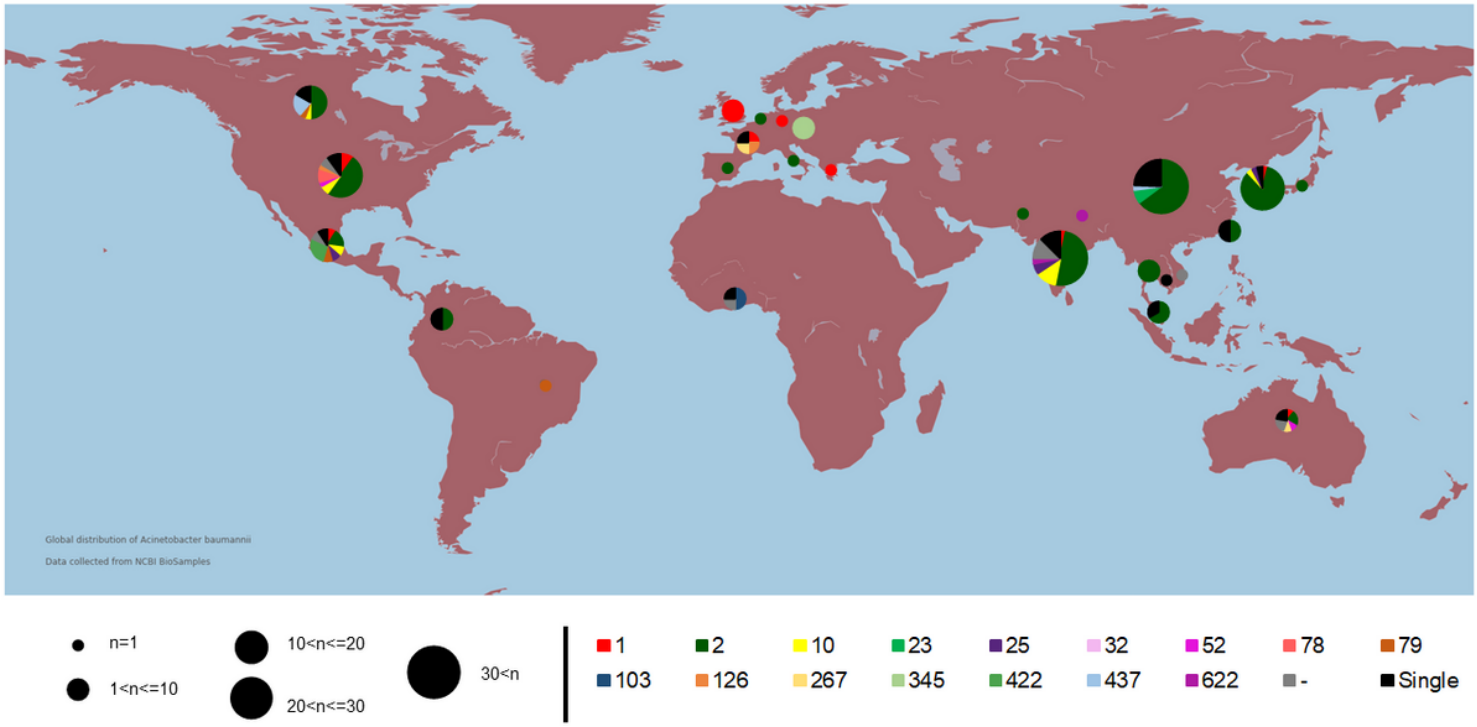

Figure 1

Graphical representation of the global distribution of isolation sites of different strains of Acinetobacter baumannii in a grouped way. The colors represent the sequence type of the strains in this study. The size of the circle indicates the amount of isolated strains. The figure was plotted in Python 3 using the Basemap library and manually edited based on BioSample data extracted directly from NCBI. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

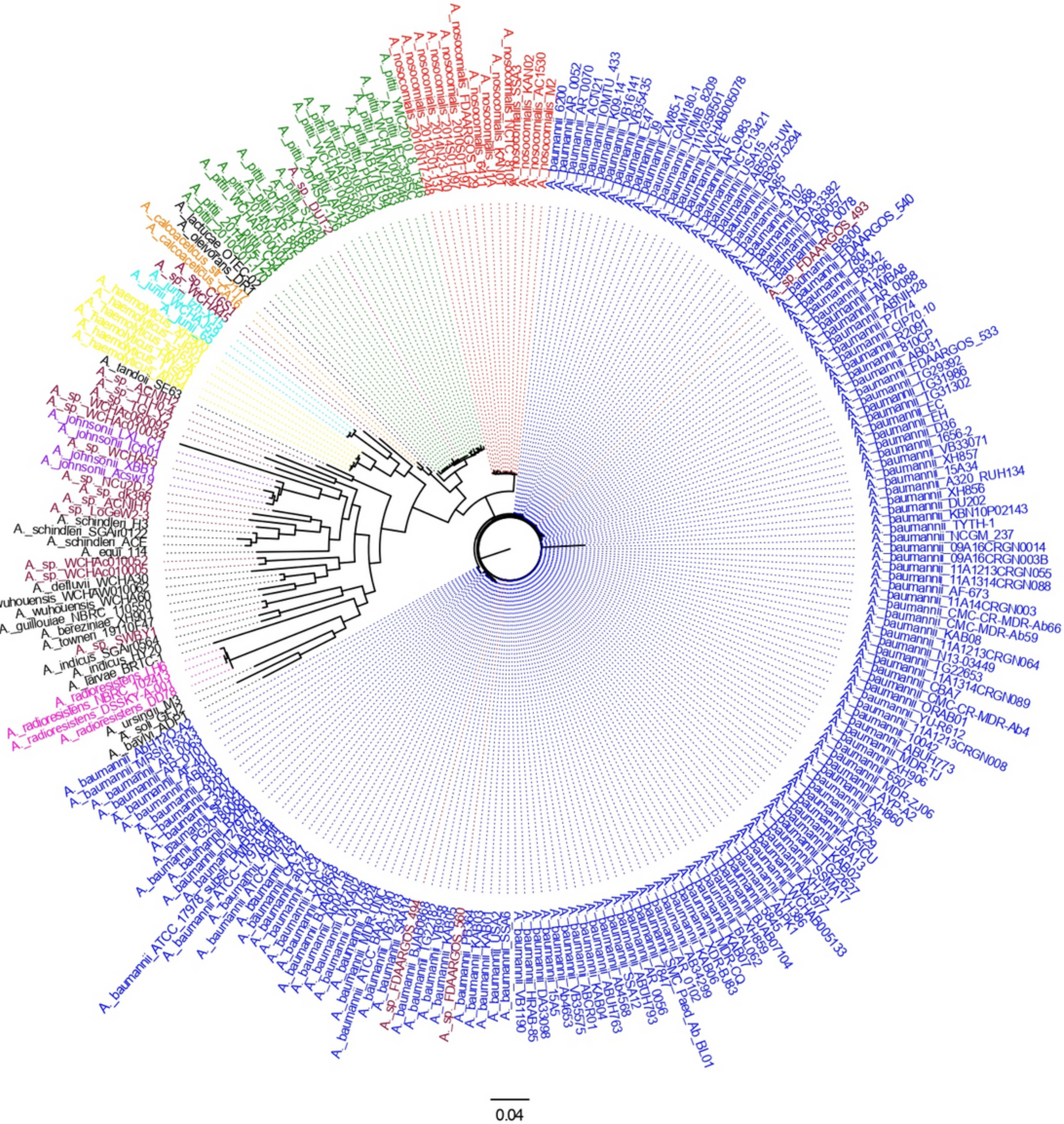

Figure 2

Phylogenetic tree based on the concatenated sequences of the 16S rRNA and rpoB genes representing the positioning of the Acinetobacter baumannii strains compared to the genus. Statistical support of 10,000 bootstraps was applied. The colors represent different species within the genus Acinetobacter, being: blue - A. baumannii; pink - A. radioresistens; green - A. pittii; purple - A. johnsonii; light blue - A. junii; yellow - A. haemolyticus; red - A. nosocomialis; dark red - A. sp; black - underrepresented species.

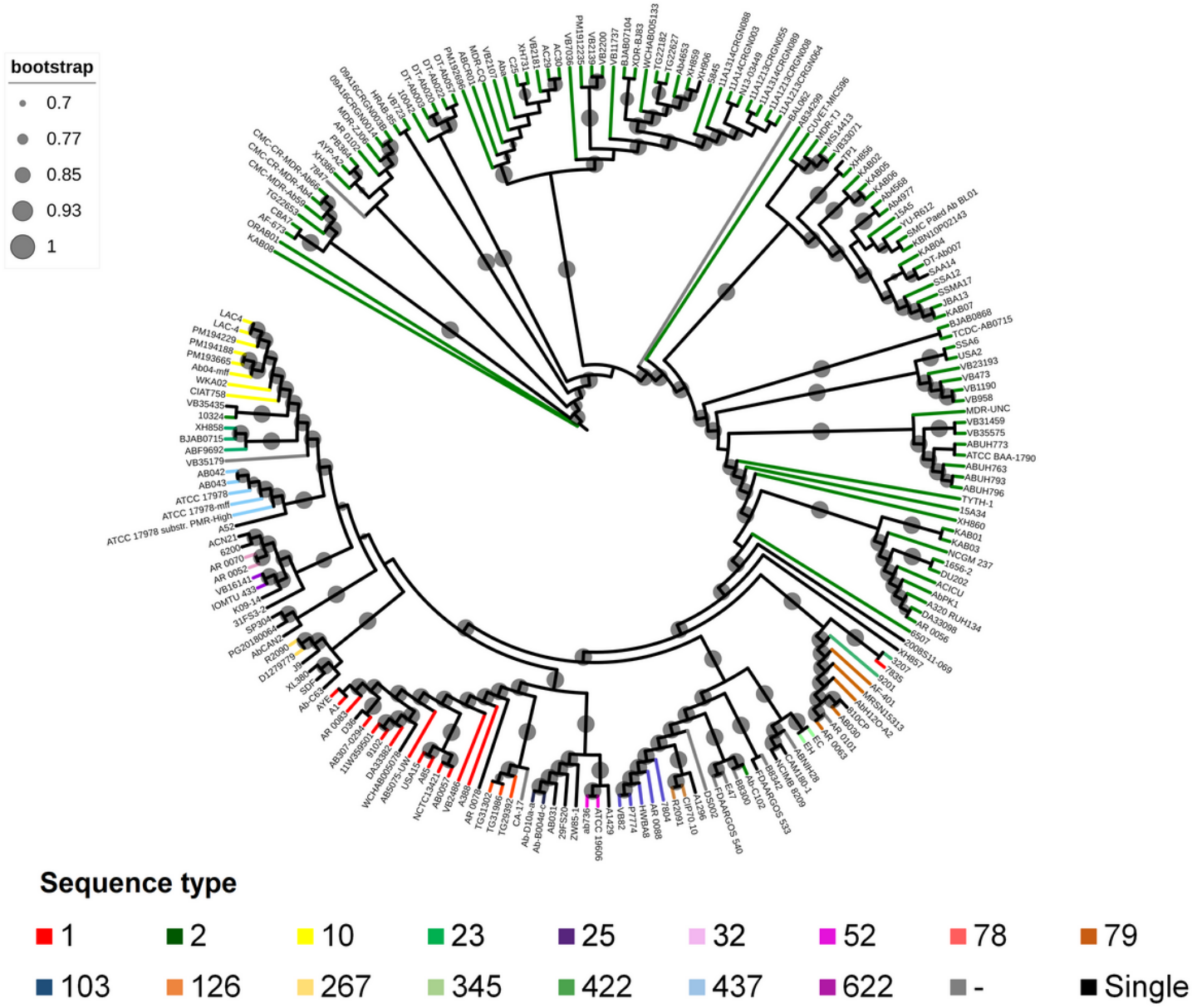

Figure 3

Phylogenomic tree based on the core-genome of 206 Acinetobacter baumannii strains. The colors represent the grouping by sequence type. The method used was maximum likelihood with statistical support of 1000 bootstraps with 1373 genes present in the core-genome.

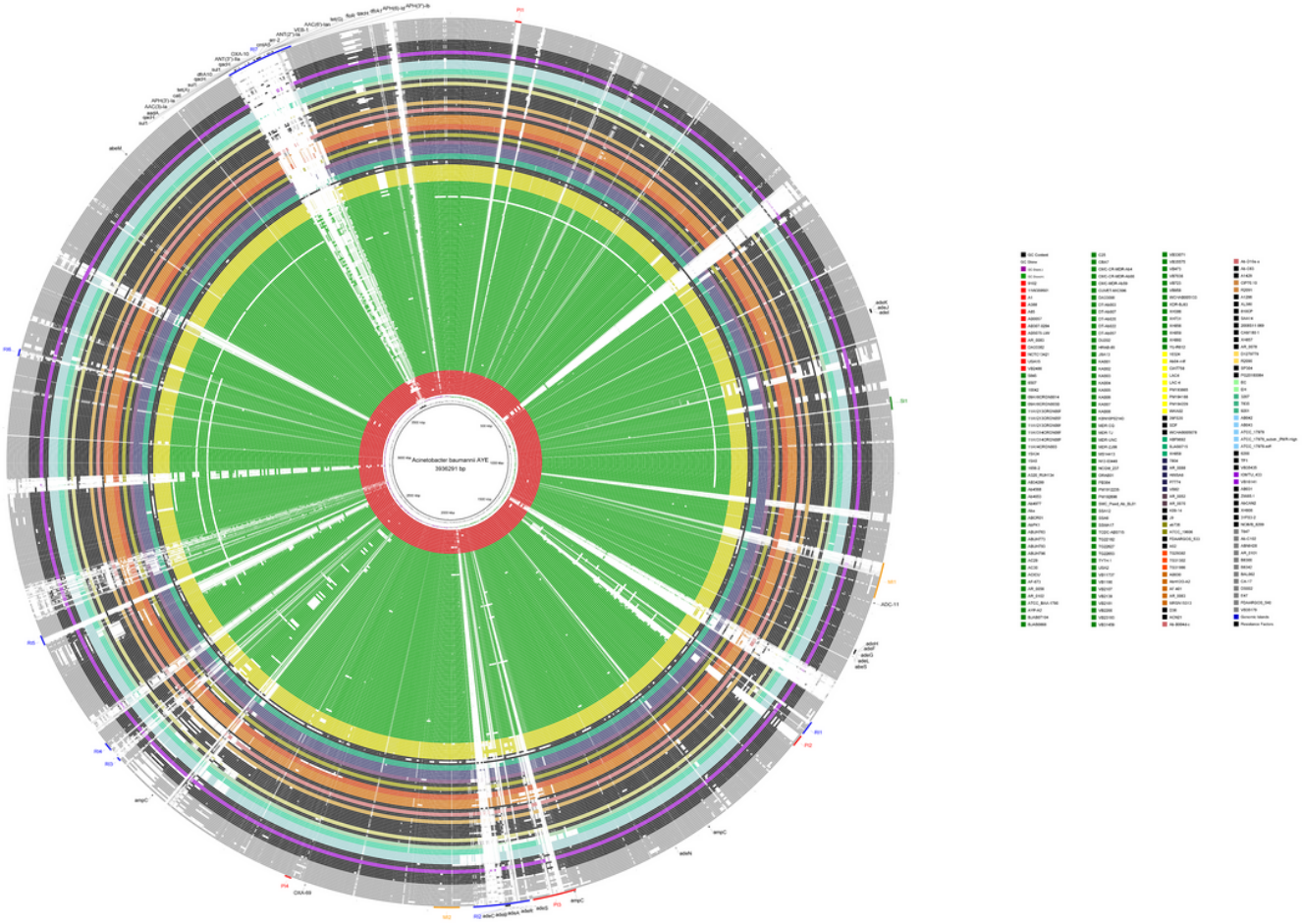

Figure 4

Representation of the circular genome of the A. baumannii AYE strain as a central genome. The compared strains were grouped according to the sequence type and visually segregated by differentiating based on the applied color. It is possible to observe the presence of resistance islands highlighted in blue using the A. baumannii SDF genome as a reference. Metabolic islands are represented in yellow, Symbiotic islands are represented in green and Pathogenicity islands are represented in red.The position of each resistance gene on the chromosome was evidenced in black.

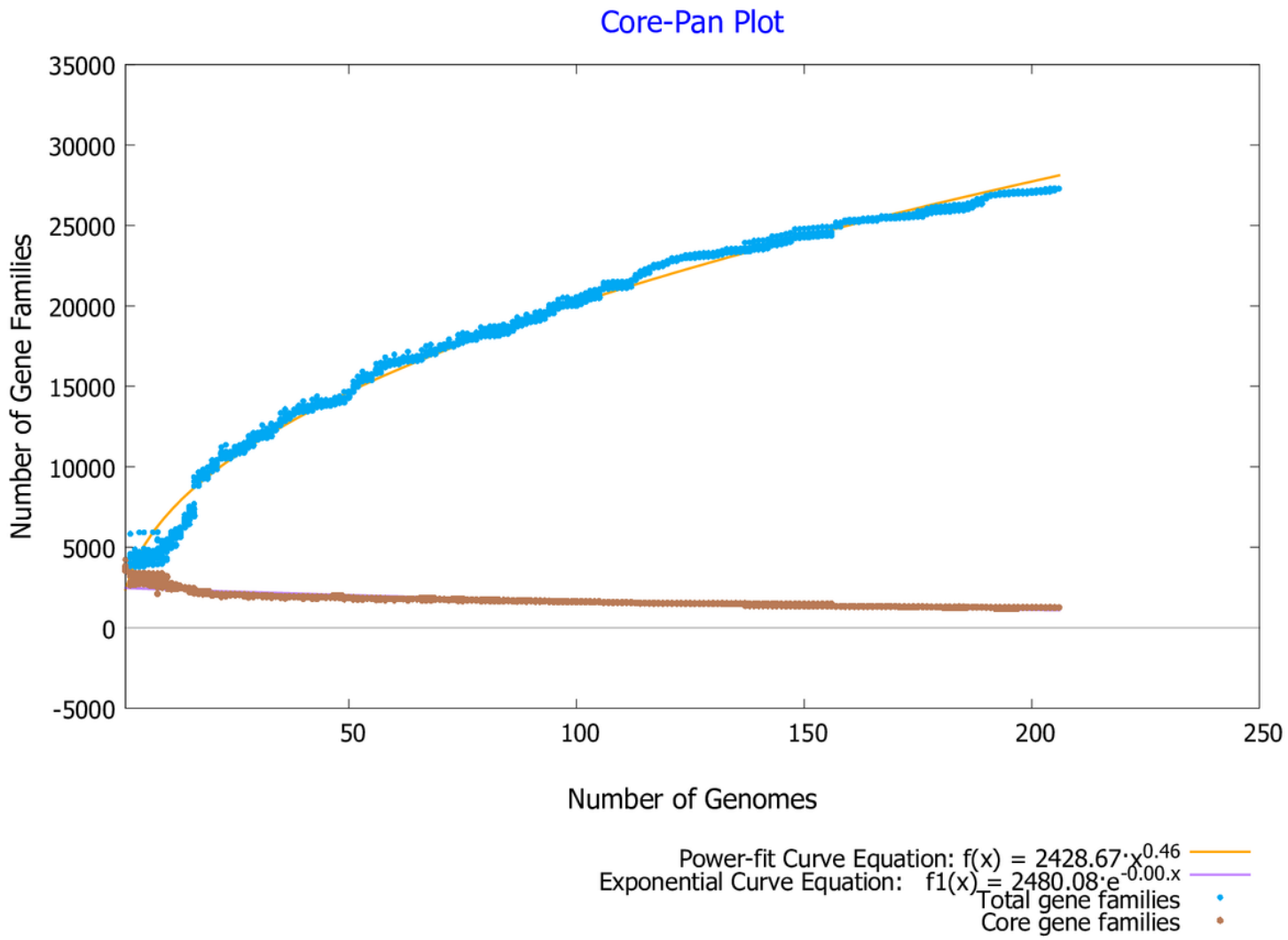

Figure 5

Graphical representation of the development of the pan-genome related to the number of genomes used. Total number of gene families (in blue), and the predicted growth curve of the pan-genome based on the power-law regression model (in yellow) are highlighted. In brown, the numbers of genes present in the core-genome are drafted, and, in purple, the core-genome stabilization curve based on the exponential curve fit model is highlighted.

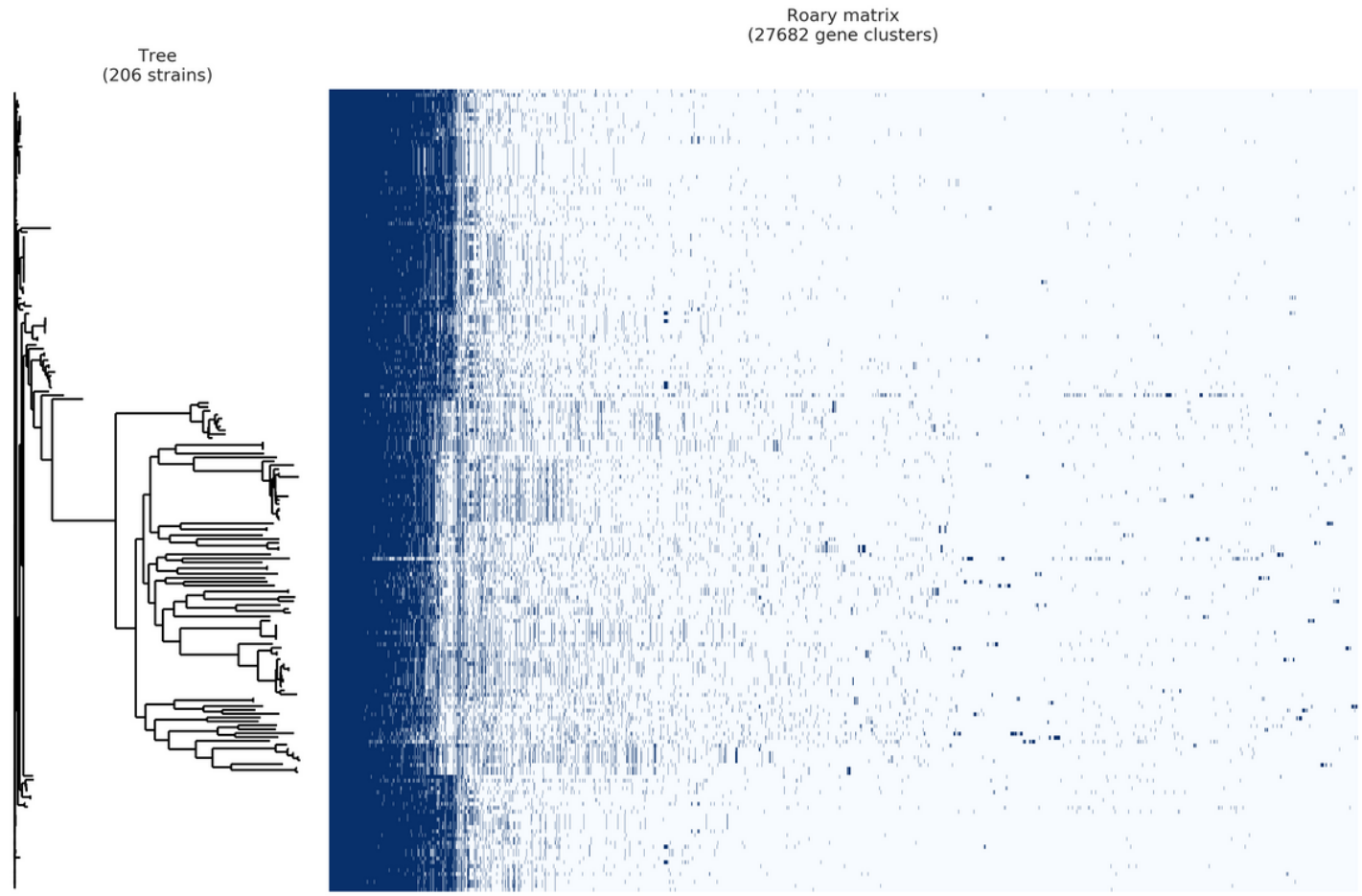

Figure 6

Matrix of presence and absence of pan-genome genes associated with the cladogram generated by the analysis of the species core genome. Distribution of pan-genome genes in A. baumannii strains. The matrix is indicating the presence (blue) or absence (white) of pan-genome genes. Each row in the matrix represents one genome, and each column represents the pangenome genes. This cladogram was generated by applying a maximum likelihood method. The phylogeny based on the core genome (1373 genes) shows a major clade concerning the gene content.

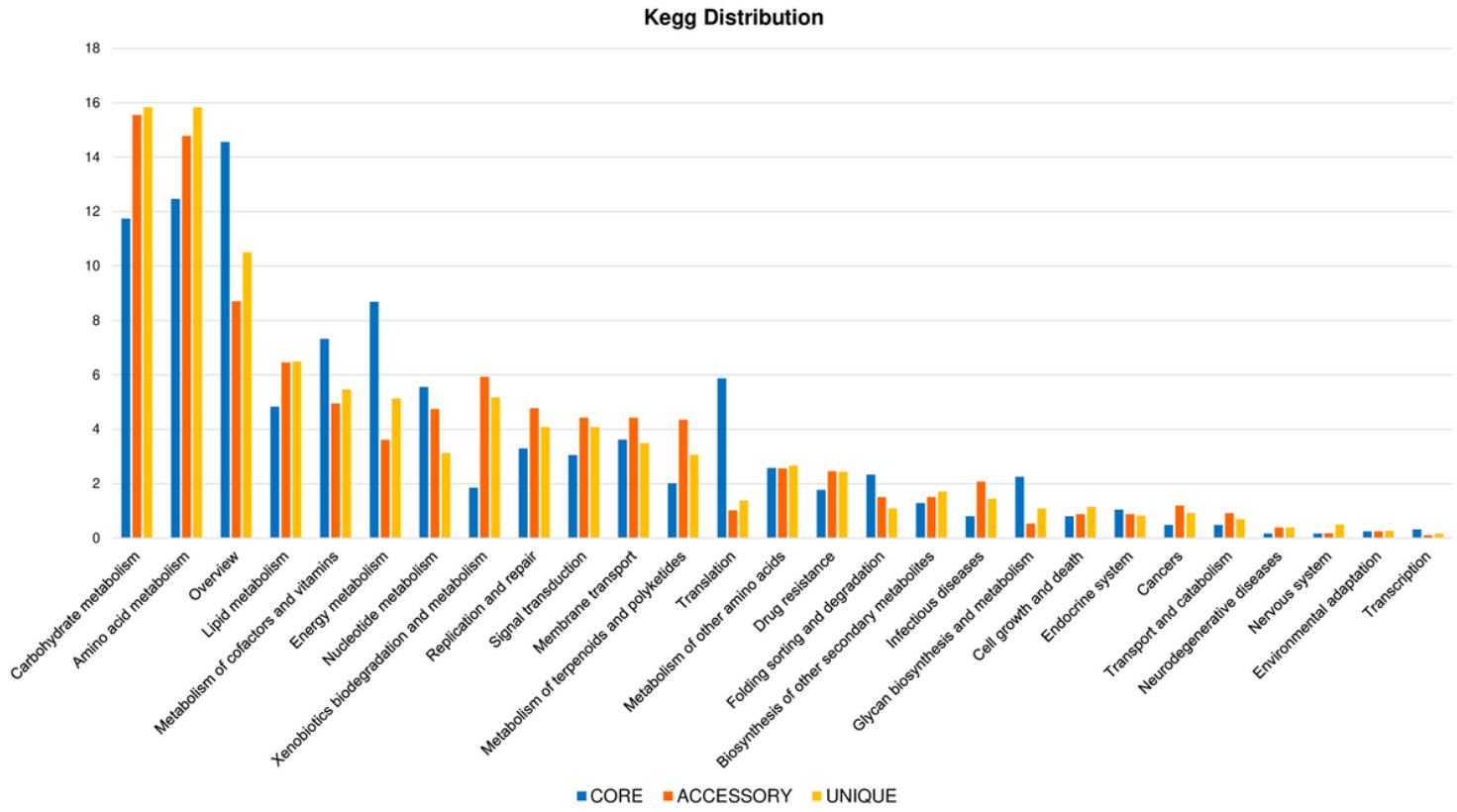

Figure 7

Graphical representation of the gene distribution by metabolic pathway within each subpartition of the total pan-genome. On the X-axis the metabolic pathways distributed according to the KEGG are shown, and, on the Y-axis, the relative number of genes related to each path are indicated. Only pathways that have at least 0.1% of the genes represented in each subpartition of the pan-genome were considered.

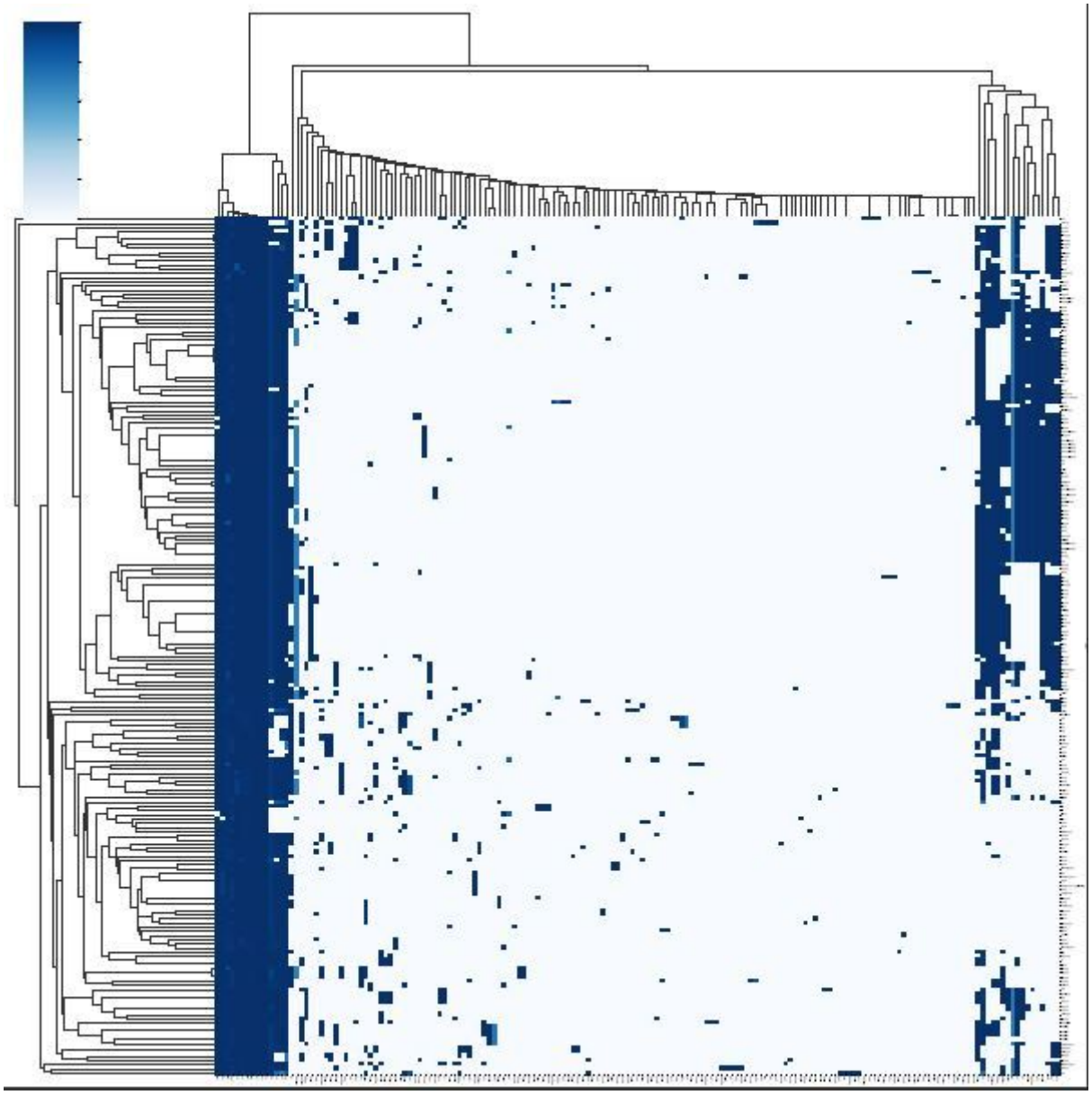

Figure 8

Clustermap representing the presence of resistance genes (X-axis) in all genomes (Y-axis) were addressed. In the case of existence, the color intensity represents the sequence similarity to the database used, with a minimum similarity of 70% versus CARD database. The cladograms used are based on the Euclidean distance between the data.

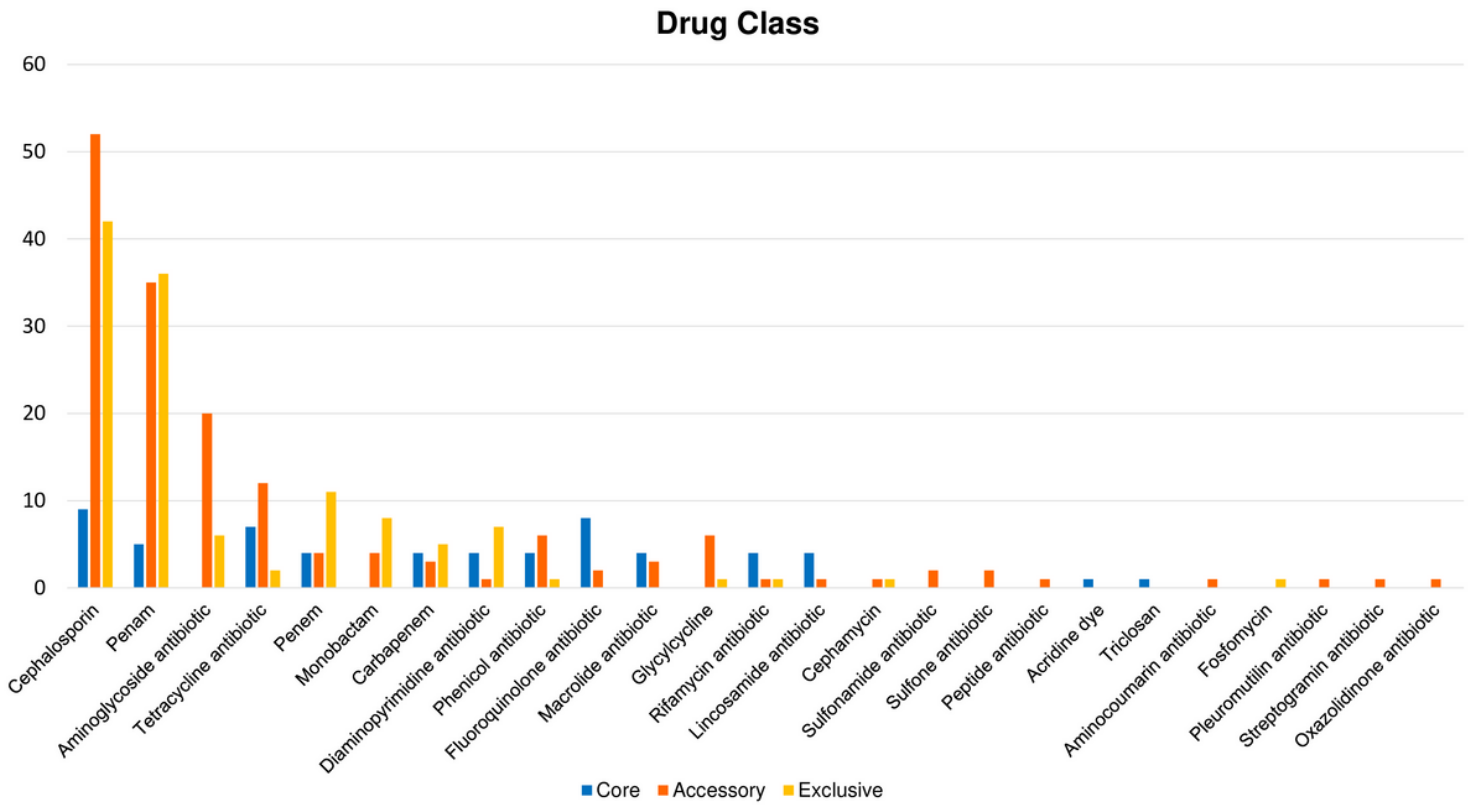

Figure 9

Distribution of the resistance mechanisms related to each antimicrobial found in the database used in the predicted total pan-resistome. On the X-axis, the antimicrobials related to the resistance found in the pan-resistome are shown. On the Y-axis, the number of genes related to the resistance to each antimicrobial raised in each subpartition of the resistome are depicted.

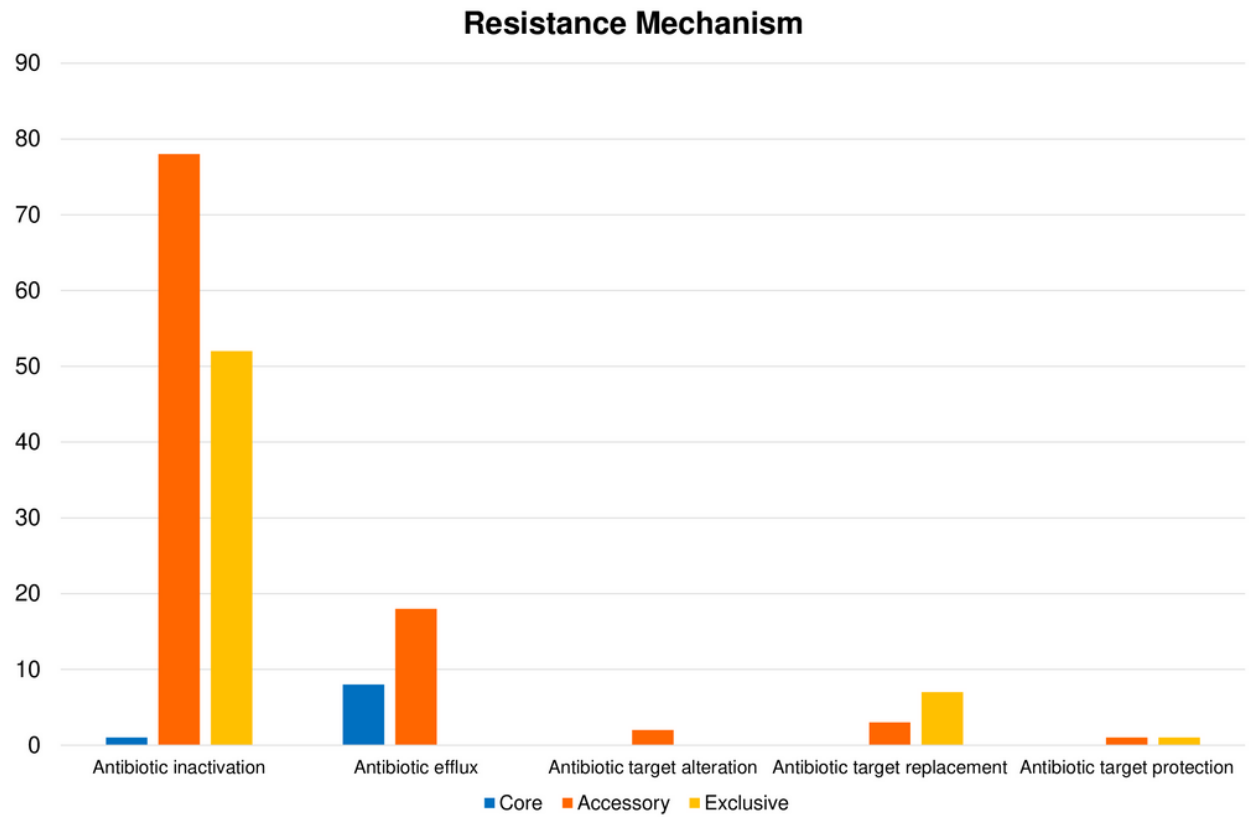

Figure 10

Distribution of the resistance mechanisms related to each type of action provided by the translated protein. On the X-axis the mechanisms of action related to the resistance found in the pan-resistome are shown, and, on the Y-axis, the number of genes related to the resistance mechanisms raised in each subpartition of the pan- resistome is exhibited.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile3.xlsx
- Additionalfile2.pdf
- Additionalfile1.xlsx