

# The Genomic Diversification of the Whole *Acinetobacter* Genus: Origins, Mechanisms, and Consequences

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## Abstract

Bacterial genomics has greatly expanded our understanding of microdiversification patterns within a species, but analyses at higher taxonomical levels are necessary to understand and predict the independent rise of pathogens in a genus. We have sampled, sequenced, and assessed the diversity of genomes of validly named and tentative species of the *Acinetobacter* genus, a clade including major nosocomial pathogens and biotechnologically important species. We inferred a robust global phylogeny and delimited several new putative species. The genus is very ancient and extremely diverse: Genomes of highly divergent species share more orthologs than certain strains within a species. We systematically characterized elements and mechanisms driving genome diversification, such as conjugative elements, insertion sequences, and natural transformation. We found many error-prone polymerases that may play a role in resistance to toxins, antibiotics, and in the generation of genetic variation. Surprisingly, temperate phages, poorly studied in *Acinetobacter*, were found to account for a significant fraction of most genomes. Accordingly, many genomes encode clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems with some of the largest CRISPR-arrays found so far in bacteria. Integrons are strongly overrepresented in *Acinetobacter baumannii*, which correlates with its frequent resistance to antibiotics. Our data suggest that *A. baumannii* arose from an ancient population bottleneck followed by population expansion under strong purifying selection. The outstanding diversification of the species occurred largely by horizontal transfer, including some allelic recombination, at specific hotspots preferentially located close to the replication terminus. Our work sets a quantitative basis to understand the diversification of *Acinetobacter* into emerging resistant and versatile pathogens.

**Key words:** comparative genomics, bacterial genus, evolution, mobile genetic elements, nosocomial pathogens.

## Introduction

In the last few years, a number of studies have harnessed the power of high-throughput sequencing to study epidemiological and evolutionary patterns within bacterial species. Such studies have uncovered patterns of transmission of multidrug resistant clones, the emergence of virulent strains, and their within-host evolution (e.g., Harris et al. 2010; Kennemann et al. 2011; Mather et al. 2013; McGann et al. 2014). There has been considerably less emphasis on the large-scale

sampling and sequencing of broader taxonomic units such as genera. Yet, this is an important level of analysis to understand the emergence of pathogenic species, because genera often include pathogens, commensals, and free-living (environmental) bacteria. The genus *Acinetobacter* is a good example of this as it includes a broad group of biochemically and physiologically versatile bacteria that occupy different natural ecosystems and play an increasing causative role in opportunistic human infections. The taxonomy of the genus consists

currently of 33 distinct validly named species (<http://www.bacterio.net/acinetobacter.html>, last accessed June 2014), about ten provisionally termed genomic species delineated by DNA–DNA hybridization (DDH) (Dijkshoorn et al. 2007), several putative new species but not validly named, and a number of strains of an as-yet unknown taxonomic status (Rodriguez-Bano et al. 2006; Yamahira et al. 2008; Smet et al. 2012). Following seminal DDH measures of relatedness (Bouvet and Jeanjean 1989; Tjernberg and Ursing 1989), several distinct phylogenies of the *Acinetobacter* genus have been estimated using one or a few phylogenetic marker genes (Rainey et al. 1994; Yamamoto et al. 1999; Krawczyk et al. 2002; La Scola et al. 2006; Diancourt et al. 2010). These analyses suggested the existence of several groups encompassing phylogenetically close species, including the species of the *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* (ACB) complex (Nemec et al. 2011), proteolytic genomic species (Bouvet and Jeanjean 1989; Nemec et al. 2009), *Acinetobacter guillouiae* and *Acinetobacter bereziniae* (Nemec et al. 2010), or *Acinetobacter nectaris* and *Acinetobacter boissieri* (Alvarez-Perez et al. 2013). However, these previous studies showed very diverse phylogenetic scenarios at higher taxonomic levels with weakly supported internal nodes (Yamamoto et al. 1999; Krawczyk et al. 2002; La Scola et al. 2006; Diancourt et al. 2010). Recent genome-wide analyses produced phylogenies showing that *A. baumannii* is well separated from the other strains of the ACB complex and these from other species in the genus (Chan et al. 2012). Nevertheless, the lack of a robust phylogenetic scenario encompassing all known *Acinetobacter* spp. and the unknown position of their last common ancestor (root of the tree) seriously hampers the understanding of the diversification of this genus.

*Acinetobacter* spp. are among the most frequent causes of hospital-acquired bacterial infections (Peleg et al. 2008). Community-acquired infections are less frequent but have also been reported (Falagas et al. 2007; Eveillard et al. 2013). Although *A. baumannii* is the most frequently identified nosocomial pathogen in the genus, several other species cause occasionally infections in humans including *Acinetobacter nosocomialis*, *Acinetobacter pittii*, and less frequently *Acinetobacter ursingii*, *Acinetobacter haemolyticus*, *Acinetobacter lwoffii*, *Acinetobacter parvus*, and *Acinetobacter junii* (Nemec et al. 2001, 2003; Dijkshoorn et al. 2007; Peleg et al. 2008; Turton et al. 2010; Karah et al. 2011). Clinical cases typically involve not only ventilator-associated pneumonia and septicemia, but also endocarditis, meningitis, burn and surgical wound infections, and urinary tract infections. Importantly, *Acinetobacter* spp. are isolated in the environment and asymptotically associated with humans, albeit the precise environmental reservoirs are unknown. Some species, notably *Acinetobacter baylyi*, are becoming emerging model organisms because of their transformability, metabolic versatility, and genome plasticity

(Barbe et al. 2004; Metzgar et al. 2004; de Berardinis et al. 2008). Interestingly, these same traits, together with intrinsic resistance to toxins and antibiotics, are thought to cause the increasing frequency of *Acinetobacter* spp. as agents of nosocomial infections (Peleg et al. 2012).

In the last decade, a small number of complete genome sequences and a large number of draft sequences of *Acinetobacter* spp. have become available (Barbe et al. 2004; Fournier et al. 2006; Antunes et al. 2013). These studies focused heavily on *A. baumannii* and the ACB complex and on what distinguishes them. Although the diversity of the genus remains largely unexplored, several studies have shown that *A. baumannii* gene repertoires are very diverse, with fewer than half of the genes being part of the species' core-genomes (Adams et al. 2008; Imperi et al. 2011; Sahl et al. 2011; Farrugia et al. 2013). Like for many bacterial clades, a large fraction of the genes of the pan-genome are of unknown function. Accessory functions involved in transport and genetic regulation are also highly variable between strains (Adams et al. 2008; Imperi et al. 2011). Finally, genome-wide comparisons between *A. baumannii* and other species suggest the existence of high genetic diversity in the genus (Vallet et al. 2008; Fondi et al. 2013).

Somewhat surprisingly, most known virulence factors of *A. baumannii* are found in its core-genome and are present in other species of the genus (Antunes et al. 2011). The multifactorial basis of virulence and the independent emergence of pathogenic strains in the genus suggest that the genetic background has an important role in *Acinetobacter* evolution. To understand the emergence of pathogenic *Acinetobacter*, it is therefore important to study the mechanisms of genetic diversification of the genus. For this, we have carefully sampled a large number of representative strains. Following the sequencing of their genomes, we characterized their genetic diversity and built a robust phylogeny of the entire genus. This information was used to assess pending taxonomic issues, to guide evolutionary studies, and to sample the genus for key mechanisms generating genetic variability. With these data at hand we focused on the genome dynamics of *A. baumannii*.

## Materials and Methods

### Choice of Strains

We analyzed 13 complete genomes retrieved from GenBank RefSeq in February 2013 (Pruitt et al. 2007), two *A. baumannii* genomes sequenced at the Pasteur Institute and at Walter Reed, and 118 genome sequences derived from 116 strains (two sequences were obtained from each of the type strains of *Acinetobacter indicus* and *Acinetobacter brisouii*) which were sequenced at the Broad Institute (see details in [supplementary table S1, Supplementary Material](#) online) (Perichon et al. 2014). The 116 strains were selected from the collections

of *A. Nemece* (strains designated NIPH or ANC) or of the Institut Pasteur (CIP strains) based on polyphasic taxonomic analyses to reflect the currently known breadth of the diversity of the genus *Acinetobacter* at the species level. Overall, 83 strains belonged to 29 validly named species (including *Acinetobacter grimontii*, a junior synonym of *A. junii*, but devoid of *A. boissieri*, *A. harbinensis*, *Acinetobacter puyangensis*, and *Acinetobacter qingfengensis*, which were unavailable at the time), 16 strains to eight genomic species as defined by DDH, and ten strains to seven tentative novel species termed *Acinetobacter* taxons 18–23 and 26. The name “*Acinetobacter bohemicus*” has recently been proposed for taxon 26 (Krizova et al. 2014). Seven remaining strains were closely related to one of the species/taxa but were considered taxonomically unique at the species level based on our previous taxonomic analysis and the average nucleotide identity (ANI) data obtained in this study (these strains are termed *A. calcoaceticus*-like, *A. brisouii*-like, *A. pittii*-like, or Taxon 18-like). The *Acinetobacter* taxons are working taxonomic groups as delineated at the Laboratory of Bacterial Genetics (National Institute of Public Health, Prague) based on the comprehensive physiological/nutritional testing, *rpoB* and 16S rDNA phylogenies and on whole-cell MALDI-TOF profiling. Each of these taxa is, at the species level, clearly distinct from any of the known species with a valid name, genomic species, or species with effectively published names. All validly named species were represented by the respective type strains, whereas each genomic species included a strain used as a reference in previous DDH experiments. If more strains per species or taxon were included, these differed from respective type/reference strains in their microbiological and ecological characteristics. Organisms were grown, according to their physiological requirements, at 30–37 °C in brain-heart infusion broth and agar (Difco Laboratories, Detroit, MI).

### Core-Genomes

We built core-genomes for the genus and for *A. baumannii*. Orthologs were identified as bidirectional best hits, using end-gap free global alignment, between the proteome of *A. baumannii* AYE as a pivot and each of the other proteomes (133 for the genus and 34 for the species). Hits with less than 40% (genus) or 80% (species) similarity in amino acid sequence or more than 20% difference in protein length were discarded. Genomes from the same species typically show low levels of genome rearrangements and this information can be used to identify orthologs more accurately (Dandekar et al. 1998; Rocha 2006). Therefore, the core-genome of the species was defined as the intersection of pairwise lists of strict positional orthologs (as in Touchon et al. 2009). The core-genomes consist in the genes present in all genomes of the two sets. They were defined as the intersection of the lists of orthologs between pairs of genomes.

### Pan-Genomes

Pan-genomes were built by clustering homologous proteins into families. We determined the lists of putative homologs between pairs of genomes with BLASTp (Altschul et al. 1997) and used the *e* values ( $<10^{-4}$ ) to cluster them by similarity with Silix v1.2 (Miele et al. 2011). A protein is thus included in the family if it shares a relation of homology to a protein already in the family. Silix parameters were set such that a protein is homolog to another in a given family if the aligned part has at least 35% of identity and represents more than 80% of the smallest protein. The pan-genomes are the full complement of genes in the genus and in the species. The pan-genomes of the 133 *Acinetobacter* proteomes (470,582 proteins) and of the 34 *A. baumannii* proteomes (128,266 proteins) were determined independently. We used a more stringent criterion of protein similarity (60%) to compare the pan-genomes of different species of *Acinetobacter*.

### Phylogenetic Analyses

Each of the 950 families of proteins of the *Acinetobacter* core-genome was used to produce a multiple alignment with muscle v3.8 (default parameters) (Edgar 2004). Poorly aligned regions were removed with BMGE (Criscuolo and Gribaldo 2010). The phylogenetic tree was inferred using the approximated maximum-likelihood method implemented in FastTree v1.4 with the Whelan and Goldman (WAG) matrix and a gamma correction for variable evolutionary rates (Price et al. 2009). We performed 100 bootstrap experiments on the concatenated sequences to assess the robustness of the topology. To root the genus phylogenetic tree, we used the genomes of species distant from each other and as close to *Acinetobacter* as possible. They were identified using a phylogenetic tree built with the 16S rDNA sequences of all the species of  $\gamma$ -proteobacteria with complete genomes in National Center for Biotechnology Information RefSeq (February 2013). The selection of a single strain and a single 16S copy per species resulted in 189 16S rDNA sequences that were aligned using MAFFT v7.1 (Kato and Toh 2008). Poorly aligned regions were removed with Gblocks 0.91 b (default parameters) (Castresana 2000). The phylogenetic tree was inferred using PhyML v3.0 under the Hasegawa–Kishino–Yano model and a gamma correction for variable evolutionary rates with eight classes (Gascuel et al. 2010). This tree highlighted *Moraxella catarrhalis* (GenBank ID NC\_014147) and *Psychrobacter* species (GenBank ID NC\_009524) as the two species closest to the *Acinetobacter* genus in our data set. We therefore reconstructed a core-genome of the genus plus these two species (same method as above). This core-genome included 677 protein families (supplementary table S3, Supplementary Material online) and was used to build a tree of the 135 complete genomes using the method mentioned above for the genus.

The reference phylogenetic tree of *A. baumannii* was reconstructed from the concatenated alignments of the

1,590 protein families of the core-genome obtained with muscle v3.8 (default parameters). As at this evolutionary distance the DNA sequences provide more phylogenetic signal than protein sequences, we back-translated the alignments to DNA, as is standard usage. Poorly aligned positions were removed with BMGE. The tree was inferred with FastTree v1.4 under the general time reversible model and a gamma correction for variable evolutionary rates with eight classes. We performed 100 bootstrap experiments on the concatenated sequences to assess the robustness of the topology. To root the species tree, we used two closely related strains in the genus: *A. nosocomialis* NIPH 2119<sup>T</sup> and *A. pittii*-like ANC 4052, rebuilt the core-genome of the species including these two outgroups and performed a similar analysis on this data set.

### Recombination and Population Genetic Analyses

We analyzed recombination in *A. baumannii* with RDP version 4.24 with default parameters except that RDP3 was used with the option “internal references only” and Geneconv with a G-scale mismatch penalty of 3 (Martin et al. 2010). The Phi test was computed using Phi Pack with default parameters, except that we made 10,000 permutations (Bruen et al. 2006). Analysis with or without permutations revealed highly correlated *P* values (Spearman’s  $\rho=0.96$ ,  $P < 0.0001$ ). We used the results of the analysis with permutations and applied a sequential Bonferroni correction of the *P* values. The analyses with ClonalFrame (Didelot and Falush 2007) were carried out using default options (except for the options -x 50000 -y 50000) on 30 nonoverlapping sets of approximately 51 genes contiguous in terms of their position in the genome of *A. baumannii* AYE. Analysis of Tajima’s *D* and Fay and Wu *H* was carried out with DnaSP 5.10.1 (Librado and Rozas 2009), using a sliding window of 1,000 positions and a step of 250. Statistical tests were performed on the average of the values on nonoverlapping windows. Analysis of *dN* and *dS* was carried out on the concatenate of the alignments of the genes of the core-genome using codeML from the PAML package version 4.4 (runmode=-2, model=2, CodonFreq=2) (Yang 2007).

### Evolutionary Distances

For each pair of genomes, we computed a number of measures of similarity: 1) The phylogenetic distance was computed from the length of branches in the genus phylogenetic tree using the *cophenetic* function in APE package from R (Paradis et al. 2004), 2) the gene repertoire relatedness (GRR) was computed as the number of homologs shared by the two genomes divided by the number of genes in the smallest genome (Snel et al. 1999), 3) the ANI was computed using Jspecies (Richter and Rossello-Mora 2009), and 4) the spacer repertoire relatedness was computed as the number of similar spacers shared by the two genomes divided by the number of

spacers in the smallest clustered regularly interspaced short palindromic repeats (CRISPR)-arrays of the two genomes.

### Identification of Specific Genes/Systems

CRISPR-arrays were identified with the CRISPR Recognition Tool using default parameters (Bland et al. 2007). The clusters of *cas* genes were identified as in Touchon and Rocha (2010) using the classification recently proposed (Makarova et al. 2011). Protospacers were identified using BLASTN to search for similarities between CRISPR spacer sequences and all the phage (831) and plasmid (3,861) genomes available in GenBank (default settings, *e* value  $< 10^{-5}$ ). We retained the matches showing  $\geq 90\%$  of identity and  $\leq 10\%$  difference in sequence length with the query. Prophages were detected with Phage Finder v.2.1 (Fouts 2006), discarding prophages less than 18 kb long. Loci encoding conjugative or mobilizable elements were identified with CONJscan (default parameters) (Guglielmini et al. 2011, 2014). Integrases were searched with the PFAM profile PF00589 for tyrosine recombinases and the pair PF00239 and PF07508 for Serine recombinases. The tyrosine recombinases of integrons were identified from the integrases using a protein profile for a region specific to these proteins that we built based on published data (Cambray et al. 2011). Error-prone polymerases were searched for with PFAM profiles: PF00136 (Pol2 like PolB), PF00817 (Y-Polymerases like UmuC, DinP, or ImuB), PF07733 (DnaE2), and PF00717 (UmuD). The cassettes *imuABC* were searched with the pair of profiles PF00817–PF07733 and *umuCD* using PF00817–PF00717 (Galhardo et al. 2005; Norton et al. 2013). All the protein profiles were searched with hmmer 3.0 with default parameters (Eddy 2011). We removed from further analysis the hits with low *e* values ( $e > 0.001$ ) or those for which the alignment matched less than half of the protein profile.

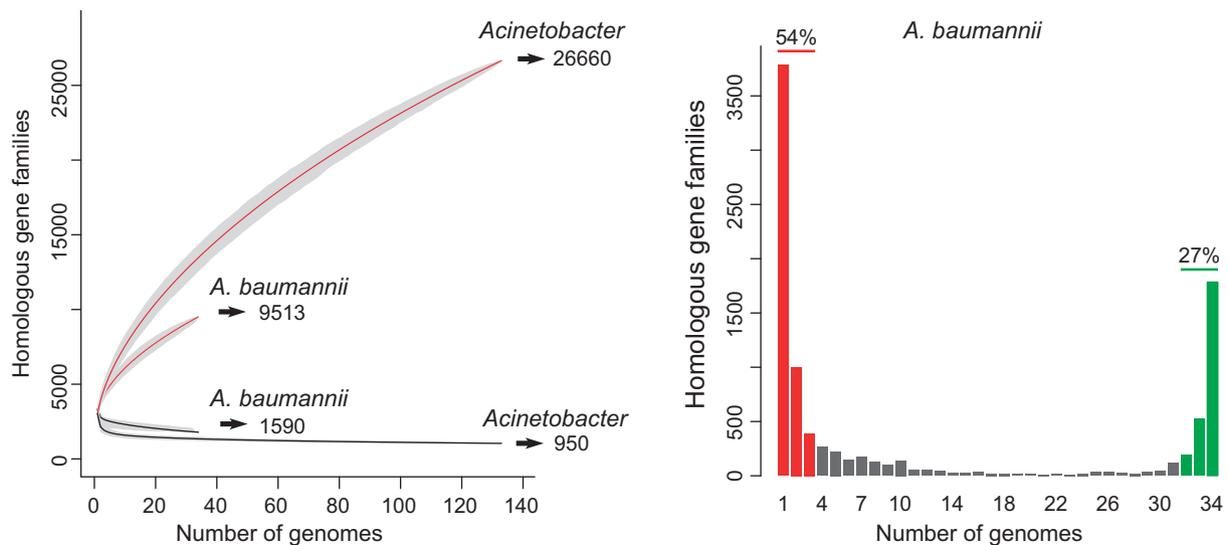
### Identification of Integration/Deletion Hotspots

The *A. baumannii* core-genome was used to identify and locate large integration/deletion (indel) regions. All regions including more than ten genes between two consecutive core genes of the species were considered as large indel regions. The relative positions of these regions were defined by the order of the core genes in *A. baumannii* AYE. This strain was used as a reference to order *A. baumannii* genes, because it represents the most likely configuration of the chromosome in the ancestor of the species. Regions located between two nonsuccessive core genes, that is, with rearrangements in between them were removed.

## Results and Discussion

### Genetic Diversity of the Genus

We analyzed a panel of 133 genomes of *Acinetobacter* spp. covering the breadth of the known taxonomic diversity of the



**FIG. 1.**—Core- and pan-genomes of the genus and of *A. baumannii* (left) and spectrum of frequencies for *A. baumannii* gene repertoires (right). The pan- and core-genomes were used to perform gene accumulation curves using the statistical software R (R Core Team 2014). These curves describe the number of new genes (pan-genome) and genes in common (core-genome) obtained by adding a new genome to a previous set. The procedure was repeated 1,000 times by randomly modifying the order of integration of genomes in the analysis. The spectrum of frequencies (right) represents the number of genomes where the families of the pan-genome can be found, from 1 for strain-specific genes to 34 for core genes. Red indicates accessory genes and green the genes that are highly persistent in *A. baumannii*.

genus (supplementary table S1, Supplementary Material online). All validly named species were represented by the respective type strains. The provisionally designated genomic species or tentative novel species were represented by strains selected based on polyphasic analysis of multiple strains belonging to the given taxon. Additional strains of *A. baumannii* representing different genotypes were identified based on multilocus sequence typing (MLST) and other typing methods (Diancourt et al. 2010). In the case of clinically relevant species other than *A. baumannii*, one or two additional strains were included to study intraspecies variation. These strains were selected to differ from the type strains as much as possible in terms of relevant genotypic/phenotypic properties and origin (e.g., clinical vs. environmental). Some strains were added to study their taxonomic position. We sequenced 120 genomes with high coverage to which we added the complete sequences of the 13 genomes available from GenBank (see Materials and Methods and supplementary fig. S1, Supplementary Material online). The average genome in the data set had 13 scaffolds for 31 contigs and an average size of 3.87 Mb (range between 2.7 and 4.9 Mb). Genomic Guanine-Cytosine (GC) content showed little variation around the average value of 39.6%. The density of protein-coding sequences was found to be homogeneous between genomes at an average of 94%. To test the completeness of the incompletely assembled genomes, we carried out two tests. First, we merged the lists of essential genes reported in *Escherichia coli* (Baba et al. 2006) and *A. baylyi* (de Berardinis et al. 2008)

(including “double band” mutants, which may not be essential). A total of 414 of the resulting 533 putatively essential genes had homologs in all *Acinetobacter* genomes (78%). Only 38 of these genes were present in less than 121 and more than 9 genomes (7%), among which only one deemed essential in both *A. baylyi* and *E. coli* (*ligA*, absent in two genomes) (supplementary table S2, Supplementary Material online). Second, we compared the fully and partially assembled *A. baumannii* genomes in terms of genome size and number of genes. The two groups of genomes were indistinguishable on both accounts (both  $P > 0.5$ , Wilcoxon tests), suggesting that not fully assembled genomes miss very few genes. Hence, our collection provides a unique and comprehensive reference data set of high quality genomes of the genus *Acinetobacter*.

To quantify the diversity of the gene repertoires, we computed the set of ubiquitous genes (core-genome) and the set of different homologous gene families (pan-genome) in the genus and in the 34 genomes of *A. baumannii* (fig. 1, supplementary tables S3 and S4, Supplementary Material online). The *Acinetobacter* core-genome contained 950 orthologous protein families corresponding to 37% of the size of the smallest proteome (*A. nectaris* CIP 110549<sup>T</sup>) and more than twice the number of essential genes in *A. baylyi*. The *A. baumannii* core-genome had 1,590 orthologous protein families corresponding to 44% of the size of the smallest proteome of the species (i.e., *A. baumannii* AB307 0294). Gene rarefaction analyses showed that core-genomes vary

**Table 1**

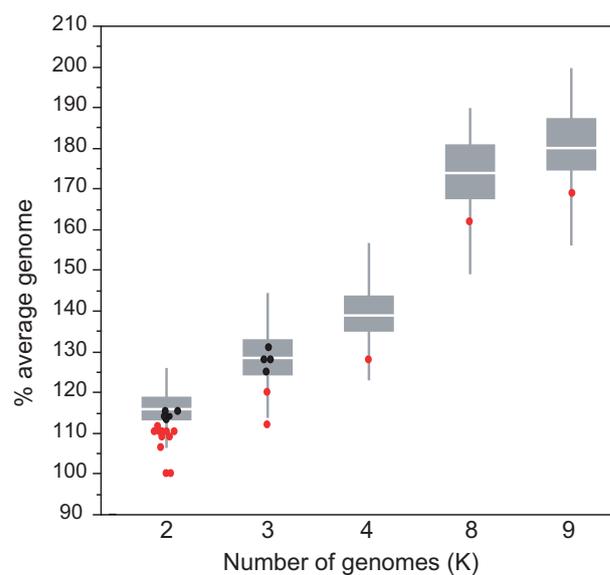
Core and Pan-Genomes for Species of the *Acinetobacter* Genus with At Least Three Sequenced Genomes (see [supplementary table S5](#), [Supplementary Material](#) online, for all species)

| Species                            | #  | Core-Genome |    | Pan-Genome |     |
|------------------------------------|----|-------------|----|------------|-----|
|                                    |    | Size        | %  | Size       | %   |
| <i>Acinetobacter calcoaceticus</i> | 4  | 2,951       | 81 | 4,677      | 128 |
| <i>Acinetobacter indicus</i>       | 3  | 2,340       | 79 | 3,309      | 112 |
| <i>Acinetobacter lwoffii</i>       | 9  | 2,161       | 66 | 5,557      | 169 |
| <i>Acinetobacter parvus</i>        | 8  | 1,810       | 64 | 4,576      | 162 |
| <i>Acinetobacter pittii</i>        | 3  | 2,926       | 81 | 4,282      | 120 |
| <i>Acinetobacter schindleri</i>    | 3  | 2,391       | 76 | 3,929      | 125 |
| <i>Acinetobacter ursingii</i>      | 3  | 2,458       | 72 | 4,353      | 128 |
| <i>Acinetobacter junii</i>         | 3  | 2,293       | 70 | 4,292      | 131 |
| Genomic sp. 13BJ/14TU              | 3  | 2,782       | 73 | 4,839      | 128 |
| Genomic sp. 16                     | 3  | 3,222       | 78 | 5,210      | 125 |
| <i>Acinetobacter baumannii</i>     | 34 | 1,590       | 42 | 10,849     | 288 |

NOTE.—For each species, the number of genomes (#), size of the core- and pan-genome, and percentage of the two relative to the size of the average genome in the clade are indicated.

little with the addition of the last genomes (fig. 1), suggesting that our estimate of the core-genome is robust. Both the pan-genomes of the *Acinetobacter* genus and of *A. baumannii* were very large with, respectively, 26,660 and 9,513 gene families. Gene rarefaction analyses showed that in both cases the addition of new genomes to the analysis still significantly increases the size of the pan-genome. This was confirmed by the spectrum of gene frequencies for the *A. baumannii* pan-genome (fig. 1), which showed that the vast majority of gene families were either encoded in a few genomes (54% in three or less) or in most of them (27% in more than 31 genomes). Over a third of the pan-genome (40%) corresponded to gene families observed in a single genome, that is, strain-specific genes. These analyses confirm that the genus and *A. baumannii* have an extremely large pan-genome. Furthermore, the shape of the accumulation curves showed that we are yet very far from having sampled it enough. Further work will therefore be necessary to characterize the genetic diversity of the species in the genus.

We took advantage of the possibility offered by our data set to compute the genetic variability of all the other validly names species or genomic species in the genus (table 1 for clades with at least three genomes and [supplementary table S5](#), [Supplementary Material](#) online, for all). With the exception of *A. baylyi*, *A. indicus*, and *A. brisouii*, for which the genomes are very similar, all genomes showed large variability of gene repertoires. The comparison of the size of the pan-genomes of these species with the distribution of random samplings of the pan-genome of *A. baumannii* with an equivalent number of genomes showed systematic larger values for the latter (fig. 2,  $P < 0.001$ , Binomial test). Hence, although

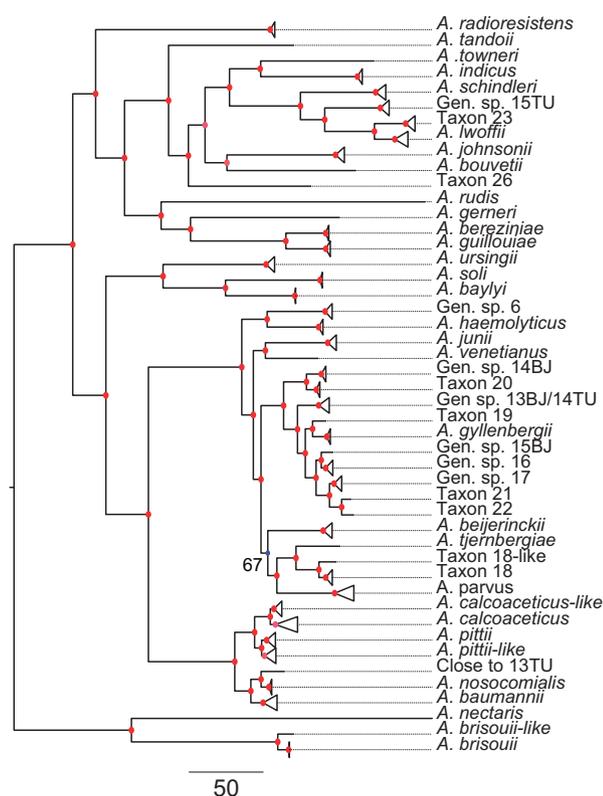


**Fig. 2.**—Comparisons of the pan-genome of *A. baumannii* computed with random samples of different size (boxplots) with those of the other species or genomic species. Each species, except *A. baumannii*, is only represented once, in the graph corresponding to the full number of available genomes for the taxa (e.g., nine genomes for *A. lwoffii*). The boxplots show the distribution of the size of the pan-genome of *A. baumannii* using random samples of  $K$  *A. baumannii* genomes ( $K = \{2, 3, 4, 8, 9\}$  genomes). Black dots correspond to pan-genomes of other species that are within the 25–75 percentiles of the distribution of the pan-genomes of *A. baumannii*, that is, these are pan-genomes approximately the size of *A. baumannii* given the same number of genomes. Red dots correspond to species with pan-genomes smaller than 75% of the *A. baumannii* pan-genomes (see [supplementary table S5](#), [Supplementary Material](#) online, for full data).

we have chosen the genomes outside *A. baumannii* to maximize known biochemical and ecological diversity within these species, these were found to be less diverse than *A. baumannii*.

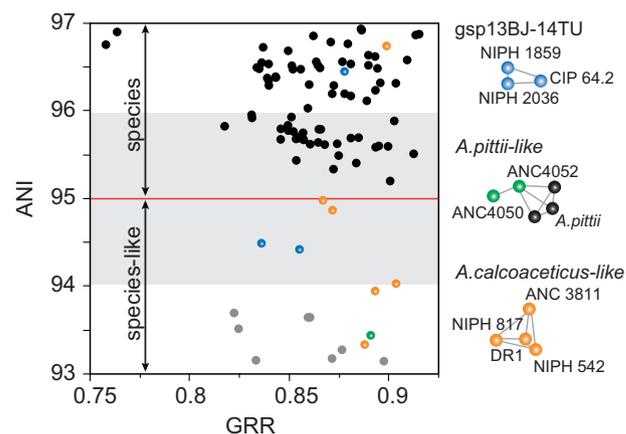
### Phylogeny and Systematics of the Genus

Despite recent progress, the understanding of the *Acinetobacter* evolution is incomplete at the phylogenetic and taxonomic levels. We used the 950 core protein families of the genus to build its phylogeny (see Materials and Methods). The resulting genus phylogenetic tree is extremely well supported from the statistical point of view, showing only one bifurcation with a bootstrap support lower than 95% (67%, see fig. 3). To root this tree and thus infer the order of evolutionary events in the genus, we used two genomes from the two most closely related genera for which complete genomes were available (*Moraxella* and *Psychrobacter*). This tree (topologically very similar to the previous one, see [supplementary fig. S2](#), [Supplementary Material](#) online),



**Fig. 3.**—Phylogeny of the *Acinetobacter* genus based on the alignment of the protein families of the core-genome (see Materials and Methods). Triangles mark groups of taxa that are from the same species or have more than 95% ANI values and therefore might be regarded as coming from the same species. The nodes in red have bootstrap supports higher than 95%. The tree was rooted using two outgroup genomes (see main text).

positions *A. brisouii* and *A. nectaris* as the taxa branching deeper in the genus, that is, the taxa most distantly related with the remaining *Acinetobacter* spp. Relative to the rest of the genus, the small (~3.2 Mb) genomes of *A. brisouii* showed average G+C content (41.5%). The genome of *A. nectaris* was among the smallest in size (2.9 Mb) and lowest in GC content (36.6%). The small size and extreme GC content of the genome and the very long terminal branch of *A. nectaris* in the phylogenetic tree suggest rapid evolution for this species. This feature is typical of bacteria enduring strong ecological niche contractions (Ochman and Moran 2001). Following this split, the phylogeny separates two very large groups of taxa: One including species such as *A. baumannii*, *A. parvus*, and *A. baylyi*; the other including *A. lwoffii*, *Acinetobacter johnsonii*, and *A. guillouiae*. Among these taxa, two are more isolated in the phylogenetic tree. *Acinetobacter radioresistens* is believed to be highly resistant to gamma-ray irradiation and might be the origin of the OXA-23 carbapenem resistance determinant in *A. baumannii* (Poirel et al. 2008; Perichon et al. 2014). It branched deep in

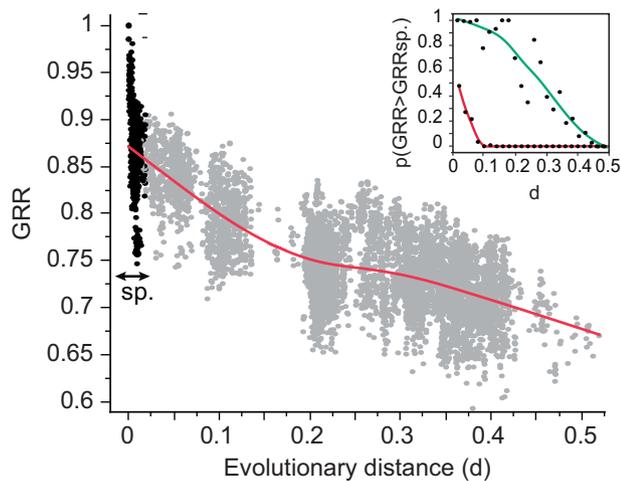


**Fig. 4.**—Analysis of the association between ANI and GRR (see Materials and Methods). The points in black correspond to the clades in triangles in figure 3. The points in gray correspond to comparisons between genomes that are closely related but not of the same species. We highlight three clades where some strains are closely related to the genomic species 13BJ-14TU, *A. pittii*, and *A. calcoaceticus*.

the tree and lacked closely related species (Nishimura et al. 1988; Poirel et al. 2008; Sahl et al. 2013). *Acinetobacter rudis* showed a long branch in the tree, even if its position is very well supported, suggesting higher evolutionary rates of this bacterium (isolated from raw milk and wastewater) (Vaz-Moreira et al. 2011).

To assess the age of the genus, we computed the average protein similarity of positional orthologs of the core-genome between the earlier branching species (*A. brisouii*) and *A. baumannii*. We did not use *A. nectaris* for this analysis because its long external branch would lead to an overestimate of the distances within the genus. The orthologs between *A. brisouii* and *A. baumannii* show an average sequence similarity of 80.1% (interval of confidence [IC], IC95%: 79.5–80.7%). As a matter of comparison, the same analysis between the orthologs of the core-genomes of *E. coli* and *Yersinia pestis*—placed in extreme opposites of the *Enterobacteriaceae* (after removing the fast-evolving *Buchnera* clade) (Williams et al. 2010)—shows an average protein similarity of 80% (IC95%: 79.3–80.7%). Hence, the genus of *Acinetobacter* is very ancient and its last common ancestor was close contemporary of the last common ancestor of *Enterobacteriaceae*. Accurate dating of bacterial genus is impossible given the lack of fossil records. Nevertheless, *Enterobacteriaceae* are thought to have diverged from the *Pasteurellaceae* over 500 Ma (Battistuzzi and Hedges 2009) and *Acinetobacter* might therefore be as ancient. The ancient history of the *Acinetobacter* genus contributes to explain its metabolic and ecological diversity.

The taxonomy of *Acinetobacter* still suffers from unclear taxonomic position and/or confusing nomenclature of some provisional species, high number of unidentifiable environmental strains (Nemec A and Krizova L, unpublished data),



**Fig. 5.**—Analysis of the association between GRR and the phylogenetic distance. Points in black indicate comparisons between pairs of genomes of the same species/genomic species (triangles in fig. 3) and points in gray indicate the other pairs. The red line is a spline fit of the data. The inset shows the relation between the evolutionary distance and the probability that comparing two genomes will result in a GRR value higher than the average within-species GRR (red) and higher than the minimal within-species GRR (green).

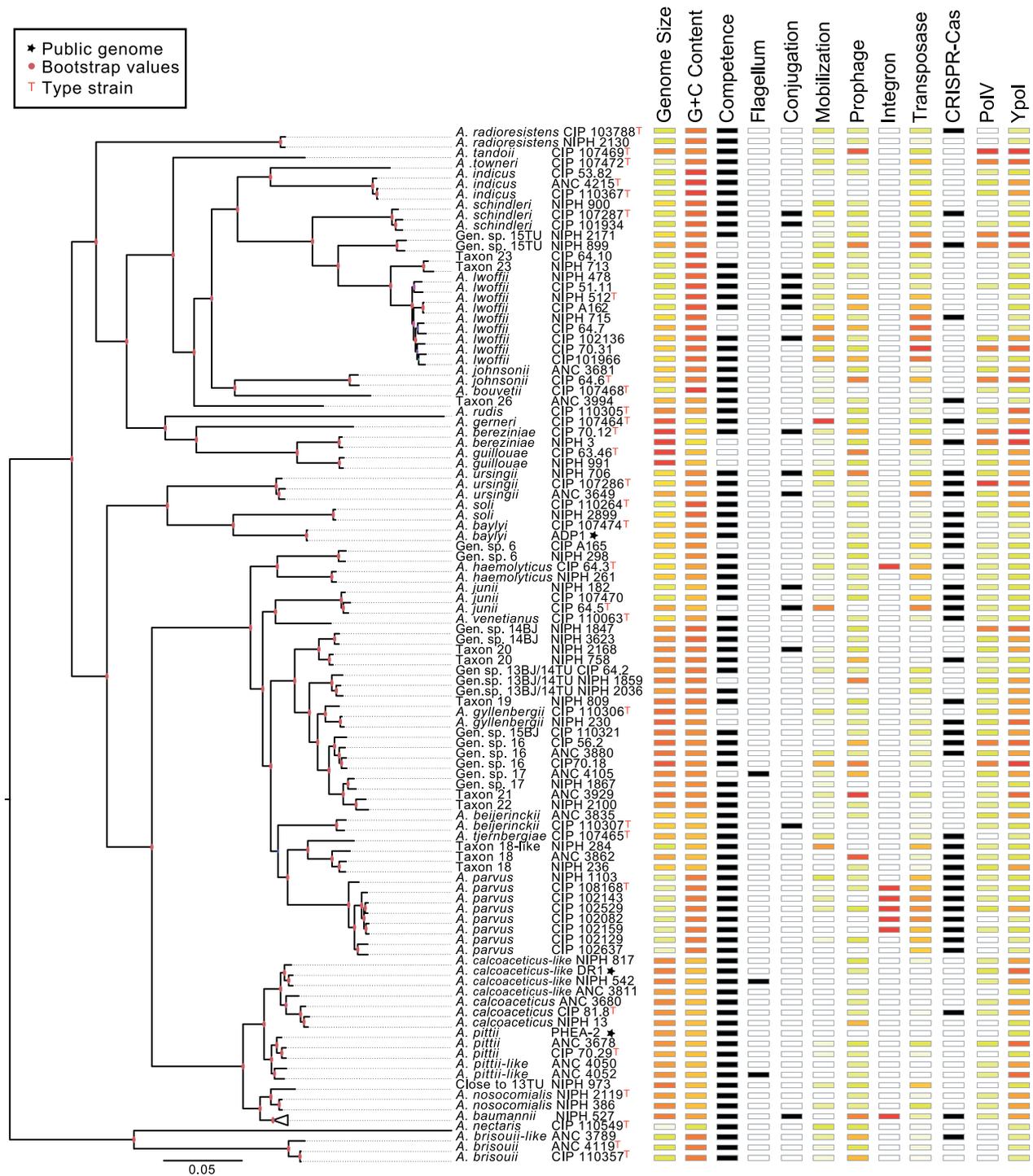
and a number of controversial interpretations of taxonomic data (Nemec et al. 2008, 2011; Vanechoutte et al. 2008). The availability of complete genomes and a robust phylogenetic history allow the identification of these taxonomical problems and the preliminary identification of taxa that might be regarded as good candidates for new species. We therefore put together the information on the core-genome tree and the ANI. Values of ANI between 94% and 96% have been proposed to be a good threshold for the definition of a bacterial species and to replace DDH measurements in preliminary identification of bacterial species from genome data (Konstantinidis et al. 2006; Richter and Rossello-Mora 2009). Most named species and genomic species in this analysis showed intraspecies ANI values higher than 95%. This is in close agreement with the idea that they represent bona fide species (fig. 4). ANI analysis also shed some light on the taxonomical status of several strains that were previously the subject of taxonomic controversies (see values in [supplementary table S1, Supplementary Material](#) online). First, high (>97%) ANI values unambiguously corroborated that *A. grimontii* CIP 107470<sup>T</sup> belongs to *A. junii* and “*Acinetobacter septicus*” ANC 3649 to *A. ursingii* as previously suggested based on DDH data (Nemec et al. 2008; Vanechoutte et al. 2008). On the other hand, strain CIP 64.10 which was believed to be derived from *A. lwoffii* NCTC 5866<sup>T</sup> (Bouvet and Grimont 1986) is clearly distinct from it (ANI of 88.3%). This finding explains previous controversial DDH results for these organisms (Tjernberg and Ursing

1989). Previous studies have also pointed out taxonomic problems with some closely related provisional species, notably among proteolytic and hemolytic strains (Bouvet and Jeanjean 1989). DDH values found by these authors for genomic sp. 15BJ and 16 are in agreement with the observed ANI reference strains of these species (92.6%), suggesting that the genetic distance between these taxa is lower but close to the thresholds underlying species definition. Genomic sp. 13BJ (Bouvet and Jeanjean 1989) and 14TU (Tjernberg and Ursing 1989) have also been considered as a single species based on DDH data, whereas their ANI values (~94.5%) are close to the threshold used to define a species. In such cases, rare in our data set, clear taxonomic conclusions will require analyses of biochemical and genetic data from more comprehensive sets of strains (to be published separately).

We studied the association between phylogenetic distance and the GRR (fig. 5). GRR was defined for each pair of genomes as the number of orthologs present in two genomes divided by the number of genes of the smallest genome (Snel et al. 1999) (see Materials and Methods). It is close to 100% if the gene repertoires are very similar (or one is a subset of the other) and lower otherwise. Consistent with the large pan-genome of most species in the genus, we observed highly variable gene repertoires for genomes within the same species (short phylogenetic distances). The most extreme differences were found when comparing the susceptible *A. baumannii* SDF strain with other strains of the same species. This strain endured a process of genome reduction concomitant with proliferation of insertion sequences (IS) (Vallenet et al. 2008). After its removal from the data set, the lowest within-species GRR (78%) was still found between *A. baumannii* strains (ATCC 17978 and ANC 4097), in line with our previous observation that this species is particularly diverse. As expected, pairs of genomes of the same species tended to have higher GRR than distantly related genomes. Some of the latter had only around 60% GRR. Yet, there were many exceptions to this average trend, and comparisons between distant genomes often showed higher GRR than comparisons between closely related strains of the same species (inset in fig. 5). For example, *A. baumannii* NIPH 146 and *Acinetobacter soli* CIP 110264 were very distant in the phylogenetic tree and have more than 82% GRR, which is more than many within-species comparisons. This shows the importance of sampling bacterial diversity using complete genome sequences and not just using MLST or core-genome-based analyses. In fact, given these patterns, some strains of distantly related *Acinetobacter* species might have more similar phenotypes than strains within the same species.

### Mechanisms of Genomic Diversification

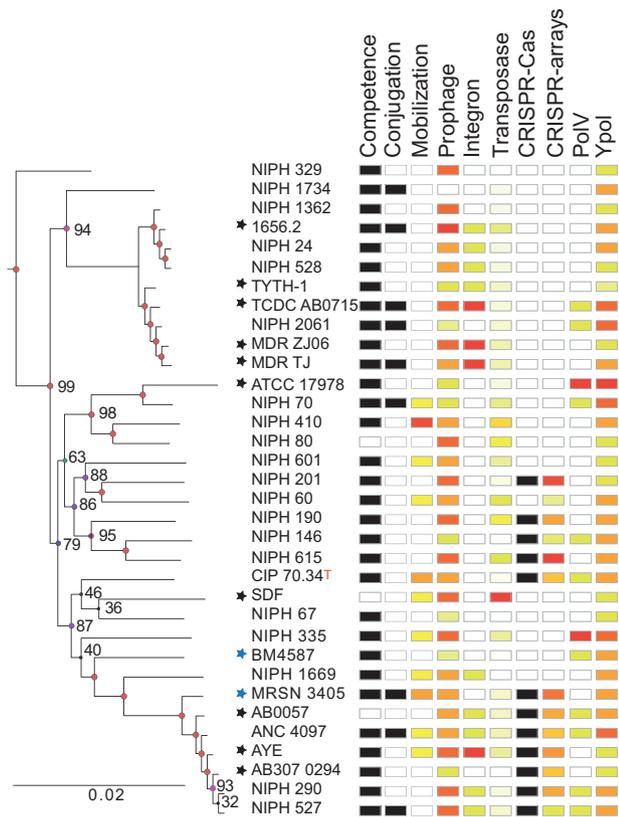
The emergence of antibiotic resistance genes in *Acinetobacter* is facilitated by conjugative elements (Goldstein et al. 1983),



**FIG. 6.**—Distribution of elements potentially related with genetic diversification in the genus. White indicates absence of the trait and black its presence. Genomes with many elements of a given type are indicated in red and those with few elements are indicated in yellow. Intermediate values are indicated in shades of orange. Black asterisks indicate complete genomes from GenBank.

integrons (Ploy et al. 2000; Huger et al. 2006), IS (Turton et al. 2006), and natural transformation (Wright et al. 2014). Accordingly, we searched for the genes associated with horizontal gene transfer or its control (figs. 6 and 7,

supplementary table S6, Supplementary Material online). We have found 23 proteins matching the profiles of tyrosine recombinases and the specific profiles for integrases of integrons. Interestingly, 17 of these were of the type *intI1*



**FIG. 7.**—Distribution of elements potentially related with genetic diversification in *A. baumannii*. White indicates absence of the trait and black its presence. Genomes with many elements of a given type are indicated in red and those with few elements are indicated in yellow. Intermediate values are indicated in shades of orange. Black asterisks indicate complete genomes from GenBank, blue asterisks indicate genomes sequenced at Pasteur Institute and at Walter Reed.

(associated with a 3'-conserved segment) and were found in 13 strains of *A. baumannii* (four strains had two copies). The abundance of integrons in this species was much higher than would be expected if it were random in the genus ( $P < 0.0001$ ,  $\chi^2$  test). The six hits for integrases of integrons in other species do not match *Int1*, *Int2*, or *Int3* and require further functional study. Analysis of the integron cassette contents in *A. baumannii* showed an *In0* structure, that is, no cassettes, in one strain (Bissonnette and Roy 1992), whereas the others contained two to five cassettes. We observed an atypical inverted organization where *sul1* was upstream from *int1* in *A. baumannii* 1656-2.

IS have also been implicated in antibiotic resistance. Most notably, *ISAbal* provides a promoter allowing expression of a downstream carbapenemase gene (Turton et al. 2006). IS are very diverse in type and abundance in the genus (from 0 to ~400 per genome), even when comparing closely related strains. The genomes of some species are particularly enriched in IS—*A. lwoffii*, *A. parvus*, *A. junii*, *A. ursingii*—and these

may have contributed to their reduced size. We found at least one copy of *ISAbal* (*IS4* family, group *IS10*) in 36% of all genomes and at least ten copies in 10%, suggesting an important role of this type of IS in the genus.

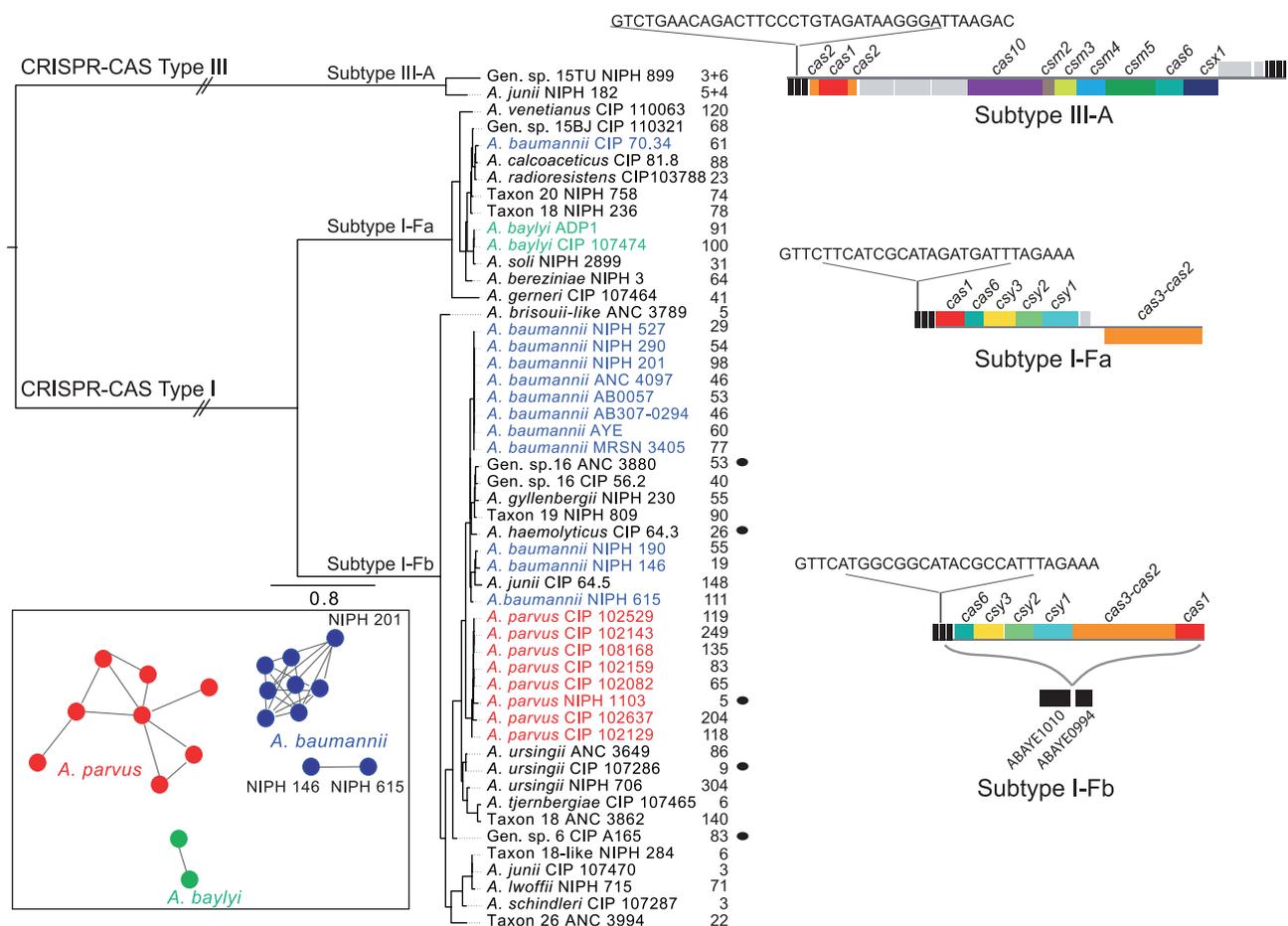
Although some *Acinetobacter* lytic phages have been studied for typing and phage therapy purposes (Bouvet et al. 1990; Ackermann et al. 1994; Shen et al. 2012), there is very little information in the literature on temperate phages infecting *Acinetobacter*. We found 260 prophages of dsDNA phages in the genomes of *Acinetobacter*. We have made a classification of these elements based on the available phages of gamma-proteobacteria (given the lack of information on *Acinetobacter* temperate phages) as in Bobay et al. (2013). More than 98% of the prophages were classified as Caudovirales, among which *Siphoviridae* (41%) and *Myoviridae* (37%) were by far the most abundant. The prophages accounted for a total of 10.4 Mb of genomic sequence in our data set, that is, an average of 2% of the genomes. Only 18 genomes lacked prophages. Hence, most *Acinetobacter* are lysogens. Among the 72 genomes with more than one prophage, *Acinetobacter* ANC 3929 stood out with six prophages (supplementary table S1, Supplementary Material online). Only a minority of these prophages (51) integrated next to tRNAs, as is common in other clades (Williams 2002), even if all identified phage integrases were tyrosine recombinases. The genes of these prophages will be studied in detail in a subsequent work. Nevertheless, as these data suggest an unsuspected role of transduction in driving horizontal gene transfer in the genus we have searched for putatively adaptive traits among prophages. Among other genes, we found one coding for a beta-lactamase in *A. baumannii* ANC 4097 and one coding for a chloramphenicol resistance protein in *A. baumannii* BM4587. Recent findings suggest that phages favor the horizontal transfer of antibiotic resistance determinants (Muniesa et al. 2013; Billard-Pomares et al. 2014). These results suggest that they may indeed contribute to antibiotic resistance in *Acinetobacter*.

Many conjugative elements have been described in association with the spread of antibiotic resistance genes between distant species (Doucet-Populaire et al. 1992; Juhas et al. 2008). We scanned genomes for genes encoding components of the conjugation machinery: Relaxases, coupling proteins, and type 4 secretion systems (T4SS) (Guglielmini et al. 2014). We identified 23 putative conjugation systems in the genus, of which 11 were classified as *MPF<sub>F</sub>* (family of the F plasmid), 4 *MPF<sub>I</sub>* (family of the R64 plasmid), and 8 *MPF<sub>T</sub>* (family of the Ti plasmid). As most genomes in our sample were not in a single contig, and breakpoints were typically found at mobile genetic elements, it is difficult to unambiguously distinguish integrative (ICE) from extrachromosomal (plasmids) conjugative elements. Yet, some information can be retrieved from the abundance of *MPF<sub>F</sub>* and *MPF<sub>I</sub>* types. These systems are much more frequently associated with plasmids than with ICE (Guglielmini et al. 2011) and they typically correspond to

narrow host-range mobile genetic elements (Encinas et al. 2014). Interestingly, the long flexible pili of these two families of elements endow them with the ability to engage in conjugation at high frequency in liquid (Bradley 1984). This suggests that liquid media may be relevant for the spread of genetic information in *Acinetobacter*. We identified 211 relaxases distant from any T4SS, mostly MOBQ (140) and MOBP1 (47) (Garcillan-Barcia et al. 2009), which presumably are part of elements mobilizable by conjugation in *trans*. Mobilizable elements are particularly abundant in the genome of *Acinetobacter generi* (16) and in certain strains of *A. junii* (up to 10 in a genome) and *A. lwoffii* (up to 9). They are less frequent in *A. baumannii* (between 0 and 3 per genome). Mobilizable plasmids are in general smaller than conjugative plasmids (Smillie et al. 2010) and it has been observed that *A. baumannii* plasmids tend to be small (Gerner-Smidt 1989; Fondi et al. 2010). Our observations suggest that mobilizable small elements may predominate over large

conjugative elements in the genus, a much stronger overrepresentation than in other prokaryotes (Smillie et al. 2010).

Competence for natural transformation has been described in a few strains of *A. baylyi* (Gerischer and Ornston 2001), *A. baumannii* (Harding et al. 2013; Wilharm et al. 2013), and *A. calcoaceticus* (Nielsen et al. 1997) but is thought to be rare in the genus (Towner 2006). In *A. baumannii*, competence and twitching motility are tightly linked and depend on the same type 4-pilus (T4P) (Harding et al. 2013; Wilharm et al. 2013). We searched for the 13 key T4P and competence-associated components and found most of them in all genomes. Only 16 genomes lacked one of the components, of which 13 lacked the *comP* gene that encodes a pilin (supplementary table S7, Supplementary Material online). These absences probably result from recent gene losses, as they are scattered in the phylogenetic tree of the genus. For example, *comP* is missing in only 3 of the 34 *A. baumannii* strains. It was suggested that *comP* was also absent from *A. baumannii*



**Fig. 8.**—Molecular phylogeny of the Cas1 protein across the genus. Phylogenetic tree for the Cas1 proteins was performed using PhyML with the WAG model and a Gamma correction. Cluster of cas genes organization, the most common repeat sequence, and the number of repeat sequences in each genome are indicated on the right part of the figure. Black circles indicate incomplete CRISPR-Cas systems. The left inset shows the genomes sharing spacers, each edge corresponds to the spacer repertoire relatedness (see Materials and Methods). Each color corresponds to a given species.

ATCC 17978 (Smith et al. 2007) but our reannotation procedure revealed a very good hit to the corresponding PFAM domain (profile coverage 99%,  $e$  value  $< 10^{-21}$ ). The very frequent specific deletion of *comP* is intriguing as it is one of the essential components of the natural transformation machinery in *A. baylyi* (Porstendorfer et al. 2000). It is tempting to speculate that the important antigenic potential of pilins (Nassif et al. 1993; Miller et al. 2014) might frequently favor selection for *comP* loss in host-associated bacteria. The conservation of the entire transformation machinery in the vast majority of the genomes suggests that most bacteria in the genus are naturally transformable under certain conditions. Further work will be required to understand the conditions leading to the expression of this trait.

CRISPR, together with associated sequences (*cas* genes and Cas proteins), form the CRISPR-Cas adaptive immune system against transmissible genetic elements such as plasmids and viruses (Sorek et al. 2013; Barrangou and Marraffini 2014). Fifty-one of the genomes encoded CRISPR-Cas systems (fig. 8). A type III-A system was associated with a cluster of 18 genes and a 37-bp repeat sequence and was present in only two distant strains (*A. junii* NIPH 182 and genomic sp. *Acinetobacter* 15TU NIPH 899). In both cases, the CRISPR-arrays located at each side of the *cas* gene clusters were very small (fig. 8). These traits, strain-specific small CRISPR-arrays found in few strains, suggest that the type III-A system has been recently acquired and/or accumulates few spacers. Most CRISPR-Cas systems in the genus were of type I-F. They included 37% of all genomes. The *cas* operon was composed of six to seven genes and the CRISPR repeat was 28 nt long. Based on the phylogenetic tree of Cas1 and the organization of the cluster of *cas* genes, we identified two I-F subtypes (I-Fa and I-Fb) that correspond to the subtypes identified in *A. baumannii* strains ADP and AYE, respectively (Hauck et al. 2012). Interestingly, the I-Fb subtype is probably very ancient as it was integrated at the same genomic locus in very distant species, for example, *A. parvus*, *A. junii*, *A. ursingii*. This CRISPR-Cas system contained some very large CRISPR-arrays in some genomes, up to 304 repeats, with highly conserved repeat sequences and highly variable spacers. This suggests the existence of a strong selective pressure on the activity of CRISPR-Cas systems of this type.

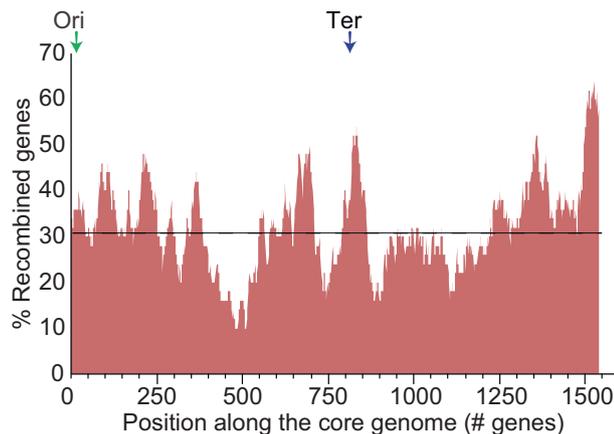
We identified approximately 3,000 spacers in CRISPR-arrays, most of which are unique (80%), that is, they are strain-specific. The vast majority of these spacers (88%) do not match other sequences in the *Acinetobacter* genomes. We found very few genomes having similar spacers and most of these cases corresponded to genomes of the same species among *A. baumannii*, *A. parvus*, or *A. baylyi* (fig. 8). Most spacers matched genes of unknown function, but some matched phage-related functions. As prophages and integrative elements are hard to delimit precisely, we searched for similarity between the spacers and the 831 complete phage and 3,861 complete plasmid genomes available in GenBank.

Only 2% of the spacers showed sequence similarity with elements of this data set. This is not surprising, given the paucity of *Acinetobacter* phages in GenBank. Notwithstanding, we identified ten spacers that match bacteriophages infecting *Acinetobacter* species (Bphi-B1251, AP22 phage), and 47 matching *Acinetobacter* plasmids (e.g., pABTJ1, pNDM-BJ0, pNDM-BJ02). Interestingly, among the few spacers matching known genes, we found homologs of VirB4, VirB5, VirB8, and resolvase proteins, which are all key components of the conjugation machinery. Nevertheless, we found no significant statistical association between the presence of the CRISPR-Cas system (or the number of repeat sequences) and the number of prophages, mobilizable, and conjugative elements in the genus (all  $P > 0.1$  Spearman's rho associations). The same negative results were obtained when the analysis was restricted to *A. baumannii*. The variability of CRISPR spacers might be used to type certain species, but only in combination with other markers, as many strains are devoid of such systems. Some of the CRISPR-arrays we have identified are among the largest ever found among bacteria. CRISPR-Cas systems are therefore likely to have an important role in the genome dynamics of the genus and in particular in controlling the transfer of conjugative elements.

Point mutations also account for the emergence of new traits in *Acinetobacter*, including antibiotic resistance (Yoon et al. 2013). The dynamics of adaptation by point mutations is accelerated when bacteria endure hypermutagenesis, for example following the implication of error-prone DNA polymerases in replicating damaged DNA (Tenaillon et al. 2004). The SOS-response of *A. baumannii* does not involve LexA, the typical key regulator of this response (Robinson et al. 2010). Accordingly, we searched and found no ortholog of LexA in the genus. It has been suggested that error-prone polymerases, which have multiple homologs in certain genomes, facilitate the rapid emergence of antibiotic resistance in *Acinetobacter* spp. by stress-induced mutagenesis (Norton et al. 2013). We screened the genomes for homologs of PolB and Y-polymerases and found no homolog of PolB, nor of the *imuABC* operon, which is implicated in damage-induced mutagenesis (Galhardo et al. 2005). In contrast, we identified 345 Y-polymerases in the genus, that is, an average of almost three polymerases per genome (fig. 6). No single genome lacked Y-polymerases and certain harbored up to five copies of the gene. The pair *umuCD* (encoding PolV in *E. coli*) was present in nearly all genomes, often in multiple copies. The multiplicity of genes encoding Y-polymerases in these genomes is intriguing and suggests that they play important roles in *Acinetobacter*, for example, in acquiring tolerance to toxins and antibiotics and/or in their genetic diversification.

#### Origin and Diversification of *A. baumannii*

The large pan-genome of *A. baumannii* showed that this species has highly diverse gene repertoires suggestive of frequent

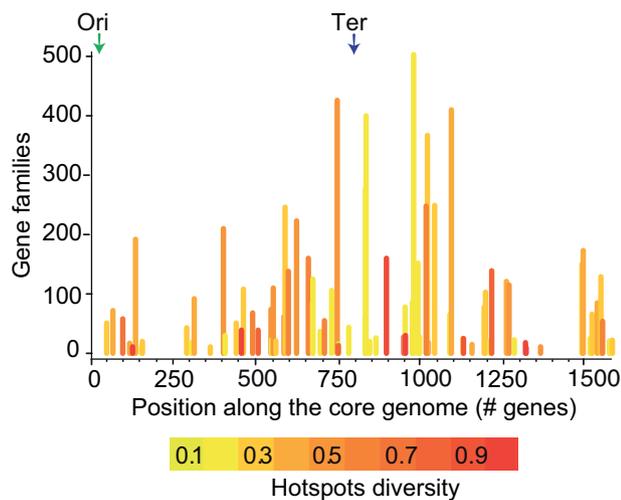


**Fig. 9.**—Distribution of genes of the core-genome of *A. baumannii* presenting significant evidence of recombination using Phi ( $P < 0.05$  after sequential Bonferroni correction) computed in sliding windows of 50 core genes. The dashed line indicates the average.

horizontal gene transfer (fig. 1). Genetic diversification can also result from allelic exchange by homologous recombination in the core-genome. We estimated the impact of this type of recombination in *A. baumannii* with Phi, a conservative and robust method to detect recombination (Bruen et al. 2006). We found that 32% of the core gene families are significantly affected by recombination ( $P < 0.05$ ) (see Materials and Methods). To quantify the number and size of recombination tracts, we concatenated the multiple alignments following the order of the *A. baumannii* ATCC 17978 strain and fetched 688 recombination events significantly highlighted by three procedures (RDP3, CH12, and GENECONV, see Materials and Methods). We were able to precisely delimit the tracts for 526 events of recombination. Their size averaged 2.1 kb (95% of the tracts were between 367 bp and 16 kb long). This size is an underestimate because of the presence of sequences separating core genes and because multiple events of recombination lead to shorter tracts. We also confirmed the presence of recombination using ClonalFrame (Didelot and Falush 2007). This program estimated that recombination contributed to the observed polymorphisms more than mutations (1.37 times). This value is very close to the one observed for MLST data (1.3) (Diancourt et al. 2010). Homologous recombination near the origin of replication was recently associated with the diversification of three outbreak strains of *A. baumannii* (Snitkin et al. 2011). We therefore quantified the distribution of recombination rates along the chromosome of *A. baumannii*. The highest density of recombining genes among the 34 genomes was indeed found close to the origin of replication, but only on the counterclockwise sense (end of the published sequence). Several other regions showed high frequency of recombination whereas others were nearly clonal (fig. 9). These results showed that a large fraction of the genes in *A. baumannii* are significantly affected by recombination,

that rates of recombination vary along the chromosome, and that recombination tracts tend to be small.

*Acinetobacter baumannii* has become a significant clinical problem in the 1970s (Bergogne-Berezin and Towner 1996), but whether this reflects adaptation of a small number of clones to hospital environments or population expansion is not known. The presence of short internal nodes close to the last common ancestor of the species and its large pan-genome have led to suggestions that *A. baumannii* might have endured one wave of population expansion during the diversification of the species and another very recently after the introduction of antibiotics at the hospital (Diancourt et al. 2010; Antunes et al. 2013). The assessment of the hypothesis for a recent population expansion will require a larger sample of closely related genomes. To test the hypothesis of an ancient population expansion, we computed Tajima's  $D$  in sliding windows along the genome of *A. baumannii* (see Materials and Methods) (Tajima 1989). We observed systematically negative values of  $D$  (average  $D = -0.50$ ,  $P < 0.001$ , Wilcoxon signed-rank test). Tajima's  $D$  is affected by recombination (Thornton 2005), but purging the alignments of genes for which Phi identified significant evidence of recombination resulted in even more negative values (average  $D = -0.9$ ,  $P < 0.001$ , same test). Negative  $D$  is consistent with population expansion and/or purifying selection. To separate between these two possibilities, we analyzed separately 4-fold degenerate synonymous ( $D_4$ ) and strictly nonsynonymous ( $D_0$ ) positions (supplementary fig. S3, Supplementary Material online). The two measures are equally affected by sampling biases, recombination, and population expansion. Differences between  $D_4$  and  $D_0$  pinpoint selective processes because nonsynonymous changes are much more deeply imprinted by natural selection than synonymous ones. The  $D_0$  values are significantly lower than those of  $D_4$  (resp. average  $D_0 = -1.5$  and  $D_4 = -0.4$ , difference significant  $P < 0.001$  Wilcoxon signed-rank test) even if both are significantly negative ( $P < 0.001$ ). This suggests that negative values of Tajima's  $D$  are driven by selection against nonsynonymous substitutions, a clear sign of purifying selection. To further test this conclusion, we measured the ratio of nonsynonymous and synonymous substitutions ( $dN/dS$ ). The average within-species  $dN/dS$  was only 0.05 ( $P < 0.001$ ) and even very closely related strains ( $dS < 0.001$ ) showed  $dN/dS$  lower than 0.2 (supplementary fig. S4, Supplementary Material online). This confirms that natural selection purges the vast majority of nonsynonymous mutations in the genome (Rocha et al. 2006). We then computed Fay and Wu  $H_4$  at 4-fold degenerate positions ( $H_4$ ) (Fay and Wu 2000). We found very low  $H_4$  average values ( $-51$ ,  $P < 0.001$ ). Negative  $H_4$  is an indication of selective sweeps or ancient population bottlenecks and negative  $D_4$  suggests population expansion. These results are thus consistent with the hypothesis of a population bottleneck in *A. baumannii* in the early stages of speciation



**Fig. 10.**—Distribution of integration/deletion hotspots along the core genome of *A. baumannii* using gene orders of *A. baumannii* AYE strain as a reference (see Materials and Methods). The bars represent the number of different gene families in all the genomes found between two consecutive genes of the core-genome. The colors represent the diversity of these gene families, that is, the number of gene families divided by the number of genes found between two consecutive genes of the core-genome. If the number of genes is identical to the number of gene families (1, maximal diversity), then every genome has a different set of genes in the hotspot indicating many different insertions in the region. If the number of families equals the number of genes per genome (close to 1/33, minimal diversity), then most genomes have the same genes in the hotspot. This last scenario typically corresponds to strain-specific large deletions.

with subsequent population expansion under a regime dominated by purifying selection.

Acquisition of resistance often results from the transfer of a mobile element encoding several resistance genes. For example, the AYE multiresistant strain has a genomic island (AbaR1) containing 45 resistance genes including numerous determinants of antibiotic resistance (Fournier et al. 2006). This island was probably acquired in multiple steps of accretion and deletion of genetic material (Sahl et al. 2011). We studied the general patterns of integration of horizontally acquired genes in *A. baumannii* to quantify how many regions in the genome were integration/deletion hotspots. We identified 1,083 regions in the genomes that were flanked by two consecutive core genes and included more than ten genes inserted or deleted (indel) in at least one genome (fig. 10). These loci were not distributed randomly. Instead, the 1,083 regions with indels occurred at the same 78 hotspot regions (5% of all possible loci), that is, they were flanked by the same 78 pairs of core gene families. A third of these loci corresponded to a single indel in one single genome, typically a strain-specific deletion (indicated by light colors and a large number of families with low diversity in fig. 10). Other loci included many different protein families in different genomes. These corresponded to hotspots that endured multiple

integrations/deletions in different lineages. Hotspots tended to be concentrated closer to the terminus of replication and symmetrically distributed around this position. This tendency has previously been observed in other species (Bobay et al. 2013) and might result from a compromise between selection for genome plasticity and organization (Rocha 2004). Intriguingly, some large regions of the chromosome showed no signs of genome plasticity suggesting that they are less plastic (fig. 10). The 78 hotspots contain 5,203 families, that is, 5% of locations in the genome accumulated 66% of the accessory genome. Hence, most genetic diversification takes place at very few loci in the genome. Querying these regions might be an efficient means of typing *Acinetobacter* strains for specific genotypes. Understanding the mechanisms leading to hotspots should enlighten how new genetic information is accommodated in the genome of *A. baumannii*.

## Conclusions

We have proceeded to an extensive characterization of the molecular and evolutionary mechanisms driving the genetic diversification of *Acinetobacter*. Interestingly, we observed that temperate phages are much more abundant than conjugative elements, even though their role as vectors for horizontal transfer has been neglected in the past. Accordingly, we observed the presence of very complex and fast-evolving CRISPR-Cas systems in the genomes of *Acinetobacter*. Population genetic analyses are consistent with the notion that *A. baumannii* arose from an ancient population bottleneck. Nevertheless, this species is extremely diverse in terms of gene repertoires and shows strong effects of natural selection on protein evolution.

Our study sets a solid basis for the understanding of the evolution of the *Acinetobacter* genus. Further work will be necessary to understand how genetic diversification leads to the key features of the genus, notably high metabolic diversity, antibiotic resistance, and virulence. The confrontation between the genetic and the phenotypic data should facilitate predicting how multiple pathogens rise within a genus by virtue of their genetic backgrounds and genetic plasticity.

## Supplementary Material

Supplementary tables S1–S7 and figures S1–S4 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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