Within-host Evolution of Acinetobacter Baumannii in Clinical Bacteremia Patients

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Abstract

Background: *Acinetobacter baumannii* colonizing the respiratory tract has been established as an independent risk factor for bacteremia. However, within-host evolution of *A. baumannii* in bacteremia has not been extensively investigated. Here we performed whole genome sequencing to discover the evolutionary characteristics that accompany the transition from respiratory tract carriage to bloodstream infection in three *A. baumannii* bacteremia patients.

Results: Within-host genetic diversity was identified. A total of twenty-one SNVs were detected. Genic and intergenic evolution occurred particularly in secretion system, DNA recombination and cell motility genes. Intergenic SNVs occurred more frequently than synonymous and non-synonymous SNVs, which indicated potential transcription or translation regulation. Non-synonymous mutations mostly occurred during the transition from the respiratory tract carriage to the bloodstream infection. Isolates of clonal complex 208 (CC208) had lower substitution rate with approximately 10^{-6} nucleotide substitutions per site year^{-1}, compared with non CC208 isolates (approximately 10^{-5}). We found evidence for the occurrence of recombination in one patient. Gene content showed patient specificity, and isolates within a single host had constrained gene content diversity.

Conclusions: Our results indicated that high within-host diversity was driven by rapid mutation rates and limited effect of recombination in *A. baumannii* from respiratory tract carriage to bacteremia.

Background

Acinetobacter baumannii is an important opportunistic pathogen that causes severe nosocomial infections worldwide [1]. Bacteremia is a prevalent cause of patient mortality, and the mortality rate in patients with *A. baumannii* bacteremia is 32.5–63.5% [2, 3]. Furthermore, due to increased exposure to antibiotics, multidrug resistant (MDR) and carbapenem-resistant *A. baumannii* have been predictably increasing in recent years. The incidence of imipenem-resistant *A. baumannii* increased from 31.0% in 2005 to 70.7% in 2017 in China [4]. With limited treatment options available, infections caused by MDR and carbapenem-resistant *A. baumannii* may result in higher mortality. A meta-analysis showed that patients with carbapenem-resistant *A. baumannii* had a significantly higher risk of mortality than patients with carbapenem-susceptible *A. baumannii* [5].

A. baumannii is widely distributed in natural and hospital environments and can colonize the human skin, mouth, and nasopharynx [6]. Colonization on human mucosal surfaces and medical devices can lead to the formation of biofilms, increasing the risk of developing *A. baumannii* respiratory infections [7, 8]. The progression of bacteria from colonization to infection is the result of within-host evolution [9]. A surveillance study found that MDR *A. baumannii* may be carried for long durations, up to 42 months [6]. Meanwhile, a case-control study showed that previous respiratory tract colonization with species including *A. baumannii* was an independent risk factor for bacteremia [10].

Several studies have demonstrated the evolution of *A. baumannii* colonization *in vivo*. A study of four patients with long-term colonization of *A. baumannii* found that multilocus sequence typing remained unchanged during colonization in three patients. The study found that *A. baumannii* demonstrates parallel evolution during colonization. One efflux pump gene and two phage-related genes showed genetic variation in all patients. Studies have shown that antibiotic use and the host environment exert selective pressure on colonized *A. baumannii*, prompting rapid molecular evolution *in vivo* [11]. Another genomic analysis of long-term infection with *A. baumannii* showed that mutations acquired during infection were over-represented in transcriptional regulators, notably pmrAB and adeRS, which can mediate resistance to the last-line therapies colistin and tigecycline, respectively [12].

However, the molecular evolution of *A. baumannii* bacteremia *in vivo* remains unclear. Dynamic genomic variations during respiratory tract carriage and transition to bloodstream infection have not yet been demonstrated. In our study, we analyzed

genomic variation in different isolates isolated from the same patient in order to elucidate the molecular mechanism of within-host evolution of *A. baumannii* bacteremia.

Results

Description of isolates

The number of isolates per patient was in the range of 6 ~ 15, over 12 ~ 44 days between the first respiratory tract isolate and last bloodstream isolate. The pattern of antimicrobial susceptibility indicated that all isolates were carbapenem-resistant *A. baumannii*, and only susceptible to colistin (Table S1). We detected two Pasteur sequence types (STs) and six Oxford STs from the isolates. All isolates belonged to the predominant clonal complex CC2. Two patients' isolates (C and L) and 11 isolates in Patient A belonged to Pasteur ST2. Four isolates in Patient A belonged to a new Pasteur ST of CC2. Different Oxford STs were identified in three patients, including ST1968 and a new Oxford ST in Patient A, ST469 and ST436 in Patient C, and ST195 and ST208 in Patient L (Table S1). A total of twelve isolates were selected for further analysis, including five bloodstream isolates in the three patients, and other isolates which have the same Oxford ST with bloodstream isolates in each patient (Fig. 1).

Within-host genetic diversity from respiratory tract carriage to bloodstream infection

To facilitate detailed analysis of strain relationships, we developed a robust phylogeny based on single nucleotide variants (SNVs) present in core regions of the genome to represent ancestral relationships among the isolates (Fig. 2). The phylogenetic tree analysis indicated that isolates collected from different patient were grouped into distinct clades. Isolates in each patient showed different genomic characteristics. Isolates which had the same ST were clustered together. Bloodstream isolates A14, C4, C6, L9 and L10 were clustered with isolates from respiratory tract in each patient.

We defined the amount of genetic diversity as the number of SNVs between 12 selected isolates within the patients. SNVs of the genomes were identified by mapping sequence reads for each isolate against the first isolates with the same Oxford ST as the bloodstream isolate in each patient (A1 for patient A, C2 for patient C, and L6 for patient L). A total of twenty-one SNVs were identified (Table S2). A complete list of all the genic mutations differing between the isolates was given in the Table 1. Except for a hypothetical protein, the SNVs were found in *srpA, gspJ, srmB, fimV, sca1, transposase, pilR, pcaJ, tniA* genes, which encodes for organic peroxide-dependent peroxidase, general secretion pathway protein, DEAD box helicase family, Hep Hag repeat protein, Tfp pilus assembly protein FimV, phage-related minor tail protein, transposase IS66 family, sigma-54 interaction domain protein, 3-oxoadipate CoA-transferase, mu transposase respectively. Two genes encode the same protein Tfp pilus assembly protein FimV. The genes encoding for 3-oxoadipate CoA-transferase PcaJ had the highest density of SNVs. Two missense variants and one synonymous variant were detected in the gene.

Table 1

The genic SNVs found from respiratory tract carriage to bloodstream infection in three *A. baumannii* bacteremia patients.

No.	SNV type	Coding region change	Gene name/function	Patient ID (reference isolate ID)	Oxford ST (*gltA-gyrB-gdhB-cpn60-recA-gpi-rpoD*)	Isolates ID
1	Stop gained	Gln113*	*srpA*, has an organic peroxide-dependent peroxidase activity	A (A1)	New (122-3-3-2-2-97-3)	**A14**
2	Disruptive inframe insertion	Phe120_Lys121 ins Asn	*gspJ*, general secretion pathway protein	A (A1)	New (122-3-3-2-2-97-3)	**A14**
3	Conservative inframe insertion	His458_Ala459 ins ValValLysValValLeuLysT hrValHisValValAlaSerValValLysIleValHisVal AlaSerSerThrGlnThrValHisValValLysValVal LeuLysThrValGlnAsnValAlaSerValValLysIle ValArgValAlaSerSerThrGlnIleValHis	*srmB*, belongs to the DEAD box helicase family	C (C2)	469 (1-12-3-2-2-103-3)	**C4, C6**
4	Upstream gene variant		hypothetical protein	C (C2)	469 (1-12-3-2-2-103-3)	**C6**
5	Upstream gene variant		Hep Hag repeat protein	C (C2)	469 (1-12-3-2-2-103-3)	**C4, C6**
6	Missense variant Synonymous variant	Thr4Lys Asp5Asp	*fimV*, Tfp pilus assembly protein FimV	C (C2)	469 (1-12-3-2-2-103-3)	C2
7	Synonymous variant	Asp18Asp	*fimV*, Tfp pilus assembly protein FimV	C (C2)	469 (1-12-3-2-2-103-3)	**C4, C6**
8	Upstream gene variant		*sca1*, phage-related minor tail protein	L (L6)	195 (1-3-3-2-2-96-3)	L6, L8, **L10**
9	Missense variant	Gly184Ser	Transposase IS66 family	L (L6)	195 (1-3-3-2-2-96-3)	L6, L7, **L9, L10**

SNVs of the genomes were identified by mapping sequence reads for each isolate against the first isolates with the same Oxford ST type as the bloodstream isolate in each patient. Isolates from bloodstream were in bold.

No.	SNV type	Coding region change	Gene name/function	Patient ID (reference isolate ID)	Oxford ST (*gltA-gyrB-gdhB-cpn60-recA-gpi-rpoD*)	Isolates ID
10	Upstream gene variant		*pilR*, sigma-54 interaction domain	L (L6)	195 (1-3-3-2-2-96-3)	L8, **L9, L10**
11	Missense variant Synonymous variant	Ile91Leu Phe90Leu Arg89Arg	*pcaJ*, 3-oxoadipate CoA-transferase activity	L (L6)	195 (1-3-3-2-2-96-3)	L7, L8, **L9, L10**
12	Synonymous variant	Leu16Leu	*tniA*, mu transposase, C-terminal	L (L6)	195 (1-3-3-2-2-96-3)	L7, **L10**

SNVs of the genomes were identified by mapping sequence reads for each isolate against the first isolates with the same Oxford ST type as the bloodstream isolate in each patient. Isolates from bloodstream were in bold.

The mean number of SNVs between consecutively sampled isolates ranged from 0.67 to 8 for different patient (Fig. 3A). To assess whether the accumulation of substitutions was time-dependent, we fitted a linear regression model of the number of accrued substitutions against the corresponding time (Fig. 3B). In general, *A. baumannii* isolates accumulated mutations over time at different rates in three patients. With the exception of Patient C, who's within-host substitution rate was 4.35114×10^{-5} SNVs site^{-1} year^{-1}, the other two patients showed lower within-host substitution rate ranging from 6.46753×10^{-6} to 7.52486×10^{-6} SNVs site^{-1} year^{-1} (Table 2). Oxford STs of Patient A (new) and L (ST195) belonged to clonal complex 208 (CC208), which was the predominant CC in China. Isolates of CC208 had similar substitution rate, and such within-host substitution rate resulted in the introduction of up to ≈ 29 substitutions per year. Non CC208 isolates showed faster substitution rate (182.5 substitutions per year).

Table 2
Within-host nucleotide substitution rates from respiratory tract carriage to bacteremia.

Patient ID	CC208	R^2	Estimate	Substitution rate	SNV year^{-1}
A	Y	0.9944	0.08647	7.52486×10^{6}	31.56155
C	N	1	0.5	4.35114×10^{5}	182.5
L	Y	0.8176	0.07432	6.46753×10^{6}	27.1268

R^2 denoted coefficient of determination. The estimated value for the regression coefficient was expressed as SNVs per week. The substitution rate were expressed SNVs site^{-1} year^{-1}.

In two of three patients, intergenic SNVs occurred more frequently than synonymous and non-synonymous SNVs (Fig. 3C). Five intergenic SNVs were identified in patient C, including 2 upstream gene variants and 3 intergenic variants. In Patient L, we discovered a total of 5 intergenic SNVs, comprising 3 upstream gene variants and 2 intergenic variants. Non-synonymous mutations mostly occurred during the transition from respiratory tract carriage to bacteremia. In Patient A and C, non-synonymous variants were only detected during the transition. In Patient L, we discovered a total of 3 missense variants among the respiratory tract isolates. Functional analysis suggested that most of the mutations were in genes

associated intracellular trafficking, secretion, and vesicular transport, DNA replication, recombination, and repair and cell motility (Fig. 3D).

Homologous recombination during within-host evolution

Homologous recombination is the major driver of evolution inbacterial pathogens. To identify or rule out the occurrence of recombination, we aligned the genomes of the isolates from each patient to assess whether we could identify genomic regions with high density of SNVs, a well-known signature for recombination. We found evidence for the occurrence of within-host recombination in one patient (Patient L). The range of recombination blocks was 1 ~ 3, while the number SNVs within each block was 14 (range: 8 ~ 36) per recombination block. We then assessed the ratio of imported SNVs via recombination relative to random substitutions (r/m) and total recombination blocks relative to random substitutions (ρ/θ), which are widely used statistics for quantifying the contribution of recombination to genomic diversification. The r/m and ρ/θ averaged across all phylogenetic branches where recombination had occurred were 3.02 (range: 0 ~ 26) and 0.35 (range:0 ~ 3) respectively.

Patient specific gene content during within-host evolution

Gene content showed patient specificity. Only 1.1% ~ 9.4% of the *A. baumannii* gene clusters observed in a specific patient (i.e., the subject-specific pan-genome) and 88.5% ~ 92.8% of the gene clusters observed in all isolates of a specific patient (i.e., the subject-specific core-genome) were shared between all three patients, and 0.5% ~ 6.3% of core gene clusters were entirely unique to a patient (Fig. 4). Antibiotic resistant gene and virulence gene profiling showed that most genes were consistent in the same MLST isolates in each patient (Table S3). Only one isolate A2 contained the fosfomycin resistance gene *fosB4*. The gene *armA* was only absent in isolate L7. The gene *aph(3')-Ia* lost in the L2, L4 and L5 isolates. For the virulence genes, only the *bap* gene showed difference within the the same MLST isolates. The isolates C4 and C7 had two biofilm-associated *bap* gene, which C2 had only one. These findings suggested a personalization of gene content at the population level. The *A. baumannii* population found in a single host retains the inherent diversity from multiple founder lineages, further evolution of the *A. baumannii* gene repertoire occurred in a host-specific manner. Our results showed that, despite their wide distribution in the SNV-based phylogeny, *A. baumannii* within a single host had constrained gene content diversity.

Discussion

Previous colonization with causative microorganisms is significantly associated with bacteremia [10]. Previous studies have shown that nasal carriage is a source of *Staphylococcus aureus* bacteremia [13]. However, previous studies did not systematically investigate the link between *A. baumannii* isolated from bloodstream and the respiratory tract before bacteremia, using the whole-genome sequencing method. Therefore, we conducted this study to assess the correlation between isolates from the respiratory tract and bloodstream in *A. baumannii*. Our results indicated that high within-host diversity appears to be driven by rapid mutation rates and limited effect of recombination.

In our study, within host diversity was identified, which suggested an interplay of both the host and pathogen factors on within-host genetic diversity. We found genic mutations in twelve genes. Genic and intergenic evolution occurred particularly in secretion system, DNA recombination and cell motility genes. Four genes were related with secretion system, including *srpA*, *gspJ* and two *fimV* genes. SrpA plays versatile regulatory functions by binding directly to the promoter region of the type III secretion system and type VI secretion system [14]. GspJ is general secretion pathway protein of type II secretion system. In *Burkholderia cenocepacia*, gspJ is required for multihost pathogenicity [15]. FimV was shown to interact with T2SS protein Vfr by a mechanism that acts as a modulator of the Vfr level [16]. Recent studies have shown that the type II secretion system (T2SS) supported survival of *A. baumannii* in a bacteremia mouse model through extracellular secretion of a variety of enzymes, including the glycan-specific metalloprotease CpaA. CpaA interferes with blood coagulation through

cleavage of factor XII (fXII) of the contact activation system. Preventing fXII-mediated generation of fibrin may allow *A. baumannii* to escape containment by intravascular clots and to disseminate [17]. The Cag type IV secretion systems (T4SSs) in *Helicobacter pylori* facilitates the delivery of the CagA effector protein and pro-inflammatory signal transduction through translocated ADP-heptose and chromosomal DNA, while various structural pilus proteins can target host cell receptors such as integrins or toll-like receptor-5. T4SSs was involved in *H. pylori* adaptation to the hostile environment in the human stomach [18]. Further research is needed to clarify the function and mechanisms of secretion system in *A. baumannii* during within host evolution. Besides, FimV was required for type IV pilus-mediated twitching motility in *Pseudomonas aeruginosa*, arising from extension and retraction of pili from their site of assembly in the inner membrane [19]. Another cell motility gene was *pilR*. In *P. aeruginosa*, the PilS-PilR two-component system regulated the expression of the type IV pilus major subunit PilA. These pilus related proteins controlled the cell motility, which may influence the transition of *A. baumannii*. DNA recombination was detected in one patient. Several DNA recombination related genes were also identified, such as phage-related gene *sca1*, and two transposase genes. DEAD box helicase family gene *srmB* was essential to genome replication and repair. These proteins may relate with DNA replication, recombination and repair during within-host evolution. Further studies are needed to clarify the mechanism of these within-host related genes.

We have some limitations in this study, only three patients were involved. The other hand, since we predominantly sequenced single colonies, these may have failed to capture temporal dynamics of co-colonising strains especially those present at low frequency. Therefore, future studies sequencing either multiple colonies or better yet the entire culture will be required to fully unravel within-sample genetic diversity and temporal dynamics of the wild-type and recombinant strain variants. Intergenic mutations occupied a large proportion at all stages, which indicated potential transcription or translation regulation during within host evolution of *A. baumannii*. Transcription level research should be carried out in future work, since regulatory networks was important for survival of *A. baumannii* within the host [20].

Conclusions

Our findings showed rapid within-host evolution of A. baumannii from respiratory tract carriage to bacteremia with evidence of adaptations through mutations in intergenic and genic regions association with secretion system, DNA recombination and cell motility proteins, which may promote efficient and prolonged survival in the host. Gene content diversity between different patient was identified, which suggested an interplay of both the host and pathogen factors on within-host genetic diversity. Our findings enhanced our understanding of within-host evolution of A. baumannii during respiratory tract carriage and provided a framework for discovering novel genomic changes and pathogenicity genes important for extended bacteremia which will be validated in future studies.

Methods

Bacterial strain isolation and phenotype tests

The *A. baumannii* isolates were obtained from three *A. baumannii* bacteremia patients sequentially during hospitalization at Peking University People's Hospital (Table S1). *A. baumannii* was identified using the VITEK2 system (bioMérieux, Marcy l' toile, France), and confirmed by the presence of $bla_{\text{OXA51-like}}$ gene by PCR and sequencing. The isolates were subjected to additional analysis to characterize the antibiotic resistance phenotypes and genotypes via multilocus sequence typing (MLST) (http://pubmlst.org/abaumannii/) [21, 22] (Table S1).

Genome sequencing and bioinformatic analysis

Bacterial DNA was extracted using a DNA purification kit (Qiagen, Hilden, Germany). Illumina sequencing libraries were prepared using Nextera kits with indexed encoded adapters from Illumina, according to the manufacturer's instructions. Libraries were pooled for sequencing on a MiSeq sequencer. The isolate A1 was also extracted by the QIAGEN Large-

Construct kit (Qiagen Sciences, Germantown, MD, USA) and sequenced by the PacBio RS II system (Pacific Biosciences) with a 10-kb size library and P6/C4 chemistry. The resulting FASTQ files were quality-trimmed and assembled de novo using Velvet (Ridom GmbH, Münster, Germany). The assembled contigs were annotated with Prokka [23]. The output was used to construct a pan-genome with Roary [24]. After recombination region removal, RAxML [25] was used to build a phylogenetic tree. The final tree was constructed using R ggtree [26] and the ggplot2 package. Multiple sequence alignments diagrams were generated using progressiveMAUVE [27].Homologous recombination was assessed using Gubbins [28]. The COG functional categories were identified using eggNOG-Mapper [29]. Detailed comparative analysis of single nucleotide variants was performed using SnpEff [30].

Abbreviations

CC: clonal complex, MDR: multidrug resistant, ST: sequence type, SNV: single nucleotide variant, MLST: multilocus sequence typing, T2SS: type II secretion system, fXII: factor XII, T4SS: type IV secretion system, COG: clusters of orthologous groups

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Ethical Review Committee of Peking University People's Hospital (No. 2018PHB187). Individuals younger than the age of 16 were not involved. Informed consent was waived by the Ethical Review Committee of Peking University People's Hospital. The retrospective study was considered exempt from ethical review as only the bacterial isolates were taken from the patients and the study with confidentiality fully guaranteed.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the NCBI Biosample repository, https://www.ncbi.nlm.nih.gov/biosample. The data can be accessed using the accession numbers SAMN11513659 (L6), SAMN11513660 (L7), SAMN11513661 (L8), SAMN11513662 (L9), SAMN11513663 (L10), SAMN15778733 (A1), SAMN15778734 (A2), SAMN15778735 (A3), SAMN15778736 (A14), SAMN15778737 (C2), SAMN15778738 (C4), and SAMN15778739 (C6).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HL and HW conceived and designed the experiments. HL, JZ, ZW, YY, HG, RW and LJ performed the experiments. QW, CZ and ZW contributed the materials. HL, JZ, ZW, YY, and HG conducted the bioinformatics analyses. HL drafted the manuscript. HW edited the manuscript. All authors read and approved the final manuscript.

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Figures

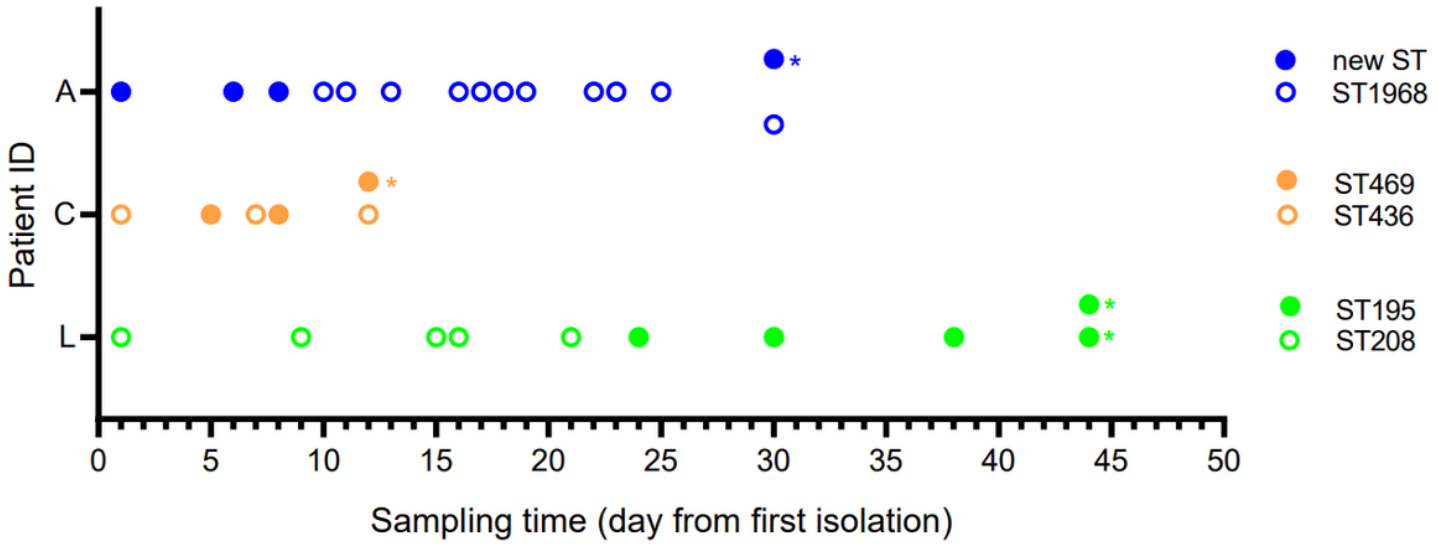

Figure 1

Isolate sampling point in three A. baumannii bacteremia patients. Bacterial isolates were represented by the colored symbols. The solid circle indicates the isolates consistent with the Oxford ST of bloodstream isolates in each patient (asterisk on the right), and which were analyzed for within-host evolution.

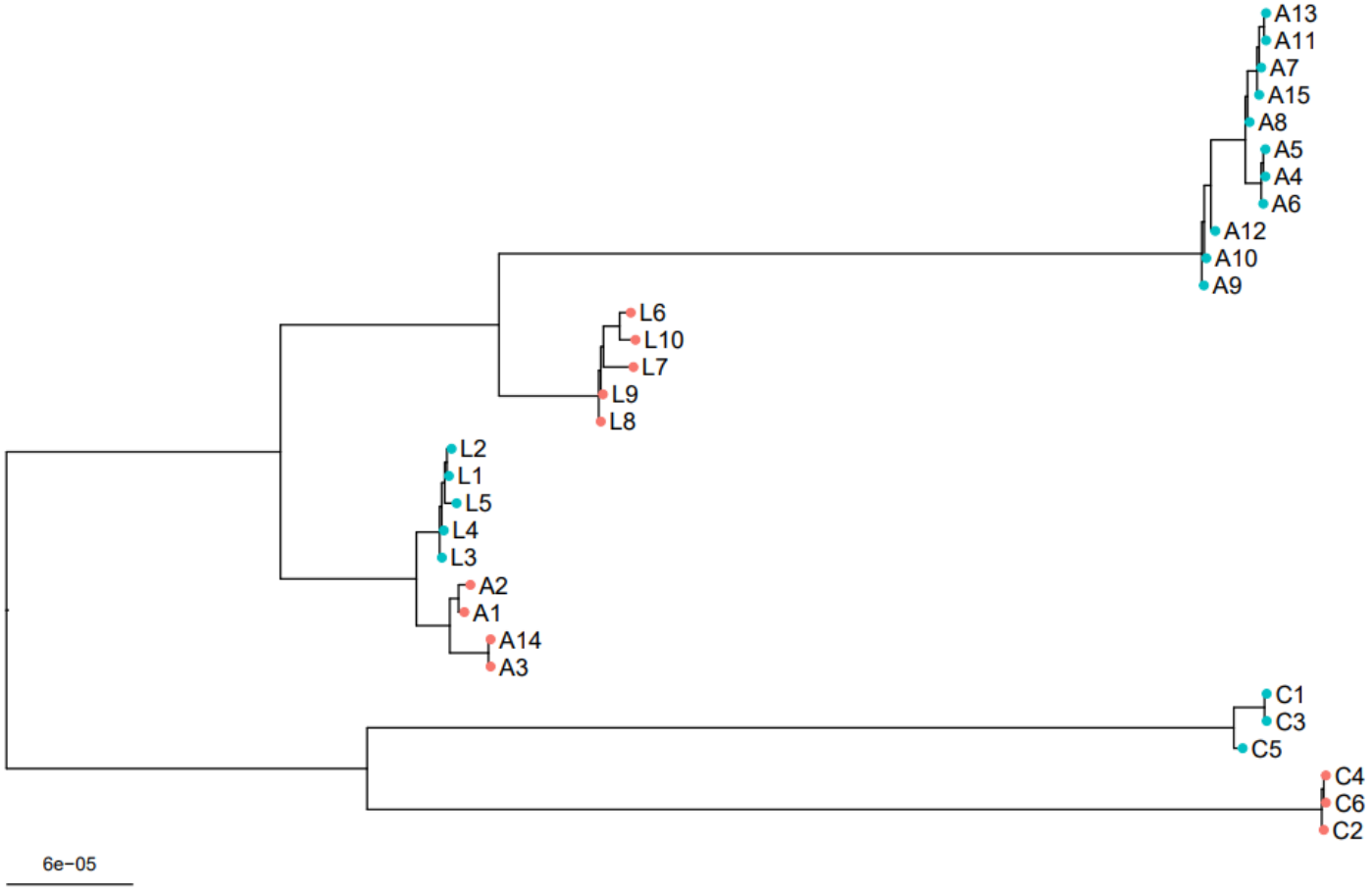

Figure 2

Phylogenetic analysis of A. baumannii collected from respiratory tract carriage to bloodstream infection. The phylogenetic tree was constructed based on core single nucleotide variants and rooted with ATCC19606. Red color indicated isolates selected for within-host evolution analysis, the other isolates were showed in blue. The scale bar represented the number of nucleotide substitutions per sites.

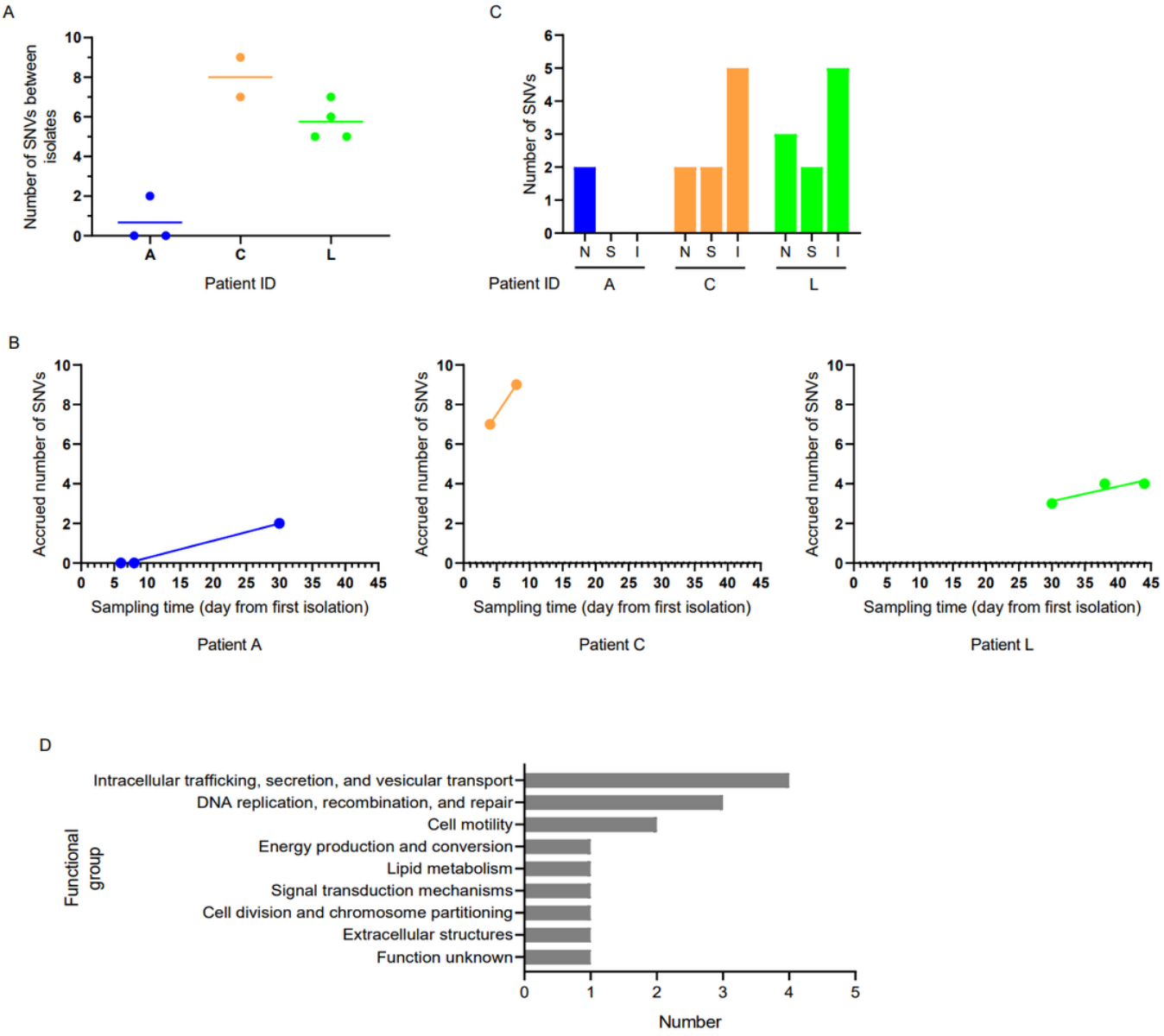

Figure 3

Characteristics of SNVs from respiratory tract carriage to bacteremia in three A. baumannii bacteremia patients. SNVs of the genomes were identified by mapping sequence reads for each isolate against the first isolates with the same Oxford STs as the bloodstream isolate in each patient (A1 for patient A, C2 for patient C, and L6 for patient L). (A) Within-host A. baumannii genetic diversity. The plots showing the number of SNVs calculated between isolates of the same Oxford ST within the same patient. (B) Within-host mutation rates. Linear relationship between the number of accrued SNVs and sampling time was assessed using linear regression. (C) The genic and intergenic SNVs identified during within-host evolution. The letters N, S and I stand for non-synonymous, synonymous and intergenic SNVs respectively. (D) Functional classification of genes with SNVs. The genes were analyzed the clusters of orthologous groups (COG) functional categories.

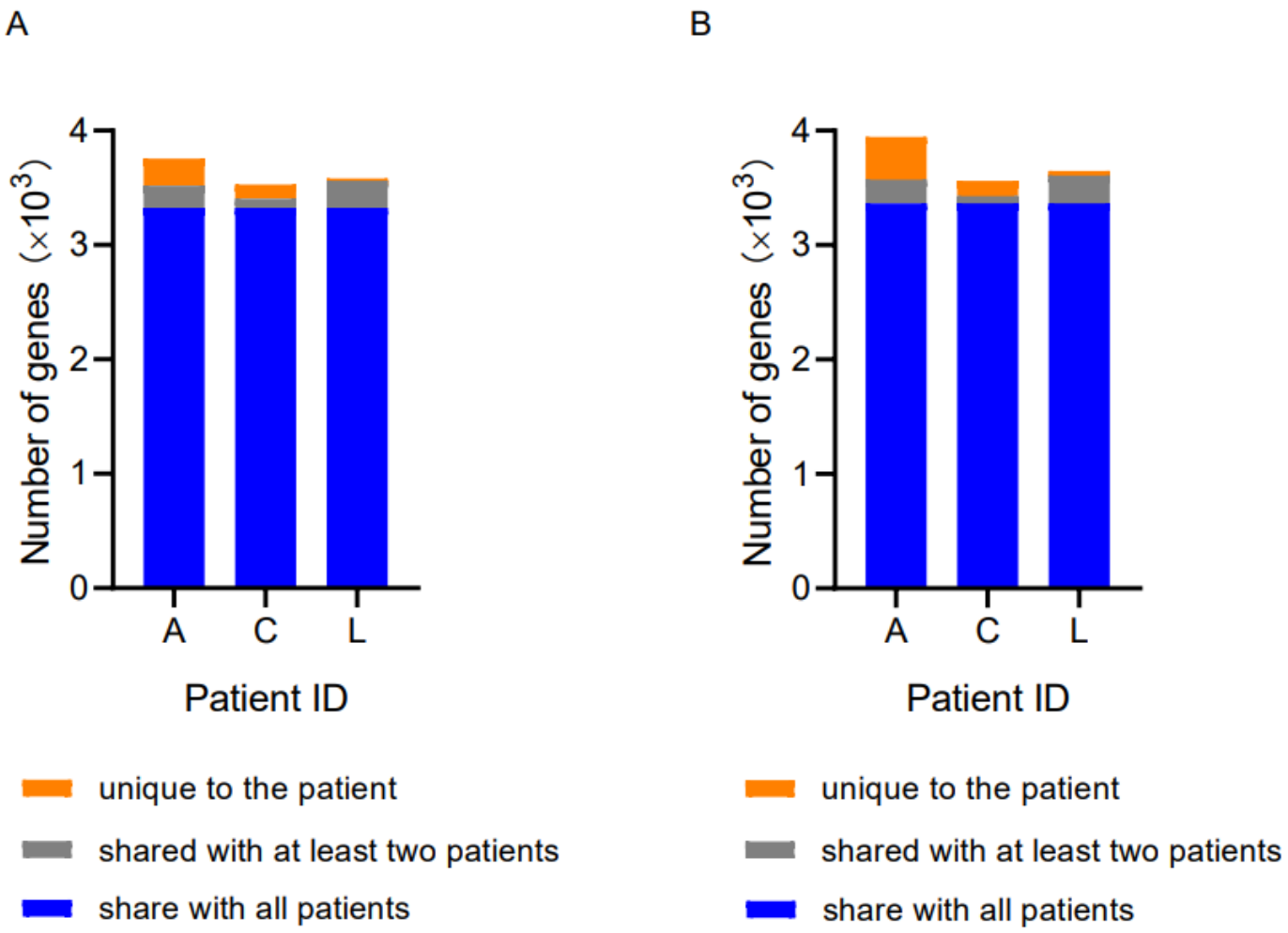

Figure 4

Shared versus unique patient-specific core (A) and pan (B) genes. The blue, grey and orange color indicated the genes share with all patients, shared with at least two patients, and unique to the patient respectively.

Supplementary Files

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

- TableS3200902.xlsx
- TableS2200902.xlsx
- TableS2200902.xlsx
- TableS1200902.xlsx
- TableS1200902.xlsx
- TableS3200902.xlsx