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Novel Variants of AbaR Resistance Islands with a Common Backbone in *Acinetobacter baumannii* Isolates of European Clone II

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In this study, the genetic organization of three novel genomic antibiotic resistance islands (AbaRs) in *Acinetobacter baumannii* isolates belonging to group of European clone II (EC II) *comM* integrated sequences of 18-, 21-, and 23-kb resistance islands were determined. These resistance islands carry the backbone of AbaR-type transposon structures, which are composed of the transposition module coding for potential transposition proteins and other genes coding for the intact universal stress protein (*uspA*), sulfate permease (*sul*), and proteins of unknown function. The antibiotic resistance genes *strA*, *strB*, *tetB*, and *tetR* and insertion sequence CR2 element were found to be inserted into the AbaR transposons. GenBank homology searches indicated that they are closely related to the AbaR sequences found integrated in *comM* in strains of EC II (*A. baumannii* strains 1656-2 and TCDC-AB0715) and AbaR4 integrated in another location of *A. baumannii* AB0057 (EC I). All of the AbaRs showed structural similarity to the previously described AbaR4 island and share a 12,008-bp backbone. AbaRs contain Tn*1213*, Tn*2006*, and the multiple fragments which could be derived from transposons Tn*3*, Tn*10*, Tn*21*, Tn*1000*, Tn*5393*, and Tn*6020*, the insertion sequences IS*26*, IS*Aba1*, IS*Aba14*, and IS*CR2*, and the class 1 integron. Moreover, chromosomal DNA was inserted into distinct regions of the AbaR backbone. Sequence analysis suggested that the AbaR-type transposons have evolved through insertions, deletions, and homologous recombination. AbaR islands, sharing the core structure similar to AbaR4, appeared to be distributed in isolates of EC I and EC II via integration into distinct genomic sites, i.e., *pho* and *comM*, respectively.

The occurrence of a major nosocomial infectious agent, antibiotic-resistant *Acinetobacter baumannii*, was attributed to the epidemic spread of international lineages European clones (EC) I and II (6, 7, 10, 11, 14). Multiple antibiotic resistance regions, inserted into the target gene *comM* and termed resistance islands (AbaR), were reported recently in *A. baumannii* strains (1, 9, 16, 21, 22). The backbone of AbaR was shown to be comprised of five open reading frames constituting the so-called transposition module (*orf1*, *tniA*, *tniB*, *orf2*, and *orf3*), together with two other genes encoding the universal stress protein (*uspA*) and sulfate permease (*sul*) (3). Bioinformatic analysis showed that three open reading frames encoded by transposition module showed similarity to transposition-associated proteins of Tn*7*, which suggested that AbaR had arisen from the ancestral transposon, distantly related to Tn*7* (24). Several studies showed the gene *uspA* in AbaR backbone was intact (AbaR4) or interrupted by a large composite transposon bracketed by two copies of IS*Ppu12* (renamed as Tn*6018* [22]) carrying the resistance genes in AbaR1, AbaR3, and AbaR5 to AbaR19 (3, 16, 21, 22). Recent analysis of these new variants of resistance islands suggested that an AbaR3-like structure was found frequently in strains of EC I and the new variants evolved through deletions or homologous recombination creating truncated derivatives (16).

Little is known regarding the AbaRs in epidemic strains of EC II. The truncated variant of AbaR1, designated AbaR2, which consisted of only the right hand of the island and carried a single copy of IS*Ppu12*, was found in EC II strain ACICU (13). However, a recent study reported that an IS*Ppu12* composite transposon was absent in the AbaR backbone and that the *uspA* gene was intact in EC II isolates (3). In the present study, the characterization of three novel AbaRs structures from *A. baumannii* isolates belonging to EC II is reported.

MATERIALS AND METHODS

Bacterial isolates. *A. baumannii* isolates LT-3, LT-11, and LT-V1 were isolated from respiratory tract and joint aspirate samples from patients hospitalized in the intensive care units of two Lithuanian hospitals in 2010 (Lithuanian University of Health Sciences Kauno Klinikos Hospital, Kaunas, Lithuania, and Vilnius University Emergency Hospital, Vilnius, Lithuania). The isolates were identified as *A. baumannii* by using automated microbiology system Phoenix (BD, USA) and amplified rDNA restriction analysis (ARDRA) (8). Antibiotic susceptibility was tested by using BD Phoenix system and interpreted accordingly the Clinical and Laboratory Standards Institute guidelines.

PFGE and MLST-IP. Pulsed-field gel electrophoresis (PFGE)-ApaI restriction analysis was performed, and gel images were analyzed using Bionumerics software with the Dice coefficient and a band tolerance set at 0.5% (30). The relatedness of isolates to EC I and II was determined by the multiplex PCR assays, as described previously (29). Genotyping by multilocus sequence typing according to the scheme of the Institut Pasteur (MLST-IP) was undertaken with the primers and conditions described on the Pasteur website (http://www.pasteur.fr/mlst), with the exception of *rpoB* amplification, which was undertaken with the primers FrpoB (5′-GTTGCTGCTGCAATCAAAGA-3′) and RrpoB (5′-TCTCACCAAAAATTGCACGA-3′), designed on the basis of a published sequence (CP001921). The sequences of *A. baumannii* AB210 (AEOX00000000), 1656-2 (CP001921), and TCDC-ABO715 (CP002522) were retrieved from GenBank for determination of the sequence type *in silico*.

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TABLE 1 Characteristics of *A. baumannii* isolates

Isolate	Hospital/clonality[a]	MLST	Resistance profile[b]	$bla_{OXA-51-like}$	Acquired CHDL	Gene cassettes of class 1 integron	Other genes conferring antibiotic resistance	AbaR length (kb)
LT-3	K/clonal	ST2	MEM IPM TZP CAZ AN CIP PIP	OXA-66	OXA-72		*tetB*, *strA*, *strB*	18
LT-11	K/sporadic	ST2	MEM IPM TZP SAM CAZ GM AN CIP PIP	OXA-66	OXA-23	*aacA4-catB8-aadA1*	*tetB*, *strA*, *strB*, *aphA1*, *armA*, *adeABC*	23
LT-V1	V/clonal	ST2	MEM IPM TZP CAZ GM AN CIP PIP	OXA-66	OXA-72	*aacC1*[c]	*tetB*, *strA*, *strB*, *adeABC*	21

[a] K, Lithuanian University of Health Sciences Kauno Klinikos Hospital; V, Vilnius University Emergency Hospital. LT3 and LT-V1 were representative isolates of clonal outbreaks in hospitals in 2010, whereas LT-11 was a sporadic isolate. Clonality was determined by PFGE-ApaI restriction analysis.
[b] Antimicrobial susceptibility to MEM (meropenem), IPM (imipenem), TZP (tazobactam/piperacillin), SAM (sulbactam/ampicillin), SCF (sulbactam/cefoperazone), CAZ (ceftazidime), GM (gentamicin), AN (amikacin), CIP (ciprofloxacin), and PIP (piperacillin) was tested.
[c] The 3′-conserved segment (CS) of integron was missing.

DNA extraction. Genomic DNA was extracted using an Arrow magnetic workstation (NorDiag, Norway) according to the manufacturer's instructions.

Gene detection. Genes coding for intrinsic β-lactamases ($bla_{OXA-51-like}$ and bla_{ADC}) and acquired genes conferring narrow-spectrum resistance to β-lactams (bla_{TEM-1}) or carbapenems (acquired carbapenem hydrolyzing class D β-lactamases $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, $bla_{OXA-58-like}$, and $bla_{OXA-143}$ and metallo-β-lactamases bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}, and bla_{NDM}), aminoglycosides (*aadA1*, *aadA2*, *aadB*, *aadA5*, *strA*, *strB*, *aphA1*, *aphA2*, *aphA6 aacC1*, *aacC2*, *aacC4*, *aacA4*, *armA*, *rmtC*, and *rmtB*), tetracyclines (*tetA* and *tetB*), and fenicols (*catI*, *catII*, *catIII*, *catB2*, *catB3*, *catB8*, *cmlA*, *cmlB*, and *floR*), in addition to class 1 integron cassettes and genes coding the *adeABC* efflux pump, were detected by PCR as described previously (5, 12, 17–19, 20, 23).

AbaR mapping and sequence analysis. Analysis of characteristic AbaR junction regions and genomic surroundings upstream and downstream 3′ and 5′ parts of *comM* gene was undertaken as described earlier (15, 25). The *comM* integrated sequences were amplified by Long-Range PCR (Fermentas, Lithuania), purified and used for DNA sequencing and AbaR mapping. The *comM* integrated sequence of 18-kb AbaR4a from isolate LT-3 was sequenced by primer walking strategy (primers are listed in Table S1 in the supplemental material). PCR mapping of AbaR backbone was carried out by using the long-range PCR with primers targeting the genes in AbaR4a and DNA restriction analysis (the positions of amplicons, restriction endonucleases, and primers are listed in Fig. S1 and Table S1 in the supplemental material). DNA regions of LT-11 and LT-V1, different from those of integrated sequence in LT-3, as determined by PCR mapping, were sequenced. DNA and protein sequences were analyzed using Blastn, Blastp (www.ncbi.nlm.nih.gov [August 2011]), and CLC Main Workbench software (CLC Bio A/S, Denmark).

Nucleotide sequence accession numbers. The entire sequences of the AbaR elements have been deposited in the GenBank under the accession numbers JN129845 (AbaR4a), JN129846 (AbaR4b), and JN129847 (AbaR4c).

RESULTS AND DISCUSSION

Characterization of isolates. *A. baumannii* isolates LT-3, LT-11, and LT-V1 were resistant to meropenem, imipenem, piperacillin-tazobactam, ceftazidime, piperacillin, ciprofloxacin, and amikacin. LT-11 and LT-V1 were also resistant to gentamicin; LT-11 was resistant to sulbactam/ampicillin. According to PFGE-ApaI macrorestriction analysis, LT-3 and LT-V1 were closely related (cutoff value, 90%), being representative isolates of the clonal outbreaks in these hospitals. The LT-11 isolate was unique (showing a similarity of <70%) and sporadic (data not shown). All isolates belonged to EC II according multiplex PCR analysis and were assigned to ST2 (MLST-IP). PCR screening and DNA sequencing confirmed the presence of the acquired carbapenemase genes bla_{OXA-72} (LT-3 and LT-V1) and bla_{OXA-23} (LT-11). The characteristics of isolates are summarized in Table 1.

Structure of resistance islands. In previous studies, AbaR sequences were found to be integrated into the same position of the *comM* gene, coding for a hypothetical protein with an ATPase function (25). Long-range PCR amplification of isolates LT-3, LT-V1, and LT-11 with primers targeting the 3′ and 5′ ends of *comM* gene yielded products of 18, 21, and 23 kb, respectively. Subsequent PCR analysis confirmed the presence of the 3′ and 5′ parts of *comM* gene and the characteristic junction regions between the AbaR sequences and *comM* in all isolates. Therefore, *A. baumannii* isolates LT-3, LT-V1, and LT-11 possessed an integrated sequence located at the same position known for other AbaRs (15, 16, 22, 25). The PCR product of 18 kb from isolate LT-3 was purified and sequenced. The resistance island carries the backbone of AbaR encoding part of the transposition module (*orf1* and *tniA*), an intact universal stress protein (*uspA*), sulfate permease (*sul*), and *orf4*, coding for the protein of unknown function (Fig. 1). However, in LT-3, the transposition module is partially deleted. *orf2* and fragments of *tniB* and *orf3* were removed. The backbone of AbaR is fused to a DNA fragment carrying the insertion sequence CR2 element and the tetracycline and streptomycin resistance genes *tetB*, *tetR*, *strA*, and *strB*. DNA sequencing showed that the resistance island contains the complete transposition module in LT-V1 and carries the insertion of Tn*2006*, encoding bla_{OXA-23}, in LT-11 (Fig. 1).

Comparative analysis of the AbaR regions in LT-3, LT-11, LT-V1, and AbaR4-type islands. GenBank homology searches indicated that the AbaR sequences of LT-3, LT-V1, and LT-11 are closely related to AbaR4, integrated in AB57_0566 locus in *A. baumannii* strain AB0057 (EC I), and to the AbaR sequences of *A. baumannii* strains AB210, 1656-2, and TCDC-ABO715, which belonged to EC II and which were assigned to ST2 (MLST-IP) corresponding to international CC2 (4, 28, 29). For clarity, the structures of these related sequences, found in the GenBank DNA database, are illustrated in Fig. 2. Since the islands identified in the present study and found in the database clearly showed structural similarity to the earlier-described AbaR4, these islands were named for simplicity as follows: AbaR4a in LT-3 (JN129845, bases 1 to 17996), AbaR4b in LT-11 (JN129846, bases 1 to 22810), AbaR4c in LT-V1 (JN129847, bases 1 to 20846), AbaR4d in *A. baumannii* 1656-2 (CP001921, bases 257120 to 291743), and

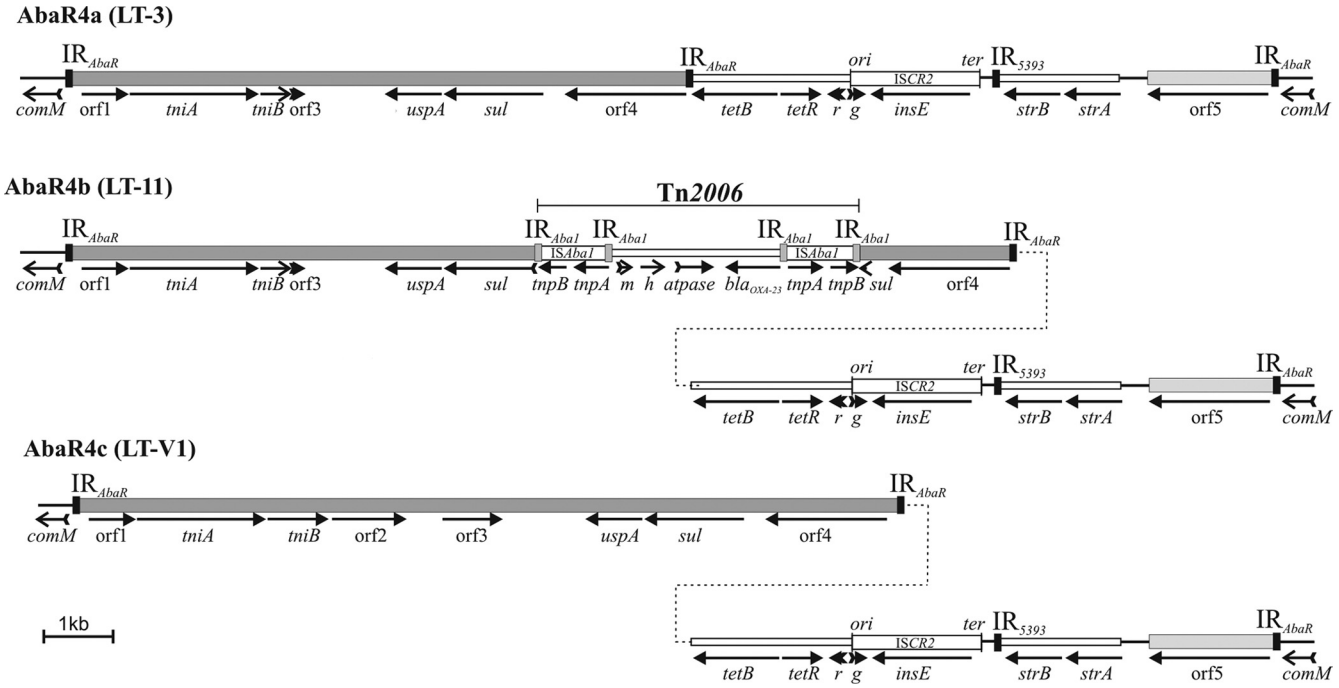

FIG 1 Structure of AbaR4a, AbaR4b, and AbaR4c islands. AbaR-type transposon backbone is shown by filled boxes bounded by inverted terminal repeats (IR_{AbaR}) shown as black bars. The additional inserted regions are indicated by vertical arrows indicating the insertion site. Boxes of different thickness distinguish the various segments: antibiotic resistance genes, IS*CR2*, and IS*Aba1*. Vertical bars indicate the *ori* and *ter* sites of IS*CR2* element and the inverted repeats (IR) with a subscript note, indicating the identity of IR. The genes are shown by horizontal arrows with the gene name below. An angled style at the beginning and the head of the arrow indicate 5′ and 3′ truncated genes, respectively. Genes named *m*, *h*, *g*, and *r* potentially encode a DNA methylase, helicase, phosphoglucosamine mutase (*glmM*), and the transcription regulator of ArsR family, respectively. Note that *orf5* is 85% identical to *orf4*. The sizes of individual AbaRs are drawn to scale. Sequences of AbaR4a, AbaR4b, and AbaR4c are available from GenBank under accession numbers JN129845, JN129846, and JN129847, respectively.

AbaR4e in *A. baumannii* TCDC-AB0715 (CP002522, bases 245943 to 291133).

Comparative structural analysis showed that AbaR4a to AbaR4e share a 12,008-bp backbone, which is composed of the transposition module, the genes *uspA*, *sul*, and *orf4*. 5′-*comM*-associated *orf5* showed significant identity in DNA (85%) to *orf4*. The partial copies of the transposition module were found in the backbone of AbaR4d and AbaR4e, which appear to have undergone multiple events of deletions, insertions, and inversions and might be generated through homologous recombination between DNA sequences carrying separate copies of AbaR backbone (Fig. 2). The direct repeats and imperfect inverted terminal repeats (ITR), flanking the AbaR4-type integrated sequences, are the same as found for the other AbaRs (2, 9, 21). ITRs of AbaR4e were found to be truncated by five and seven nucleotides, probably due to DNA recombination and deletion.

Antibiotic resistance regions of AbaR4-type islands. In contrast to EC I-related AbaRs, which carry multiple antibiotic resistance regions, inserted in the *uspA* gene and flanked by two directly repeated copies of IS*Ppu12*, a less complex structure in EC II-related AbaRs was observed. Tn*1213*, Tn*2006*, and the multiple fragments, perhaps derived from transposons Tn*3*, Tn*10*, Tn*21*, Tn*1000*, Tn*5393*, and Tn*6020*, insertion sequences IS*CR2*, IS*26*, IS*Aba1*, and IS*Aba14*, class 1 integron, and chromosomal DNA, were found inserted into distinct regions (coding for *sul*, *tniB*, and *orf3*) or fused to the backbone elements of AbaR (Fig. 2). Some genes, such as *strA*, *strB*, *tetB*, and *tetR*, have been found in most AbaR4-type islands. *sul2*, *aphA1*, *aacA4*, *catB8*, *aacC1*, *aadA1*, *sul1*, bla_{TEM-1}, bla_{PER-1}, and bla_{OXA-23} have been identified in AbaR4d or AbaR4e. A recent study showed the novel variant of AbaR4-type resistance island carrying two transposition modules, and the DNA fragments similar to those observed in AbaR4a to AbaR4c (*tetB*, *tetR*, IS*CR2*, *strA*, and *strB*) and AbaR4d (*A. baumannii* genomic DNA insertion) inserted into the backbone (31).

AbaR4-type islands contain IS*CR2*. Importantly, IS*CR2*, an atypical class of insertion sequences, was found integrated in AbaR4a-e (Fig. 1 and 2). Such sequences, which are known to move by a mechanism of rolling-circle transposition, are believed to be responsible for the mobilization of virulence and antibiotic resistance genes and thought to represent a powerful system, which can mobilize any section of DNA (27). These elements can cotranspose DNA adjacent to their terminal ter*IS* sequence, mediated by a single copy of the IS*CR* (26). IS*CR1* and IS*CR3* elements were identified in AbaR1, suggesting that they are implicated in the acquisition and transposition of the resistance genes (27).

AbaR4-type islands insert into the *pho* target in isolates of EC I. *A. baumannii* AB0057, belonging to EC I group, is known to possess resistance island AbaR3 integrated in *comM* and resistance island AbaR4 integrated in a locus AB57_0566 (2). A recent study reported that the isolates of EC I, carrying the second genomic AbaR4-type island found at a distinct insertion site, are widespread in United Kingdom (28). Bioinformatic analysis showed that this target gene encodes the putative phosphatase (similar to ABAYE3309) and, therefore, in the present study was designated as the target *pho*. Sequence analysis showed that *pho*-integrated

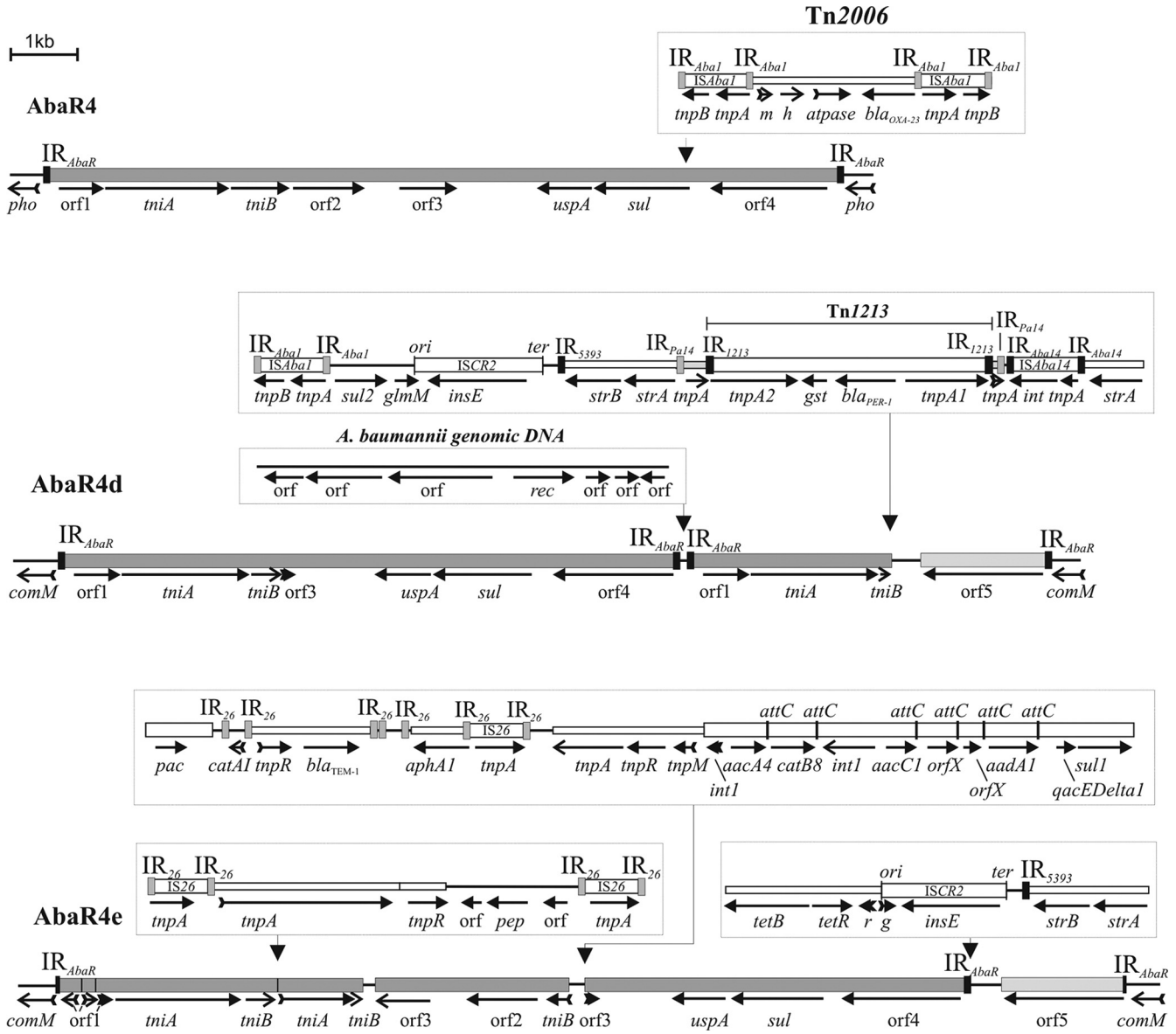

FIG 2 Structure of AbaR4, AbaR4d, and AbaR4e islands. Features are as described in Fig. 1. The gene *pac* potentially encodes a puromycin *N*-acetyltransferase (usually designated *orf5* in class 1 integrons). The sizes of individual AbaRs are drawn to scale. Sequences of AbaR4, AbaR4d, and AbaR4e are available from GenBank under accession numbers NC_011586, CP001921, and CP002522, respectively.

AbaR4 contains terminal imperfect inverted repeats, similar to *comM* specific islands and distinct direct repeats (ACTGA instead of ACCGC).

Concluding remarks. We showed here that AbaR islands found in isolates of EC II share a core structure similar to that of AbaR4 and common backbone elements for transposition. The results presented here and previously published data demonstrate the similar AbaR-type transposon structures in EC I and EC II isolates and indicate that both AbaRs originated independently from a common larger progenitor. AbaR4-type islands have evolved through the multiple events of insertions, deletions, and homologous recombination and could be distributed in isolates of EC I and EC II through the integration into distinct genomic sites: *pho* and *comM*, respectively. Recent studies report the worldwide domination of *A. baumannii* strains related to EC I and II, show the selective advantage for strains of this lineage, and require monitoring of further genetic evolution.

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