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Whole-Genome Pyrosequencing of an Epidemic Multidrug-Resistant *Acinetobacter baumannii* Strain Belonging to the European Clone II Group▽†

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The whole-genome sequence of an epidemic, multidrug-resistant *Acinetobacter baumannii* strain (strain ACICU) belonging to the European clone II group and carrying the plasmid-mediated bla_{OXA-58} carbapenem resistance gene was determined. The *A. baumannii* ACICU genome was compared with the genomes of *A. baumannii* ATCC 17978 and *Acinetobacter baylyi* ADP1, with the aim of identifying novel genes related to virulence and drug resistance. *A. baumannii* ACICU has a single chromosome of 3,904,116 bp (which is predicted to contain 3,758 genes) and two plasmids, pACICU1 and pACICU2, of 28,279 and 64,366 bp, respectively. Genome comparison showed 86.4% synteny with *A. baumannii* ATCC 17978 and 14.8% synteny with *A. baylyi* ADP1. A conspicuous number of transporters belonging to different superfamilies was predicted for *A. baumannii* ACICU. The relative number of transporters was much higher in ACICU than in ATCC 17978 and ADP1 (76.2, 57.2, and 62.5 transporters per Mb of genome, respectively). An antibiotic resistance island, AbaR2, was identified in ACICU and had plausibly evolved by reductive evolution from the AbaR1 island previously described in multiresistant strain *A. baumannii* AYE. Moreover, 36 putative alien islands (pAs) were detected in the ACICU genome; 24 of these had previously been described in the ATCC 17978 genome, 4 are proposed here for the first time and are present in both ATCC 17978 and ACICU, and 8 are unique to the ACICU genome. Fifteen of the pAs in the ACICU genome encode genes related to drug resistance, including membrane transporters and ex novo acquired resistance genes. These findings provide novel insight into the genetic basis of *A. baumannii* resistance.

Acinetobacter baumannii has emerged as an important opportunistic pathogen worldwide (15). It is responsible for large outbreaks of nosocomial infection which account for 2 to 10% of all infections caused by gram-negative bacteria, with the highest incidence occurring in intensive care units (ICUs) (17, 18). *A. baumannii* infections include nosocomial pneumonia, secondary meningitis, skin, soft tissue, and urinary tract infections, and bacteremia and result in high rates of morbidity and mortality (9, 13). *A. baumannii* has simple growth requirements and exploits a variety of nutritional sources, and it is adaptable to a range of temperatures, pHs, salinities, and levels of humidity (4). This contributes to the fitness of *A. baumannii* in the hospital, which represents the main reservoir of the bacterium. The rates of recovery of *A. baumannii* from natural environments and its incidence in the community are low, while its rate of carriage by hospitalized patients is high and its occurrence in the hospital setting is frequent (15).

Resistance to antimicrobial agents is the main advantage of *A. baumannii* in the nosocomial environment (15). Multidrug-resistant clones of *A. baumannii* are emerging and spreading throughout many geographic areas (10, 36, 40). The emergence of two pan-European epidemic clones, referred to as European clones I and II, was reported in northwestern Europe in the 1980s (15, 28), and since then these clones have been documented in many regions of Europe (8, 39). These epidemic lineages are endowed with resistance to a broad range of antibiotics, although individual strains can display different types and combinations of resistance determinants. The treatment options for multidrug-resistant *A. baumannii* infections are generally limited to carbapenems, but the emergence of imipenem and meropenem resistance is a growing source of concern (32). Recently, the OXA-58 carbapenemase has been shown to be involved in carbapenem resistance in members of the European clone II group (29).

The pathogenicity of *A. baumannii* is poorly understood, as this organism is commonly regarded as a low-virulence bacterium, despite the severity of the infections that it causes (15). Recently, novel insights into *A. baumannii* virulence have been gained from whole-genome analysis of strain ATCC 17978. About 17% of its ca. 4-Mb genome consists of 28 putative alien islands (pAs) acquired by horizontal gene transfer (HGT); and 20 of them harbor potential virulence genes involved in protein secretion, drug resistance, pilus formation, and cell wall bio-

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genesis (35). These pAs were described as large chromosome portions that had little homology with the *Acinetobacter baylyi* ADP1 genome (1) or that showed anomalies in sequence composition and codon usage. Some of them were confirmed to be important for the fitness of ATCC 17978 by insertional mutagenesis and virulence assays with the *Caenorhabditis elegans* and *Dictyostelium discoideum* infection models (35). Moreover, comparative genomics revealed the presence in the European clone I AYE strain of an 86-kb resistance island (AbaR1) carrying 45 resistance genes acquired by HGT from other gram-negative species (30). AbaR1 is inserted into an ATPase gene that is in an "empty" state in antibiotic-susceptible strain *A. baumannii* SDF. This highlights the exceptional ability of *A. baumannii* to gather and assemble foreign genes.

In this study, the genome of epidemic multidrug-resistant strain *A. baumannii* ACICU, representative of a clone that caused an outbreak in Rome in 2005 (21, 22), has been entirely sequenced. This clone belongs to the European clone II group and carries the plasmid-mediated bla_{OXA-58} gene (37). Since *A. baumannii* ATCC 17978 was isolated in the early 1950s, prior to the development of macrolides, glycopeptides, cephalosporins, and the latest beta-lactams (2), we compared the ACICU genome with the genomes of *A. baumannii* ATCC 17978 and *A. baylyi* ADP1 with the aim of identifying novel traits related to the drug resistance, virulence, and pathogenesis of *A. baumannii* European clone II.

MATERIALS AND METHODS

Bacterial strains. *A. baumannii* ACICU (referred to as isolate H34) was described elsewhere (21, 22). *A. baumannii* ACICU is characterized by resistance to ampicillin, piperacillin, piperacillin-tazobactam, ticarcillin, ticarcillin-clavulanic acid, aztreonam, cefepime, ceftazidime, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, amikacin, gentamicin, tobramycin, imipenem, and meropenem (21). This strain was assigned to molecular group 1 by the multiplex PCR-based method described by Turton et al. (37). *Escherichia coli* DH5α [ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Nal^r] was used as the host strain for the DNA cloning experiments. *E. coli* strain ATCC 25922 was used as the control for the double-disk synergy test (DDST). DDST was performed to assess the contribution of AmpC to ceftazidime resistance. Disks containing 30 μg of ceftazidime or 30 μg of cefotaxime were placed on a Mueller-Hinton agar plate on which the bacterial suspension had been spread, with a center-to-center distance of 18 mm to a 500-μg cloxacillin-containing disk or a 250-μg boronic acid-containing disk (Rosco Diagnostica A/S, Taastrup, Denmark), respectively. The plates were incubated at 37°C overnight (33, 41).

High-density pyrosequencing and sequence assembly of the *A. baumannii* ACICU genome. The complete sequencing work flow of the Genome Sequencer FLX system is described at www.454.com. In brief, preparation of the genomic DNA of strain ACICU was performed by using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI), according to the manufacturer's procedure. The genomic DNA preparation (3 to 5 μg) was fractionated into fragments ranging from 400 to 800 bp by nebulization and was ligated to adaptor A or B (4-base nonpalindromic sequences) by priming amplification and sequencing reactions. The fragments were also immobilized onto streptavidin beads by the biotin tag on adaptor B. Part of the DNA library was analyzed by the GS20 system, which produced sequences with an average length of 100 bp, while a second fraction was analyzed with the Genome Sequencer FLX system, which produced sequences of 200 to 250 bp. A total of 900,363 high-quality sequences and 96,454,548 bp were obtained with a 23-fold coverage of the genome. The 454 de Novo Assembler software was used to assemble the sequences that were read. This first automatic step produced 1,036 contigs with a maximum length of 200,179 bp.

Contigs were aligned by paired-end analysis (http://www.454.com), with 47 scaffolds obtained by semiautomatic assembly analysis with DNAStar Lasergene software (http://www.dnastar.com/products/lasergene.php). This approach permitted a manual check of every added sequence read to confirm the correct assembly.

To solve problems due to the assembly of repetitive DNA regions, short sequences that mapped into contigs carrying repeated regions were reassembled by manual rereading of the DNA sequences. If a single nucleotide variation was identified, the degenerated character defined in IUB code was used, while for larger variations (2 or more nucleotides), different contigs were created and their localization was validated by PCR. Since homopolymeric stretches into the genome can determine a high probability of frameshift error during the assembly of the sequence, potential errors were checked by visual inspection of the sequences read.

Seven contigs representing the whole genome were finally obtained and were interrupted at the 4.4-kb rRNA gene clusters. To complete the assembly of the contigs, a PCR strategy with 14 primer pairs designed on the basis of univocal sequences flanking each rRNA cluster was used. The use of all possible combinations of these primers and the seven contigs as DNA templates was attempted with the Elongase amplification system (Invitrogen, Milan, Italy). Seven amplicons were obtained and were sequenced with an ABI 3730 DNA sequencing instrument (Applied Biosystems, Monza, Italy). Adjacent contigs were then recognized and joined to obtain a full-length genome sequence.

To avoid hybrid contigs, plasmid DNA was analyzed independently from genomic DNA by using the same assembly protocols.

Genome annotation. Coding genes were identified by crossing the predictions from the FGENESB package (24) (http://www.softberry.com/), the GeneMark program (7), and the GLIMMER program (14). We considered an open reading frame (ORF) prediction to be good when it was identified by each of the three prediction tools. Discrepant ORFs were manually verified by the Artemis viewer (5) and by identification of putative ribosomal binding sites.

Each gene was functionally classified by assigning a cluster of orthologous group (COG) number (39) or a Kyoto encyclopedia of genes and genomes (KEGG) number (23), and each predicted protein was compared against every protein in the nonredundant (nr) protein databases (http://ncbi.nlm.nih.gov) (1). In order to associate a function with a predicted gene, we used a minimum cutoff of 30% identity and 80% coverage of the gene length, checking at least two best hits among the COG, KEGG, and nonredundant protein databases.

The syntenies of the *A. baumannii* ACICU, *A. baumannii* ATCC 17978 (EMBL accession no. CP000521), and *A. baylyi* ADP1 (EMBL accession no. CR543861) genomes were investigated by the use of Mauve software (12).

The rRNA genes were identified by the FGENESB tool on the basis of sequence conservation, while tRNA genes were detected with the tRNAscan-SE program (23).

The strain ACICU and ATCC 17978 genomes were submitted to the http://www.membranetransport.org/ website to determine the presence of efflux systems and for the identification of transporter families, subfamilies, and substrates by the TransAAP (Transporter Automatic Annotation Pipeline) tool. The membrane transport systems in ACICU and ATCC 17978 were also compared with the systems previously identified by the TransAAP tool in different bacterial genomes (31). The entire list of transporters in ACICU and ATCC 17978 is available at the www.itb.cnr.it/genome-project website.

Insertion sequences (ISs) were identified by submission of the whole genome to the IS Finder website (http://www-is.biotoul.fr).

Genome comparison. The BLASTp algorithm was used to search for protein similarities. The criterion used to evaluate the deduced amino acid sequence synteny was >50% similarity at the amino acid level and >50% coverage of protein length. Coding DNA sequences (CDSs) not matching these criteria were considered absent in the comparison of the *A. baumannii* ATCC 17978 and ACICU genomes.

Small-scale and large-scale genomic variations were detected in the sequence of strain ACICU compared with the sequence of strain ATCC 17978. Small-scale variations are defined as genetic changes affecting individual genes or small clusters (less than five CDSs) and will not be discussed in detail. Large-scale variations were interpreted when changes involved clusters of at least five adjacent CDSs.

HGT analysis. The strain ACICU genome was analyzed with the extended PyPhy system (the SPyPhy system) (36; T. Sicheritz-Ponten, unpublished data) to make an automated wide primary screen of the genome for HGT, as described previously (36). Briefly, each protein sequence was compared to the sequences in the UniProt database by use of the BLASTp algorithm. All compatible sequences with >30% amino acid identity and >50% coverage of the protein length were aligned by using the ClustalW program. After filtering and alignment of the sequences, the GBLOCKS program (9) was used to remove poorly aligned and diverged positions (allowed gap positions, half; minimum length of a block, 2; maximum number of contiguous nonconserved positions, 20). Neighbor-join-

ing analysis with 100 bootstrap replicates was performed with the PAUP* program (38) for those alignments for which there was a sufficient number of homologs for tree construction. Only BLAST matches involving at least five consecutive CDSs were interpreted and considered potential candidates for HGT.

Plasmid analysis. Plasmid DNA pyrosequencing was performed by using template purified with a PureLink Hipure plasmid midiprep kit (Invitrogen). Contigs containing repetitive sequences were analyzed for colinearity by restriction fragment length polymorphism analsis. SacI- and SalI-restricted plasmids were separated on a 1% agarose gel, transferred onto positively charged nylon membranes (Roche Diagnostics, Monza, Italy), and hybridized with PCR-generated specific probes with a DIG DNA labeling and detection kit (Roche Diagnostics). To complete the sequence, a SacI library was obtained in the pZErO-2 vector (Invitrogen) and transformed in competent MAX Efficiency *E. coli* DH5α cells (Invitrogen). The transformants were selected on Luria-Bertani agar plates containing 1 mM isopropyl-β-D-thiogalactopyranoside and kanamycin (40 μg/ml) alone or in combination with ampicillin (20 μg/ml). The nucleotide sequences of the SacI-generated inserts were confirmed with universal and walking primers (see details and primer sequences at the www.itb.cnr.it/genome-project website) with the ABI 3730 DNA sequencing instrument (Applied Biosystems, Monza, Italy).

Nucleotide sequence accession numbers. The *A. baumannii* ACICU chromosome and plasmid sequences (pACICU1 and pACICU2) were submitted to the GenBank database and can be found under accession numbers CP000863, CP000864, and CP000865, respectively. The general properties of this genome are summarized in the supporting information available at the www.itb.cnr.it/genome-project website.

TABLE 1. General characteristics of the *A. baumannii* ACICU genome

Characteristic	Value
Chromosome	
Size (bp)	3,904,116
Coding regions (%)	84.87
G+C content (%)	39.03
No. of genes	3,758
No. of protein-coding sequences	3,670
No. of rRNA operons	8
No. of tRNA genes	64
No. of insertion sequences	14
Mini- and microsatellite DNA (%)	0.38
Plasmid pACICU1	
Size (bp)	28,279
No. of protein-coding sequences	31
No. of insertion sequences	10
Plasmid pACICU2	
Size (bp)	64,366
No. of protein-coding sequences	64
No. of insertion sequences	1

RESULTS AND DISCUSSION

Phenotypic and genetic definition of *A. baumannii* ACICU. *A. baumannii* ACICU is an invasive multidrug-resistant strain isolated from the cerebrospinal fluid of a patient cared for at the ICU of Hospital S. Giovanni-Addolorata in Rome, Italy (21, 22). *A. baumannii* ACICU was assigned to the European clone II group; produces amplicons of 355, 702, and 599 bp; and is referred to as molecular group 1 (37). This result was confirmed by arbitrarily primed PCR and pulsotyping, and identity or a high degree of similarity to prototypic European clone II isolate RUH134 was shown (data not shown).

Basic features of ACICU *A. baumannii* genome. *A. baumannii* ACICU contains a single circular chromosome of 3,904,116 bp and two plasmids (pACICU1 and pACICU2) of 28,279 and 64,366 bp, respectively; 3,758 genes were annotated in the ACICU chromosome, including 3,670 predicted protein-encoding CDSs, 64 tRNA genes, and 8 rRNA operons (Table 1). Nearly 70% of the CDSs ($n = 2,670$) were assigned to a COG functional category; several genes belonged to more than one COG class (see Table S1 in the supplemental material). For many COG classes, the number of CDSs identified in ACICU largely exceeds the number identified in ATCC 17978, since in the latter strain only 60.1% of the genes were assigned to a COG class. The discrepancy observed is justified by the fact that almost the 7% of the ACICU CDSs do not show a significant match (>50% similarity, >50% coverage) with any CDS of ATCC 17978, explaining why many COG classes are more abundant in ACICU than in ATCC 17978.

ACICU also contains 14 ISs in the chromosome, including 7 IS*Aba125* elements, 4 IS*Aba2* elements, 2 IS*26* elements, and 1 IS*Pu12* element, and 11 on plasmids, including 3 IS*Aba3* elements, 3 IS*26* elements, 4 IS*Aba2* elements, and 1 IS*Aba125* element. IS*Aba1* elements were not identified in this strain. The chromosome is composed of 0.38% short repetitive mini- and microsatellite DNA sequences (www.itb.cnr.it/genome-project).

***Acinetobacter* synteny.** The *A. baumannii* ACICU genome was compared with that of its closest sequenced relatives, namely, *A. baumannii* ATCC 17978 (35) and *A. baylyi* ADP1 (1), with Mauve software (12). That comparison enabled the alignment of orthologous and xenologous regions. Comparison of the genomes showed that the *A. baumannii* ACICU genome had 86.4% synteny with the *A. baumannii* ATCC 17978 genome and 14.8% synteny with the *A. baylyi* ADP1 genome. An interactive graphical output made it possible to locate potential regions unique to the *A. baumannii* ACICU genome (Fig. 1).

Thirty-six putative pAs were identified in the strain ACICU genome (designated pA_{ICU} and numbered from 1 to 36; Table 2), for a total of 1,030 CDSs, by genome comparison, use of the SPyPhy program, and G+C content analyses. Of these, 24 showed partial or total correspondence with the pAs previously identified in *A. baumannii* ATCC 17978 (35), while 4 of the 28 *A. baumannii* ATCC 17978 pAs (pA3, pA4, pA20, and pA25) were absent from the ACICU genome. Four additional regions of the ACICU genome ($pA_{ICU}4$, $pA_{ICU}9$, $pA_{ICU}26$, and $pA_{ICU}34$) were identified as potential candidates of HGT, showed significantly different G+C contents compared with that for the whole ACICU genome, and are predicted to encode proteins involved in metabolism. These four regions are present in the ATCC 17978 genome, but they were not designated pAs in the previous annotation (Table 2). Finally, eight regions ($pA_{ICU}3$, $pA_{ICU}6$, $pA_{ICU}8$, $pA_{ICU}20$, $pA_{ICU}24$, $pA_{ICU}29$, $pA_{ICU}30$, and $pA_{ICU}32$) were exclusive to the ACICU genome and showed no match with any region of the ATCC 17978 genome.

The *A. baumannii* ACICU genome shows 367 genes involved in membrane transport, equivalent to 76.6 genes per Mb of genome (Fig. 2A) (http://www.itb.cnr.it/genome-project/index.php?mod=6). This value is much higher than the values for the other *Acinetobacter* genomes (275 transporters in ATCC 17978, equivalent to 57.2 genes per Mb, and 225 transporters in ADP1, equivalent to 62.5 genes per Mb), while it is

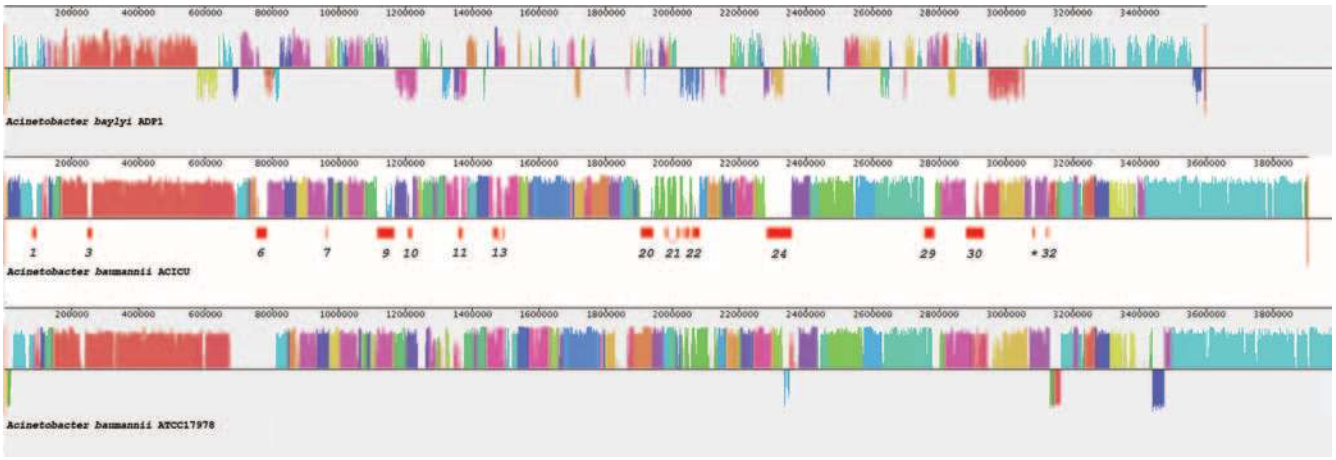

FIG. 1. Multigenome comparison between *A. baumannii* ACICU, *A. baylyi* ADP1, and *A. baumannii* ATCC 17978 obtained by use of the Mauve tool (12). The vertical bars denote the conservation level, and their upwards and downwards orientations relative to the genome line indicates colinear and inverted regions, respectively. Colors are arbitrarily assigned by the Mauve tool to each block of aligned sequences and vary to highlight that a genetic rearrangement (inversion, deletion, transfer) occurred between two contiguous blocks. Horizontal red bars under the *A. baumannii* ACICU map show regions univocally present in the ACICU genome and refer to pAs (see Table 3 for details). *, the ACICU_02910 surface adherence protein mentioned in the text.

comparable to the values for the *E. coli*, *Staphylococcus aureus*, and *Pseudomonas* sp. genomes (Fig. 2A). A feature common to all *Acinetobacter* strains is the paucity of sugar-specific phosphotransferase systems compared with the number in fermenting species like *E. coli* and *S. aureus*, which have about 20. None of the *Acinetobacter* phosphotransferase systems shows a predicted specificity for glucose, while only one fructose-specific type IIC enzyme was found in all three *Acinetobacter* genomes. Moreover, *Acinetobacter* spp. lack predicted monosaccharide-specific transporters of the major facilitator superfamily (MFS). These observations, combined with the lack of exokinase and glucokinase enzymes involved in the first glycolytic step (35; this work; http://www.itb.cnr.it/genome-project/index.php?mod=7), account for the noteworthy inability of members of the genus *Acinetobacter* to catabolize glucose. On the other hand, *A. baumannii* ACICU shows specificity for the uptake of a variety of alternative carbon sources, including benzoate, citrate, galactonate, gluconate, glycerol, lactate and 4-hydroxyphenylacetate (data not shown), which is consistent with the peculiar catabolic profile of the species (3, 34). Interestingly, 11 chromosomal ORFs were predicted to encode porins which could have a role in diffusion of substrates through the outer membrane.

pA$_{ICU}$s. The 36 pA$_{ICU}$s represent the most interesting part of the *A. baumannii* ACICU genome. The relevant features of the pA$_{ICU}$s in comparison with the features of the *A. baumannii* ATCC 17978 pAs are outlined below and are summarized in Table 2.

(i) pA$_{ICU}$1. pA$_{ICU}$1 is remarkably different from ATCC 17978 pA1. The genes involved in cell wall biogenesis (*wecC*, *mviM*, *wbbJ*, *wecE*, and the O-antigen translocase-encoding gene) detected in ATCC 17978 pA1 do not match any of the genes identified in pA$_{ICU}$1. However, newly acquired functions related to cell wall biogenesis were identified in pA$_{ICU}$1, including the UDP-*N*-acetyl-D-mannosaminuronate dehydrogenase (78% amino acid identity with the *Pasteurella multocida* homologue), the spore coat polysaccharide biosynthesis protein (44% amino acid identity with the *Alkalilimnicola erlichei* homologue), the sialic acid synthase (75% amino acid identity with the *Pseudomonas stutzeri* homologue), and the membrane protein involved in the export of O antigen and teichoic acid (35% amino acid identity with the *Pseudomonas aeruginosa* homologue). All these functions plausibly originated by HGT, since the overall G+C content of pA$_{ICU}$1 (34.0%) differs significantly from that of the ACICU genome (39.1%). Thus, pA$_{ICU}$1 is predicted to retain a general function in cell envelope biogenesis, but with a low level of gene conservation with respect to that of its counterpart in the ATCC 17978 genome.

(ii) pA$_{ICU}$3. A homologue of ATCC 17978 pA3 was not detected in the ACICU genome. At the same genomic location, the pA$_{ICU}$3 drug resistance island, which contains transposases, integrases, insertion sequences, antibiotic resistance genes, and heavy metal detoxification proteins, was identified (this is further explained below).

(iii) pA$_{ICU}$6, pA$_{ICU}$9, pA$_{ICU}$24, and pA$_{ICU}$30. pA$_{ICU}$6, pA$_{ICU}$9, pA$_{ICU}$24, and pA$_{ICU}$30 are exclusive to the ACICU genome and mostly contain genes encoding hypothetical proteins, in addition to phage-related proteins. It was not possible to predict any specific function conferred by the acquisition of these phages (designated ACICU phages 1, 2, 3, and 4, respectively), since except for pA$_{ICU}$24, the encoded proteins do not show significant similarity with any known protein. pA$_{ICU}$24 contains a toxin-antitoxin stability system whose importance for the maintenance of this region deserves further investigation.

(iv) pA$_{ICU}$13. pA$_{ICU}$13 has a predicted function in phenyl acetic acid degradation and is homologous to part of pA10, previously identified in ATCC 17978. However, strain ACICU shows 35 novel CDSs within this pA, including four MFS transporters, likely involved in drug resistance on the basis of predictions obtained with the TransAAP tool. Only one MFS transporter was identified in the corresponding pA of ATCC 17978, pA10 (outlined below and in Table S2 in the supplemental material). Furthermore, one additional transporter of

TABLE 2. pAs identified in the *A. baumannii* ACICU genome

pA_{ICU}	ACICU CDS	General function(s) predicted in ACICU	% G+C content[b]	Presumed source(s) of HGT best BLAST hits by SPyPhy system	No. of CDSs gained relative to no. in ATCC 17978	Former pA designation in ATCC 17978
1	72–88	Cell envelope biogenesis	34.0	*Pasteurella, Alkalilimnicola, Pseudomonas*	11	pA1
2	135–146	Drug resistance, autoinducer production	41.6	Gammaproteobacteria	0	pA2
3	220–241	Antimicrobial resistance island[a]	51.2	*Salmonella*, *E. coli*, *Pseudomonas aeruginosa*	21	Absent
4	494–498	Metabolism	43.3	*Burkholderia*, *Enterobacter*	1	Present but not designated as a pA
5	672–680	Amino acid metabolism	39.3	*Pseudomonadales*	0	pA5
6	681–701	Hypothetical proteins, transposases, phage 1[a]	34.2	Bacteriophage	20	Absent
7	859–891	Drug resistance, iron transport[a]	40.7	*Pseudomonas*, *Nitrosomonas*, *Stenotrophomonas*	17	pA6
8	908–932	Metabolism, membrane transport	42.1	*Burkholderia*, *Pseudomonas*	0	Present but not designated as a pA
9	997–1074	Hypothetical proteins, iron transport,[a] phage 2[a]	38.1	Bacteriophage	67	Absent
10	1,108–1,138	Amino acid metabolism	36.8	Enterobacteriaceae, *Serratia*	12	pA7
11	1,240–1,264	Drug resistance, xenobiotic degradation	36.5	*Pseudomonas*	6	pA8
12	1,277–1,294	Metabolism	39.9	Gammaproteobacteria, alphaproteobacteria	4	pA9
13	1,314–1,427	Drug resistance,[a] membrane transport,[a] phenyl acetic acid degradation	39.7	*Psychrobacter*, *Burkholderia*, *Pseudomonas*	35	pA10
14	1,438–1,459	Drug resistance, amino acid metabolism, membrane transport	40.5	*Psychrobacter*, *Burkholderia*	0	pA11
15	1,481–1,496	Arsenic resistance/taurine metabolism	36.2	*Enterobacter*, *Burkholderia*	0	pA12
16	1,539–1,554	Drug resistance, pilus biogenesis	34.1	*Moraxellaceae*	0	pA13
17	1,627–1,639	Iron transport	38.5	*Methylibium*, *Burkholderia*	0	pA14
18	1,686–1,702	Drug resistance, vitamin B_{12} metabolism	37.6	Gammaproteobacteria	0	pA15
19	1,743–1,758	Drug/metabolite resistance	39.9	*Methylobacterium*	0	pA16
20	1,786–1,823	ABC molybdate transport system,[a] coenzyme PQQ synthesis,[a] pilus biogenesis[a]	36.8	*Burkholderiaceae*	38	Absent
21	1,824–1,864	Drug resistance, hypothetical proteins[a]	37.2	Gammaproteobacteria, betaproteobacteria	18	pA17
22	1,878–1,979	Drug resistance, virulence (surface adhesion protein fused to RTX calcium-binding cytotoxin, hemagglutinin protein FhaB, hemolysin activator protein FhaC)[a]	38.8	*Pseudomonas*, *Neisseria*, *Moraxella*	24	pA18
23	2,071–2,083	Amino acid metabolism	34.4	*Alcaligenes*	0	pA19
24	2,140–2,234	Hypothetical proteins, toxin-antitoxin stability system,[a] phage 3[a]	36.7	*Burkholderia, Psychrobacter*, bacteriophage	94	Absent
25	2,268–2,287	Iron transport/metabolism	35.7	Gammaproteobacteria, alphaproteobacteria	2	pA21
26	2,337–2,341	Membrane transport	40.7	*Pseudomonas aeruginosa*	0	Present but not designated as a pA
27	2,494–2,507	Drug resistance	41.9	*Burkholderia*	0	pA22
28	2,569–2,595	Drug resistance, iron transport (siderophore acinetobactin)	38.4	*Marinomonas*	0	pA23
29	2,596–2,623	Drug/metabolite resistance,[a] membrane transport[a]	36.8	*Vibrio*, *Pseudomonas*	27	Absent
30	2,705–2,772	Hypothetical proteins, phage 4[a]	39.7	Bacteriophage	68	Absent
31	2,884–2,895	Metabolism	38.8	Gammaproteobacteria	2	pA24
32	2,939–2,944	Virulence (putative hemagglutinin/hemolysin-related proteins),[a] hypothetical proteins[a]	50.4	Gammaproteobacteria	6	Absent
33	3,205–3,214	Lipid metabolism	41.6	*Oceanospirillales*	2	pA26
34	3,330–3,335	Amino acid metabolism	41.5	*Serratia*	0	Present but not designated as a pA
35	3,449–3,465	Hypothetical proteins	35.6	Proteobacteria	0	pA27
36	3,595–3,613	Metabolism	40.0	*Pseudomonas*	0	pA28

[a] Genes and relative functions identified in the ACICU genome but absent in the ATCC 17978 genome.
[b] The G+C content of the ACICU genome, calculated by exclusion of the 36 pA_{ICU}s, is 39.2%

the nucleobase:cation symporter 1 (NCS1) was identified in ACICU but was absent from ATCC 17978, together with three ATP binding cassette (ABC) transporters, two of which showed substrate specificity for the ferric ion, and one MSF not involved in drug resistance. These findings strongly suggest that $pA_{ICU}13$ is a candidate relevant to ACICU virulence and drug resistance.

(v) $pA_{ICU}20$ and $pA_{ICU}32$. $pA_{ICU}20$ and $pA_{ICU}32$ were identified by screening with the SPyPhy system, and they are exclusive to the ACICU genome, being absent from strain ATCC 17978. A complete ABC transport system for molybdate, a cluster of genes involved in the coenzyme pyrroloquinolinequinone (PQQ) biosynthesis (73% amino acid identity with the *Pseudomonas aeruginosa* homologue), and an additional cluster for pilus biosynthesis were identified in $pA_{ICU}20$. Hypothetical proteins and a putative hemagglutinin/hemolysin-related protein (48% amino acid identity with the *Marinobacter algicola* homologue) were identified in $pA_{ICU}32$. These traits

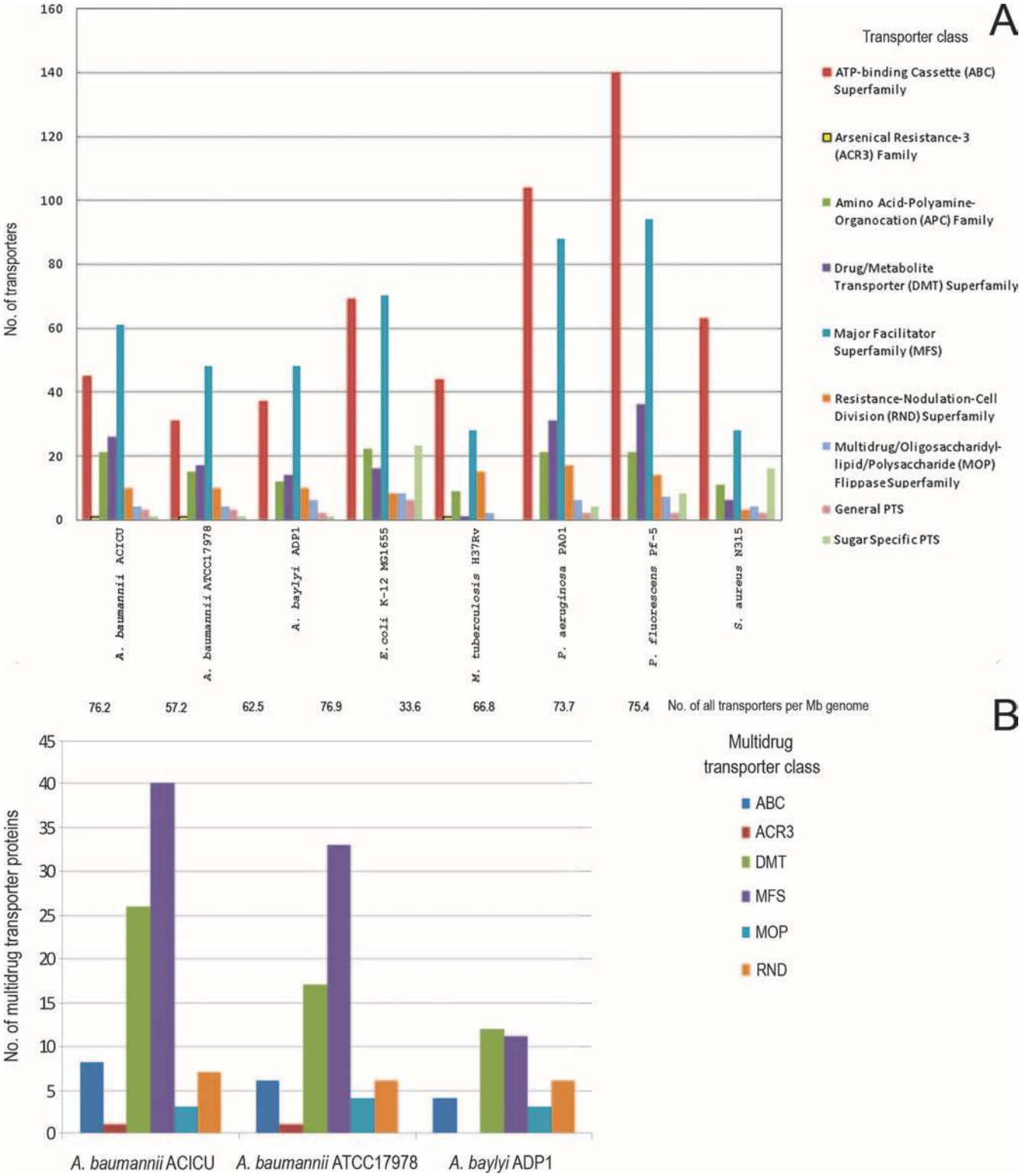

FIG. 2. (A) Comparison of the numbers of transporters in *A. baumannii* ACICU, *A. baumannii* ATCC 17978, *A. baylyi* ADP1, *E. coli* MG1655, *Mycobacterium tuberculosis* H37Rv, *P. aeruginosa* PAO1, *Pseudomonas fluorescens* Pf-5, and *S. aureus* N315. Transporter proteins were classified by use of the TransAAP tool (http://www.membranetransport.org/), based on the TransportDB program (31). Only the main transporter classes are shown, with the number of transporters per Mb of genome given for each bacterial species. The color codes for the transporter classes are given on the right. (B) Comparison of the number of predicted drug efflux systems in *A. baumannii* ACICU and ATCC 17978, and *A. baylyi* ADP1. Transporter proteins were classified by use of the TransAAP tool (http://www.membranetransport.org/), based on the TransportDB program (31). Only family members that clearly clustered with known multidrug efflux transporters were counted. Genetically associated membrane fusion or outer membrane proteins were not considered. The color codes for the transporter classes are given on the right.

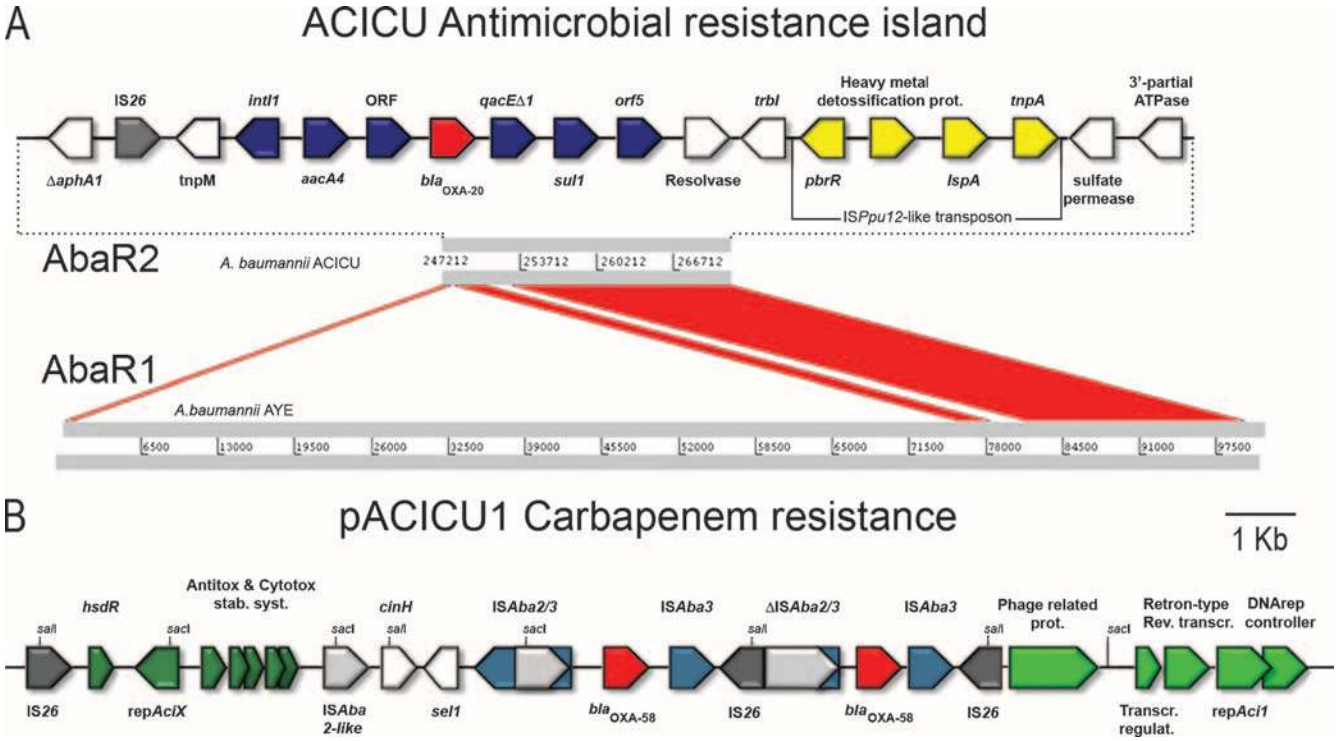

FIG. 3. Schematic representation of the genomic regions involved in multidrug resistance in *A. baumannii* ACICU. (A) Layout of the AbaR2 region inserted into the *A. baumannii* ACICU chromosome (top; gene sizes are not to scale) and comparison of the AbaR2 and AbaR1 chromosomal regions located in *A. baumannii* ACICU and AYE, respectively (bottom). (B) Physical map of the pACICU1 carbapenem resistance plasmid. Colors denote CDS categories: IS*26* and IS*Aba2-like* in gray; IS*Aba3* in light blue; bla_{OXA} in red; integron in blue; IS*Ppu12*-like transposon in yellow; plasmid scaffolds containing *repAciX* and *repAci1* in green and light green, respectively; and all other functions in white.

plausibly contribute to the virulence potential of strain ACICU. $pA_{ICU}32$ has likely been acquired by HGT, as it showed a significantly higher G+C content (50.4%) than the core ACICU genome.

(vi) $pA_{ICU}21$ and $pA_{ICU}22$. $pA_{ICU}21$ and $pA_{ICU}22$ correspond to pA17 and pA18 of ATCC 17978, respectively, and are implicated in drug resistance, as they encode specific membrane transport systems. In particular, $pA_{ICU}21$ carries the cluster encoding the AdeABC system, which has been demonstrated to be involved in antimicrobial resistance in *A. baumannii* (29). Interestingly, $pA_{ICU}22$ contains one MFS transporter and one drug/metabolite transporter (DMT) likely involved in multidrug resistance, a surface adhesion protein fused to an RTX calcium-binding cytotoxin (33% identity with the *Pseudomonas putida* homologue), a hemagglutinin FhaB-like protein, and a hemolysin activator FhaC-like protein previously identified in ADP1 but not in ATCC 17978. The surface adhesion protein is predicted to belong to the family of the RTX cytotoxins, which are involved in invasion and hemolysis. Notably, the *Vibrio vulnificus* RtxA1 toxin causes hemolysis through pore formation and helps with invasion of the bloodstream from ligated ileal loops of CD-1 mice (20). Other genes are present in this region, including a second MFS permease, esterase/lipase, and catalase enzymes, whose roles in ACICU pathogenesis are unclear at present.

(vii) $pA_{ICU}29$. $pA_{ICU}29$ is exclusive to the ACICU genome and contains several genes encoding membrane transport systems related to drug resistance. In particular, a permease of the MFS group and two DMTs were identified in this region.

Genomics of antibiotic resistance in *A. baumannii* ACICU. To gain further insight into the genetic basis of resistance, the *A. baumannii* ACICU and ATCC 17978 genomes were inspected for drug resistance determinants, and the drug resistance regions were then compared with those identified in the multiresistant strain *A. baumannii* AYE (16). An 86-kb resistance island (AbaR1) carrying 82 CDSs was recently described in *A. baumannii* AYE (16). A different resistance island, designated AbaR2, was identified in the genome of ACICU as $pA_{ICU}3$ (Fig. 3A). AbaR2 was shorter than AbaR1, showing only 17 CDSs, including seven resistance genes. Within this region, a class 1 integron carrying the aminoglycoside acetyltransferase gene *aacA4*, a putative CDS encoding an unknown function, and the bla_{OXA-20} beta-lactamase gene were identified. This gene cassette array is different from that described for AbaR1, even if genes flanking the 5′ and 3′ conserved segments of the integron perfectly match in AbaR1 and AbaR2. In both islands the integrons are flanked by the *aphA1*-IS*26* element at the 5′ conserved segment and the IS*Ppu12*-like transposon at the 3′ conserved segment. However, 116 nucleotides at the 3′ end of the *aphA1* gene are missing in AbaR2 as well as the remaining portion of AbaR1. The 3′ end of an ATPase gene, located at one boundary and considered the integration site of AbaR1 within the AYE genome, is missing in ACICU. Both strains show the 5′ end of the ATPase gene,

TABLE 3. *A. baumannii* ACICU CDSs associated with drug resistance

Drug class	Gene name, description	ACICU protein tag	GenBank match with *Acinetobacter* spp. (% similarity, % coverage)		
			ATCC 17978	ADP1	AYE
Beta-lactams	Putative class A beta-lactamase	ACICU_01260	A3M448 (97, 95)	Negative[a]	CAJ77854 (96, 100)
	Class C beta-lactamase	ACICU_02564	A3M792 (98, 88)	Negative	CAJ77859 (99, 99)
	bla_{OXA-58}, class D beta-lactamase (two copies)	AC1_0019 and AC1_0024[c]	Negative	Negative	Not available[b]
	bla_{OXA-20}, class D beta-lactamase	ACICU_00226	Negative	Negative	Not available
	bla_{OXA-66}, class D beta-lactamase	ACICU_01560	A3M4V0 (90, 70)	Negative	AAW51371 (98, 100)
Aminoglycosides	*aaCA4* aminoglycoside 6′-N-acetyltransferase	ACICU_00223	Negative	Negative	Not available
Fluoroquinolones	*gyrA* (Ser→Leu) mutation at position 83	ACICU_02869	A3M7Z9 (100, 97)	Q6F956 (91, 100)	CAJ77862 (100, 100)
Sulfonamides	*sul1* dihydropteroate synthase	ACICU_00228	Negative	Negative	CAJ77031 (100, 63) CAJ77050 (100, 100) CAJ77053 (100, 100) CAJ77089 (100, 100)
Heavy metals	Acr3, arsenite efflux pump	ACICU_01494	A3M4N8 (99, 84)	Negative	CAJ77016 (88, 100)
	Heavy metal detoxifiCAtion protein	ACICU_00235	Negative	Negative	CAJ77022 (100, 100)
	czcD, Co/Zn/Cd efflux pump	ACICU_01084	A3M3I3 (100, 66)	Negative	CAJ77829 (99, 100)
Quaternary ammonium	*qacEΔ1*, SMR family efflux pump	ACICU_00227	Negative	Negative	CAJ77030 (100, 100) CAJ77049 (100, 100) CAJ77052 (100, 100) CAJ77088 (100, 100)

[a] Negative indicates <60% similarity.
[b] Not available, DNA sequences or information not available for strain AYE.
[c] The AC1_ protein tags refer to the CDSs present in plasmid pACICU1.

located at the other boundary of the island. This configuration is compatible with a deletion event in AbaR2 involving a large portion of AbaR2 from the 3′ end of the flanking ATPase gene to a site internal to the *aphA1* gene. As a consequence of this deletion, AbaR2 lacks 69 CDSs, including the arsenic, mercury, and tetracycline resistance operons, and a large integron carrying the bla_{VEB-1} and bla_{OXA-10} genes.

A. baumannii AYE and ACICU belong to different clonal groups (European clones I and II, respectively) (37). However, the presence of related resistance islands in both lineages suggests that AbaR1 and AbaR2 derived from an island acquired by a common *A. baumannii* ancestor before their divergence into two different clonal lineages.

Besides the bla_{OXA-20} gene identified within the integron, strain ACICU contains three additional beta-lactamases, including a putative class A beta-lactamase previously reported in strain AYE (18), the previously described AmpC beta-lactamase, and the naturally occurring OXA-66 oxacillinase (Table 3). OXA-66 is predicted to be not expressed since it is not flanked by any IS previously described to be necessary to drive expression of the carbapenem resistance phenotype (38). Interestingly, the *ampC* gene is preceded by an IS*Aba125* element that could influence its expression. Notably, IS*Aba1*-directed overexpression of the naturally occurring *ampC* gene has been identified as a mechanism conferring ceftazidime resistance in *A. baumannii* clinical strains (19). However, DDST performed with class C β-lactamase inhibitors, such as cloxacillin and boronic acid, did not restore ceftazidime susceptibility in ACICU, suggesting that AmpC is not the only mechanism accounting for ceftazidime resistance and that multidrug efflux pumps could contribute to this phenotype.

Resistance to fluoroquinolones in ACICU (ciprofloxacin MIC > 16 μg/ml [21]) is likely ascribed to the previously characterized mutation occurring in the *gyrA* gene, with a substitution of Ser for Leu at position 83 of its amino acid sequence. In comparison with the sequences of strains AYE and ATCC 17978, several amino acid substitutions that were not previously described to be associated with fluoroquinolone resistance in any *Acinetobacter* strain, however, were observed in the *parC* and *parE* genes, while no mutations were observed in the *gyrB* gene of ACICU.

Transporters plausibly involved in drug efflux likely make an important contribution to the overall multidrug-resistant phenotype of ACICU (see Table S2 in the supplemental material), and these transporters are more numerous in ACICU than in other *Acinetobacter* strains (Fig. 2B). Forty MFS drug transporters were identified in ACICU, whereas 33 were identified in ATCC 17978 and only 11 were identified in ADP1. Twenty-six DMTs were also detected, with 17 detected in ATCC 17978 and 12 detected in ADP1. Furthermore, eight drug transporters of the ABC superfamily, seven of the resistance-nodulation-cell division (RND) family, and three of the multidrug/oligosaccharidyl-lipid/polysaccharide family were also identified in ACICU (the ATCC 17978 genome contained six, six, and four of these transporters, respectively) (Fig. 2B; see also Table S2 in the supplemental material). Among the RND transporters, both AdeABC (ACICU_01823, ACICU_01824, ACICU_01825) and AdeIJK (ACICU_02987, ACICU_02988, ACICU_02989) were identified in ACICU.

These RND systems were demonstrated to act as pumps for the efflux of multiple antibiotics in *A. baumannii* (11, 25). Resistance to arsenic compounds was due to one transporter of the arsenical resistance-3 family, which was also detected in ATCC 17978 but not in ADP1 (Fig. 2B; see Table S2 the supplemental material). These transporters may account for the antimicrobial resistance phenotype of strain ACICU, including the resistance to those drugs for which specific genes have not yet been identified (i.e., tetracycline, trimethoprim, and chloramphenicol).

Plasmid-mediated carbapenem resistance. The carbapenem resistance in *A. baumannii* ACICU is conferred by the carbapenem-hydrolyzing OXA-58 oxacillinase, whose gene was detected in plasmid pACICU1. Notably, the chromosomal gene encoding outer membrane protein CarO (ACICU_02813), which is responsible for the influx of carbapenem antibiotics, should not contribute to carbapenem resistance in this strain, since it is not interrupted by any IS, as is the case in other *A. baumannii* strains (27).

Plasmid pACICU1 carries two copies of the $bla_{\text{OXA-58}}$ gene, and these likely originated from direct repetition of the IS*Aba2*–$bla_{\text{OXA-58}}$-IS*Aba3* mobile element (Fig. 3B). The duplication of the $bla_{\text{OXA-58}}$ gene has been already described in other unrelated *A. baumannii* strains and is generally associated with increased resistance to imipenem and meropenem (6). The duplication of this genetic determinant has likely been facilitated by the presence of multiple IS*26* elements dispersed within this plasmid region.

Plasmid pACICU1 also contains two replication systems. It carries two replicase genes (*repAci1* and *repAciX*), both of which are flanked by *cis* regulatory elements (iterons). *repAciX* is close to genes encoding an antitoxin-toxin system, which ensures plasmid maintenance, and to the *par* gene, which confers stability during plasmid partitioning. These systems are very well known in plasmids of the family *Enterobacteriaceae* (26), but to the best of our knowledge, this is the first description of their presence in an *Acinetobacter* plasmid. *repAci1* is associated with a retro-type reverse transcriptase-like gene and a gene encoding a phage protein. This part of the plasmid could therefore derive from a bacteriophage and could be implicated in horizontal mobilization, since a conjugation system(s) was not identified in this plasmid. A complete *tra* locus, which encodes the conjugative apparatus and the type IV secretion system, was identified in plasmid pACICU2, which coresides with pACICU1 in *A. baumannii* ACICU. It is therefore plausible that pACICU2 could provide in *trans* the mobilization functions for pACICU1 conjugation. It is intriguing that the composite scaffold of pACICU1, which carries two replicons and phage-related proteins, is the vehicle of the $bla_{\text{OXA-58}}$ gene. It is worth emphasizing that the $bla_{\text{OXA-58}}$ gene has been identified worldwide as one of the most common mechanisms for carbapenem resistance in clinical *A. baumannii* isolates. Thus, elucidation of the mechanism(s) implicated in the mobilization and diffusion of a $bla_{\text{OXA-58}}$-carrying plasmid, as well as determination of the plasmid scaffold sequence, would help to set a genetic screen for the rapid monitoring for and detection of this carbapenem resistance plasmid in other strains unrelated to *A. baumannii*.

Concluding remarks. *A. baumannii* has gained a leading position among opportunistic pathogens, and epidemic clones are rapidly evolving toward antimicrobial panresistance. This feature greatly facilitates their spread in health care facilities. In this study, we demonstrated the usefulness of high-density pyrophosphate DNA sequencing as a tool to achieve rapid insight into the physiology, pathogenicity, and molecular basis of antimicrobial resistance of *A. baumannii* ACICU, a pathogenic multidrug-resistant strain typed as a member of the European clone II group. Whole-genome comparison of *A. baumannii* ACICU and the *A. baumannii* ATCC 17978 reference strain revealed a number of distinctive traits. The key feature of the ACICU genome is a conspicuous number of additional functions that were not identified in ATCC 17978. In several cases these regions are clusters of genes homologous to those identified in pathogenic or commensal proteobacteria, such as *Neisseria*, *Burkholderia*, and *Pseudomonas*. These clusters were presumably acquired by HGT from these genera or, alternatively, originated in *Acinetobacter* and were then transferred to other organisms. Given the propensity of *A. baumannii* to acquire exogenous genetic material, it is tempting to speculate that HGT events may occur with phylogenetically related species sharing a common ecological niche, such as the hospital environment or the skin and mucosal surfaces of the colonized human host. Among the novel functions gained by *A. baumannii* ACICU, there are (i) improved membrane transport potential (76.2 transporters per Mb of genome compared with 57.2 transporters per Mb in ATCC 17978 and 62.5 transporters per Mb in ADP1); (ii) expanded drug resistance (which accounts for the panresistant phenotype); and (iii) alteration of the cell envelope (novel genes for the synthesis of the cell wall), adherence properties (surface adhesion proteins fused to putative cytotoxin binding domains), potential changes in the pathogenic potential (hemagglutinin/hemolysin-related proteins), and a high number of hypothetical and/or functionally uncharacterized proteins, often associated with regions of prophage origin. Altogether, these findings provide a solid genetic background for future insights into the functional basis of the biology, pathogenicity, and multidrug resistance of *A. baumannii*.

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