Complete genome sequence of hypervirulent and outbreak-associated *Acinetobacter baumannii* strain LAC-4: epidemiology, resistance genetic determinants and potential virulence factors

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*Acinetobacter baumannii* is an important human pathogen due to its multi-drug resistance. In this study, the genome of an ST10 outbreak *A. baumannii* isolate LAC-4 was completely sequenced to better understand its epidemiology, antibiotic resistance genetic determinants and potential virulence factors. Compared with 20 other complete genomes of *A. baumannii*, LAC-4 genome harbors at least 12 copies of five distinct insertion sequences. It contains 12 and 14 copies of two novel IS elements, ISAba25 and ISAba26, respectively. Additionally, three novel composite transposons were identified: Tn6250, Tn6251 and Tn6252, two of which contain resistance genes. The antibiotic resistance genetic determinants on the LAC-4 genome correlate well with observed antimicrobial susceptibility patterns. Moreover, twelve genomic islands (GI) were identified in LAC-4 genome. Among them, the 33.4-kb GI12 contains a large number of genes which constitute the K (capsule) locus. LAC-4 harbors several unique putative virulence factor loci. Furthermore, LAC-4 and all 19 other outbreak isolates were found to harbor a heme oxygenase gene (hemO)-containing gene cluster. The sequencing of the first complete genome of an ST10 *A. baumannii* clinical strain should accelerate our understanding of the epidemiology, mechanisms of resistance and virulence of *A. baumannii*.

Recognized as one of the most problematic bacterial pathogens due to emergence of multidrug-resistant (MDR) strains1, *Acinetobacter baumannii* has been responsible for a significant proportion of world-wide healthcare-acquired infections, including ventilator-associated pneumonia, surgical site and urinary tract infections and septicemia2–5. Additionally, MDR clinical isolates of this species have been found to infect military personnel wounded in combat zones6,7. Hospital outbreaks of *A. baumannii* infection are often associated with multidrug resistance in the causative strains8,9. Besides their intrinsic resistance to certain antibiotics due to the presence of native β-lactamase genes, poor permeability and efflux systems, *A. baumannii* strains have acquired a large array of antibiotic resistance mutations and genes, located either on the chromosome or plasmids10,11. Furthermore, clinical strains of *A. baumannii* resistant to carbapenems, the last resort antibiotics to treat infections caused by *A. baumannii*, have been found to possess acquired *bla* genes encoding several groups of carbapenem-hydrolyzing β-lactamases, such as *bla*OXA-23, *bla*OXA-24/33/40, and *bla*OXA-58. These *bla* genes appear to have been transferred via mobile genetic elements such as insertion sequences, transposons or plasmids.
Despite the clinical significance of *A. baumannii* infections, the molecular basis on the virulence and acquisition of multidrug resistance by *A. baumannii* remains largely unknown. To better understand the genome plasticity, natural history, epidemiology and acquisition of resistance and pathogenicity islandsgenes of *A. baumannii*, the complete genomes of 21 *A. baumannii* strains, including LAC-4, became available as of December 31, 2014\(^1\)\(^{-}\)\(^{30}\). In addition, the genomes of hundreds of *A. baumannii* strains have been sequenced to scaffold or contig levels (http://www.ncbi.nlm.nih.gov/genome/genomes/403). Most of these sequenced *A. baumannii* genomes are divided into 31 groups on basis of their sequence similarity. These efforts and other incomplete Whole Genome Shotgun (WGS) projects involving *A. baumannii* strains and strains of other *Acinetobacter* species\(^{11}\)\(^{-}\)\(^{12}\) will likely offer additional insights on epidemiology, phylogenetics, evolution of pathogenic strains and gene flows among *Acinetobacter* species including *A. baumannii*.

Several years ago, we described antibiotic resistance patterns and clonal relationships of 20 MDR clinical isolates of *A. baumannii* obtained from nosocomial outbreaks in Los Angeles County hospitals (LAC-1 to LAC-20)\(^5\). Our pulsed-field gel electrophoresis (PFGE) fingerprinting analysis indicated that these isolates appeared to have originated from eight epidemiologically distinct lineages\(^5\). More significantly, we identified the LAC-4 strain as hypervirulent in a mouse model of intranarial infection\(^1\)\(^{5}\) in comparison to other clinical isolates and laboratory strains of *A. baumannii*, including the eight representative LAC isolates and the widely studied clinical strain AYE\(^2\)\(^{6}\)\(^{-}\)\(^{8}\). The LAC-4 strain reliably reproduces the most relevant features of human pulmonary *A. baumannii* infection, including significant extrapulmonary dissemination and bacteremia\(^7\). Subsequent studies showed that LAC-4 exhibits high serum resistance, expresses a highly efficient heme utilization system\(^8\) and contains some unique structure and composition in its surface polysaccharide\(^9\), which may contribute to its hypervirulence. However, the precise mechanism of the hypervirulence of LAC-4 remains to be determined. Here we describe the complete genome sequence of the LAC-4 strain with special emphasis on the comparative genomics analyses to identify genomic regions that may contribute to the acquisition of antibiotic resistance and establishment of superior colonization and invasion by this hypervirulent strain.

**Results and discussion**

**Phylogenetic lineages based on trilocus multiplex PCR and MLST.** To understand the epidemiology and phylogenetics of 20 clinical isolates of *A. baumannii* (including LAC-4) obtained from four apparent nosocomial outbreaks, we first attempted to determine the clonal relationships among these isolates of *A. baumannii* by Trilocus multiplex PCR (TLM-PCR) analyses. Our results indicate that we can only type four isolates (LAC-11, LAC-12, LAC-13 and LAC-14) belonging to Global Clone (GC) II (Table 1). Since the TLM-PCR method failed to resolve phylogenetic relationships of most of these *A. baumannii* isolates, multilocus sequence typing (MLST) scheme based on Pasteur Institute approach was subsequently employed. Our results showed that previously non-typable isolates belong to unusual ST types (such as ST10, ST241 and ST417) (Table 1). Previously, PFGE profiling divided these 20 outbreak isolates into eight distinct clonal groups: LAC-1 to LAC-3; LAC-4; LAC-5 and LAC-8; LAC-6; LAC-7, LAC-9, and LAC-10; LAC-11 to LAC-14; LAC-15; and LAC-16 to LAC-20\(^5\). In accordance with the PFGE grouping\(^5\), MLST typed LAC-5 and LAC-8 to a rare ST241 (Table 1). Since these two strains were isolated during two separate outbreaks in a single hospital, it appears that the ST241 lineage persisted for at least four years (1997–2001) in the same facility. More interestingly, we found that LAC-4, which was much more virulent than other LAC isolates in mice\(^5\), belongs to ST10 (Table 1). Most importantly, two series of outbreaks were caused by ST10 strains in two separate hospitals (LAC-1 to LAC-4 in Hospital A; LAC-16 to LAC-20 in Hospital C) in Los Angeles County, California, during the late 1990s (Table 1), suggesting that ST10 strains were quite dominant in causing nosocomial outbreaks in Los Angeles County at the time, with LAC-4 being their representative. While LAC-1 to LAC-4 were all typed to ST10, the PFGE profile of LAC-4 diverged from those of LAC-1 to LAC-3 sufficiently to be grouped as a separate clone\(^5\), indicative of possible divergent evolution of LAC-4 from its original clone.

There have been few reports describing clinical isolates of *A. baumannii* belonging to ST10. Among 1237 *A. baumannii* strains with assigned STs in Pasteur Institute’s MLST database as of Oct 23 2014 (the most recent update), only three *A. baumannii* ST10 strains were listed. Recently, an ST10 strain of *A. baumannii* was isolated from a

| Table 1 | Molecular tests for 20 clinical isolates of *A. baumannii* obtained from Los Angeles County, CA |
|---------|--------------------------------------------------|
| Strain code | TLM- GC | cpo60 | fusA | gltA | pyrG | recA | rplB | rpoB | MLST-ST | hemO cluster |
|----------|---------|------|-----|-----|------|------|------|------|--------|------------|
| LAC-1    | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-2    | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-3    | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-4    | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-5    | NT      | 40   | 3   | 15  | 2    | 40   | 4    | 4    | 241    | +          |
| LAC-6    | NT      | 1    | 2   | 2   | 2    | 11   | 1    | 5    | 417    | +          |
| LAC-7    | NT      | 1    | 2   | 2   | 2    | 11   | 1    | 5    | 417    | +          |
| LAC-8    | NT      | 40   | 3   | 15  | 2    | 40   | 4    | 4    | 241    | +          |
| LAC-9    | NT      | 1    | 2   | 2   | 2    | 11   | 1    | 5    | 417    | +          |
| LAC-10   | NT      | 1    | 2   | 2   | 2    | 11   | 1    | 5    | 417    | +          |
| LAC-11   | II      | 2    | 2   | 2   | 2    | 2    | 2    | 2    | 2      | +          |
| LAC-12   | II      | 2    | 2   | 2   | 2    | 2    | 2    | 2    | 2      | +          |
| LAC-13   | II      | 2    | 2   | 2   | 2    | 2    | 2    | 2    | 2      | +          |
| LAC-14   | II      | 2    | 2   | 2   | 2    | 2    | 2    | 2    | 2      | +          |
| LAC-15   | NT      | 1    | 2   | 2   | 2    | 11   | 1    | 5    | 417    | +          |
| LAC-16   | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-17   | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-18   | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-19   | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |
| LAC-20   | NT      | 1    | 3   | 2   | 1    | 4    | 4    | 4    | 10     | +          |

TLM- GC, Trilocus multiplex assay Global Clone designation.  
MLST-ST, Multi-locus sequence type.  
NT, not typable.
wound of a Canadian patient who had been previously hospitalized in India37. This strain and a strain of *Klebsiella pneumoniae* were transmitted to five other patients in an Edmonton, Alberta hospital, resulting in the colonization of four patients and the death of one patient due to septic shock caused by the OXA-23-producing *A. baumannii* strain37. Furthermore, four of five MDR strains of *A. baumannii* (LAC-6, LAC-7, LAC-9 and LAC-10) isolated during another outbreak in Hospital A in 2001 were typed to an uncommon ST417, indicating ST417 became dominant in this hospital in 2001 (Table 1)5. These results show that there was a clonal succession of outbreaks in that hospital during a five-year span (1996–2001), transitioning from ST10 dominance in 1996–1999 to ST417 dominance in 2001. Previously reported clinical outbreaks were frequently caused by strains belonging to ST1 (Global Clone I)38–40, ST2 (Global Clone II)40,41 or ST1540. To our knowledge, in this report we describe the unusual ST10 (Table 1). Previous studies have shown that LAC-4 and eight other outbreak LAC strains belong to the mutator family of transposases, IS Finder database BLASTp search identifies ISEc39 as its closest homologue, sharing 72% transposase amino acid identities. ISEc39 produces TSD of 8 bases in length (see Supplementary Table S1 online) and its inverted repeats (IRL and IRR) are 26 bp in lengths. Interestingly, two copies of ISEc39 flank a large number of continuous loci (15 genes) which are predicted to be involved in copper resistance (Fig. 1B). Moreover, 22 copies of ISEc39 are scattered around the chromosome of LAC-4 (see Supplementary Table S1 online). Among these elements, 14 copies were found to create TSD sequences of 9 bases in length as expected, while one element has an 8-base TSD (see Supplementary Table S1 online). Finally, we found 14 ISEc39 elements in LAC-4 genome, each with a TSD of 3 bases (see Supplementary Table S1 online).

LAC-4 genome sequences and general features. Our MLST analysis showed that LAC-4 and eight other outbreak LAC strains belong to the unusual ST10 (Table 1). Previous studies have shown that LAC-4 exhibits several distinctive attributes (iron utilization and unusual repeating unit composition of surface polysaccharides) that may contribute to its hypervirulence in mice33,36. Since none of the *A. baumannii* strains whose genomes were completely sequenced belongs to ST10, while one IS element (ISABC26) is located on the LAC-4 chromosome (see Supplementary Table S1 online). This IS is 1,318 bp in length and contains one gene encoding a transposase with 402 amino acids. While transposase protein sequence BLAST analysis indicates ISABC26 belongs to the mutator family of transposases, IS Finder database BLASTp search identifies ISfs39 as its closest homologue, sharing 72% transposase amino acid identities. ISABC26 produces TSD of 8 bases in length (see Supplementary Table S1 online) and its inverted repeats (IRL and IRR) are 26 bp in lengths. Interestingly, two copies of ISABC26 flank a large number of continuous loci (15 genes) which are predicted to be involved in copper resistance (Fig. 1B). Moreover, 22 copies of ISABC13 are scattered around the chromosome of LAC-4 (see Supplementary Table S1 online). Among these elements, 14 copies were found to create TSD sequences of 9 bases in length as expected, while one element has an 8-base TSD (see Supplementary Table S1 online). Finally, we found 14 ISABC13 elements in LAC-4 genome, each with a TSD of 3 bases (see Supplementary Table S1 online).

The LAC-4 genome also includes two plasmids (Table 2). Plasmid pABLAC1 contains nine predicted ORFs (ABLAC_p100010–ABLAC_p100090). Among these loci, ABLAC_p100020 (rep) encodes a replicase (Aci3) belonging to group 3 of replicases defined80. Fifty-six base pairs upstream of the *rep* gene, four copies of iterons were found with a sequence of 5′- TAAAAAGGAGTTAACCTTGCAT-3′, which is identical to those observed in replicons Ab203-Aci3 and Ab736-Aci784. Iterons have been shown to be involved in controlling plasmid replication via interacting with replicase proteins85. Additionally, an AT-rich sequence (5′-AAAAATAT-3′) identical to that found in the pRAY plasmid86 was located 37 bp downstream of the fourth iteron and 11 bp upstream of *rep* start codon. The AT-rich element and the iterons presumably serve as the oriV site of the plasmid. Many of predicted proteins of plasmid pABLAC1 loci (ABLAC_p100020 through ABLAC_p100200) share identical amino acid sequences with those encoded by genes harbored by the plasmid (pD1279779) in *A. baumannii* strain D127977933. On the other hand, plasmid pABLAC2 shares nearly 100% with a series of plasmids related to pRAY35,45, in particular pRAY*. Of particular note, two pABLAC2 loci (ABLAC_p100050 and ABLAC_p200060) encode homologues of MobA and MobC, respectively, which are known to be involved in plasmid mobilization; it is tempting to speculate that these mobilization genes may contribute to the transfer of the aminoglycoside resistance

| Table 2 | General features of the *A. baumannii* LAC-4 genome |
| Feature | Chromosome | pABLAC1 | pABLAC2 |
| Total number of base pairs | 3,954,354 | 8,006 | 6,076 |
| G + C content (%) | 39 | 32 | 39 |
| Number of protein-coding genes* | 3,788 | 9 | 8 |
| Number of rRNA operons | 6 | 0 | 0 |
| Number of tRNA/tmRNA genes | 73 | N/A | N/A |
| Number of genomic island-like regions | 12 | 0 | 0 |
| Number of Insertion Sequences (ISs) | | 19 (ISAb1) | 22 (ISAb13) |
| | | 14 (ISAb125) | 12 (ISAb29) |
| | | 22 (ISAb13) | 14 (ISAb26) |

*These features were obtained based on annotations using the Glimmer 3.02 program.

*NA, not applicable.*
gene (ABLAC_p200010, ant(2')-Ia or aadB) carried on this plasmid. Moreover, two copies of AT-rich sequence 5'-AAAAATAT-3' were found within the coding region of a predicted ORF (ABLAC_p200080). However, no potential replication gene (rep) or iterons were found on pABLAC2. Similar to pRAY series of plasmids, no plasmid partitioning or restriction/modification systems were identified in both plasmids of LAC-4.

Comparative genomics of LAC-4 with 20 other completely sequenced A. baumannii genomes. All protein-coding genes (CDSs) of LAC-4 were analyzed by mGenomeSubtractor-based in silico subtractive hybridization for presence of homologues against 20 other completely sequenced A. baumannii genomes. Our results indicate that the numbers of homologous CDSs (H-value > 0.42) among these genomes are 2,276 (Fig. 2A), representing 60% of the total CDSs in LAC-4. The LAC-4 chromosome is also presented in a continuous linear format showing the locations of all of its CDSs each with a degree of “blackness” reflecting relative conservation among the other 20 A. baumannii genomes (Fig. 2B). In particular, the LAC-4 genome exhibits the highest level of sequence identities to the BJAB0715 genome with 3,451 conserved genes (H-value > 0.42) (see Fig. 2A, 2B; Supplementary Fig. S1 online). When Acinetobacter
Figure 2 | The mGenomeSubtractor-based in silico subtractive hybridization of the LAC-4 genome against genomes of twenty other completely sequenced A. baumannii isolates. The twenty subject A. baumannii chromosomes include: ATCC 17978 (NCBI accession no. NC_009085), SDF (NC_010400), AYE (NC_010410), ACICU (NC_010611), AB0057 (NC_011586), AB307-0294 (NC_011595), 1656-2 (NC_017162), MDR-ZJ06 (NC_017171), TCDC-AB0715 (NC_017387), MDR-TJ (NC_017847), TYTH-1 (NC_018706), D1279779 (NC_020547), BJAB07104 (NC_021726), BIAB0868 (NC_021729), BJAB0715 (NC_021733) and ZW85-1 (NC_023028) available at NCBI RefSeq project; and AB031 (GenBank accession no. CP009256), AC29 (CP007535), AB030 (CP009257) and AbH120-A2 (CP009534) available at GenBank. (A) Histogram of BLASTn-based H-values for all 3,788 annotated protein-coding genes in the LAC-4 chromosome against all 20 subject chromosome sequences (color-coded). The H-value reflects the degree of similarity in terms of the length of match and the degree of identity at a nucleotide level between the matching gene in the subject genome and the query gene examined. The conserved genes were identified based on each of the obtained H-values greater than 0.42. The genome of LAC-4 shows the most significant sequence identity to the BJAB0715 genome with 3,451 conserved genes (H-value > 0.42) among all the other 20 completely sequenced genomes; whereas, the lowest sequence identity to the SDF genome with 2,511 conserved genes (H-value > 0.42). (B) Chromosome map of LAC-4 with gene black/white-shade-coded based on the number of comparator A. baumannii genomes identified as harboring a nucleotide sequence-conserved homologue. Genes shown in absolute black (‘20’) are conserved across all 20 A. baumannii comparator genomes, with genes shown in decreasing shades of black being conserved in lower numbers of A. baumannii comparator genomes, while at the other extreme those shown in white (‘0’) are unique to LAC-4. Non-coding regions are shown as gaps. The genomic island-like hyper-variable regions (also see Table 3) are marked by red rectangles.
Table 3 | Genomic island (GI)-like regions identified in the LAC-4 chromosome

| Region | Coordinates [CDS] | Length (kb) | G + C% | Features |
|--------|------------------|-------------|-------|----------|
| GI1    | 120,830–133,655 [ABLAC_01100–01230] | 12.8 | 42 | Novel composite transposon Tn6250 (formed by ISAba1 elements) encompassing entire island containing IS1006, partial ISVsaa3, streptomycin and sulphonamide resistance genes, conjugal transfer functions TraA and TraD |
| GI2    | 547,857–581,621 [ABLAC_05290–05570] | 33.7 | 38 | Novel IS elements (ISAba26) flanking a 15-gene copper resistance cluster; another novel IS element (ISAba25); integrase |
| GI3    | 788,377–800,975 [ABLAC_07480–07600] | 12.6 | 43 | Novel IS elements (ISAba25); RND-type multidrug efflux pump proteins Adel, Adej and Adek and a partial ISAba1 |
| GI4    | 940,403–951,920 [ABLAC_08880–08980] | 11.5 | 40 | ISAba25; alpha/beta hydrolase superfamily; a gene encoding for ComEC-like protein; enzymes for biosynthesis of lipoproteins; purine biosynthesis enzymes |
| GI5    | 1,229,550–1,257,942 [ABLAC_11500–11860] | 28.4 | 40 | Insertion site: Arg IRNA gene (ABLAC_100580); phage proteins |
| GI6    | 1,571,326–1,611,225 [ABLAC_14900–15460] | 39.9 | 39 | Two ISAba13 elements; mostly phage proteins |
| GI7    | 1,730,338–1,741,047 [ABLAC_16600–16720] | 10.7 | 35 | Integrase; phage proteins |
| GI8    | 1,941,480–1,993,076 [ABLAC_18640–19030] | 51.6 | 39 | Insertion site: Ser tRNA gene (ABLAC_t00260); IS |
| GI9    | 2,963,891–2,993,411 [ABLAC_28230–28700] | 29.5 | 36 | Mostly phage protein; ISAba13, ISAba125, ISAba25, ISAba26 |
| GI10   | 3,028,724–3,058,412 [ABLAC_29030–29480] | 29.7 | 35 | Insertion site: tmRNA gene (ABLAC_t00730); phage proteins; ISAba13 |
| GI11   | 3,367,720–3,386,189 [ABLAC_32320–32540] | 18.5 | 40 | ISAba26; gtsh, frmA and frmB genes; Genes similar to the plasmid p3ABAYE and pABT12 are found |
| GI12   | 3,852,499–3,882,676 [ABLAC_36850–37160] | 33.4 | 35 | The K locus, which consists of genes encoding enzymes involved in biosynthesis of capsular polysaccharides, including two series of enzymes responsible for biosynthesis of α-L-frucosamine and α-8-epi-legionaminic acid, two sugars of the surface polysaccharide repeating units; six ORFs of unknown function which are unique for LAC-4 among completely sequenced A. baumannii genomes |

* The G + C content of the LAC-4 chromosome is 39%.

baumannii genome groups are considered, LAC-4 was found to belong to Genome Group 3, represented by the genome of BJAB7015, as verified by a phylogenetic tree composed of 45 completely or partially sequenced A. baumannii genomes (see Supplementary Fig. S1 online).

A total of 12 genomic islands were identified (Fig. 2B and Table 3). The GI1 (Table 3) consists of a novel transposon (Tn6250) composed of two ISAba1 elements at the ends, and an IS1006 internally next to a partial ISVsa3 element (Fig. 1A). Additionally, this genomic island harbors resistance genes for streptomycin (strA and strB) and for sulphonamides (sul2), thus this GI is a resistance island (Table 3). The 34 kb GI2 contains two novel IS elements (ISAba26) near the ends (Table 3 and Fig. 1B). Notably, these two IS elements sandwich a long 15-gene cluster (ABLAC_05330 to ABLAC_05470) coding for various proteins involved in copper resistance (Fig. 1B), representing the second resistance island found in LAC-4. Most of the genes of this gene cluster were also present in the genome of A. baumannii ATCC 17978, and to a lesser extent, in those of A. baumannii strains AB0057 and AYE (Fig. 3A). Outside one of the ISAba26 elements (ABLAC_05500), another novel IS element (ISAba25) and a phage integrase gene (ABLAC_05570) are found (Table 3 and Fig. 3A), implicating the roles of mobile elements in the transfer of this GI. Furthermore, GI3 is bounded by two copies of a novel IS element (ISAba25; transposase ORFs: ABLAC_07480–07500 and ABLAC_0758–07600) and it harbors a partial ISAba1 element and genes for the AdeKJ, which are components of an RND-type efflux pump system involved in resistance phenotypes of multiple classes of antibiotics (Fig. 1B).

Several GIs (GI5, GI6, GI7, GI8, GI9 and GI10) contain many genes encoding phage-derived proteins of unknown functions; the significance of these GIs remains to be elucidated (Table 3). Among these GIs, nearly all of the phage-derived genes of the GI8 have no homologues in any of the 20 other completely sequenced A. baumannii genomes (except several transposase genes and Zn-dependent protease gene) (data not shown). Moreover, GI11 contains a number of genes encoding putative proteins/enzymes with functions of detoxification of a variety of xenobiotic compounds (Fig. 3B); for example, glutathione S-transferase (ABLAC_32340, gstB), S-formylglutathione hydrolase (ABLAC_32430, frmb) and S-(hydroxymethyl)glutathione dehydrogenase (ABLAC_32440, frmA) are involved in resistance to and detoxification of formaldehyde and chlorine. Interestingly, other than the frmA gene, most of the GI11 genes are unique to LAC-4 among the 21 completely sequenced A. baumannii genomes (data not shown). Finally, the GI12 contains the K (capsule) locus which encodes enzymes responsible for biosynthesis of the surface polysaccharide of LAC-4 (Fig. 4). Gene product homology analyses indicate that the K locus gene cluster harbors genes encoding two series of enzymes (FniA, FniB, FniC and LegI–6) which apparently catalyze biosynthesis of α-L-frucosamine and α-8-epi-legionaminic acid, respectively. These two sugars are the two precursor sugars (other than α-D-glucosamine, which is supplied by cellular metabolism) for the biosynthesis of three-sugar repeating unit of LAC-4 surface polysaccharide recently determined to consist of α-L-frucosamine, α-D-glucosamine and α-8-epi-legionaminic acid. Interestingly, six ORFs (orfK1-6, ABLAC_36970–ABLAC_37020) in the middle of the gene cluster encode protein products (two of which are oxidoreductases) sharing no homology with any proteins in the 20 completely sequenced A. baumannii genomes (Fig. 4). Among the 20 A. baumannii strains whose genomes were completely sequenced, several have acquired resistance islands of various sizes. For example, strain AYE harbors an 86-kb resistance island in which 45 resistance genes are located. Similarly, MDR-TJ contains a 42-kb resistance island with four drug resistance genes, while MDR-ZJ06 has a 38-kb resistance island (AbaR22) containing 40 genes, five of which are involved in antimicrobial resistance.

Antibiotic resistance of LAC-4 and resistance genetic determinants. The recent emergence and rapid dissemination of multidrug and pandrug resistant A. baumannii has caused significant burdens in clinical management of infections world wide. While we previously
described antimicrobial susceptibility profile of LAC-4 towards 17 antibiotics, minimal inhibitory concentration (MIC) values were not presented5. Our MIC results of 23 antimicrobial drugs indicate that this strain is resistant to 13 of the 23 antibiotics (six of eight classes) tested, including ampicillin, carbenicillin, piperacillin, most of the cephalosporin analogs, three of four aminoglycosides, nalidixic acid and ciprofloxacin, trimethoprim and chloramphenicol (see Supplementary Table S2 online). Compared to the most highly resistant strains (AYE, ACICU, MDR-TJ and MDR-ZJ06) among the 20 A. baumannii strains whose genomes were completely sequenced, the MDR LAC-4’s level of resistance is moderate (e.g., susceptible to imipenem and meropenem, intermediate to amikacin), which reflects the relatively early isolation of this strain (in 1997) from a hospital in Los Angeles. Nevertheless, the availability of complete genome sequences of A. baumannii strains with varied degrees of antimicrobial resistance (including the moderately resistant LAC-4) is highly useful for the scientific research community, especially from the standpoints of emergence and dissemination of antimicrobial resistance genes.

To better understand the repertoire of MDR genetic determinants and organization, in-depth analysis of the genome sequences of LAC-4 was performed. Consistent with the fact that LAC-4 was not among the most resistant strains analyzed in our 2008 report6, the LAC-4 genome harbors a moderate number of genetic determinants, some of which are linked to mobile genetic elements (IS or Tn), with a potential to encode resistance functions observed in this bacterial strain (Table 4). For example, genes potentially encoding for all four classes of β-lactamases (Classes A, B, C and D) have been found (Table 4). Two of such loci (ampC and blaOXA-23) are closely associated with ISAba1 elements (Fig. 1B and 1A, respectively) which may provide exogenous promoter functions, consistent with LAC-4 being resistant to piperacillin, older versions of the penicillins, and several cephalosporin antibiotics (see Supplementary Table S2 online). ISAba1 has also been found in the genomes of other A. baumannii strains associated with antibiotic resistance genes (including ampC), most likely driving robust expression of the resistance phenotypes17,47. While a blaOXA-51-like gene (ABLAC_23600) is found in the LAC-4 genome (Table 4) as expected, the absence of blaOXA-23-like, blaOXA-40-like and blaOXA-58-like genes (data not shown) in the genome explains its susceptible phenotypes to imipenem and meropenem (see Supplementary Table S2 online). In contrast, in MDR strains AB0057, MDR-TJ, MDR-ZJ06, BJAB07104, BJAB0868 and BJAB0715, the presence of blaOXA-23 could account for their carbapenem resistance17,18,25. Aminoglycoside 6-phosphotransferase [aph(6)-Id, i.e., strB] and/or aminoglycoside 3’-phosphotransferase [aph(3’)-Ib, i.e., strA] were known to contribute resistance to streptomycin49. The existence of several aminoglycoside modification enzyme genes [ABLAC_01120 (strA), ABLAC_01130 (strB), and ABLAC_p200010 (ant(2’)-Ia)] in the LAC-4 genome (Table 4) and an association of an ISAba1 with two of these loci (strA and strB) in the context of a novel transposon Tn6250 (Fig. 1A) correlate well with the strain’s resistance to streptomycin, gentamicin, kanamycin and tobramycin (see Supplementary Table S2 online). Finally, genes encoding a series of proteins for major facilitator superfamily (MFS), resistance-nodulation-division (RND) family, and multidrug and toxic compound extrusion (MATE) family of efflux pumps were also located in the LAC-4 genome (Table 4). Notably, there are at least five complete sets of RND type of efflux pump systems in LAC-4 (Table 4). Besides the AdeIJK genes linked with several IS elements described above in GI3 (Table 3 and Fig. 1B), four other sets of RND type efflux pump systems include AdeABC (encoded by ABLAC_26430–26450), AdeFGH (encoded...
phenotypes of clinical strains to tigecycline. Additionally, AdeIJK in over-expression of classes of antibiotics and other toxic compounds/metals in bacteria. Membrane-associated transporters (Table 4) confer resistance to diverse nonfunctional copy of IS locus of unknown function (ABLAC_07540) and an apparent nizable regulatory gene(s). Upstream of unobservable genes, there exist a gene cluster encoding resistance to a large variety of antibiotics and toxic compounds. For instance, there are three sets of RND efflux pump systems in A. baumannii strains (AdeABC, AdeIJK and AdeFGH). While LAC-4 genome harbors three gene clusters each of which separately encodes one of the above RND type efflux pumps, no apparent two-component system gene homologues adeRS was found upstream of adeABC gene. Such two-component systems (AdeRS) were found to be critical in regulating expression of AdeABC efflux pumps in several strains of A. baumannii. Interestingly, Sun and colleagues observed that the insertion of an ISaba1 into the adeS gene resulted in over-expression of adeABC operon hence the non-susceptible phenotypes of clinical strains to tigecycline. Additionally, AdeIJK appears to be regulated by AdeN, a TetR type of regulator in some strains. On the contrary, the LAC-4’s adeIJK genes exist in an unusual genomic context. These genes are not linked with any recognizable regulatory gene(s). Upstream of adeIJK genes, there exist a locus of unknown function (ABLAC_07540) and an apparent nonfunctional copy of ISaba1 element (Fig. 1B). All these genes are flanked by two copies of novel IS element ISaba1 (Fig. 1B). To our knowledge, this is the first report of a gene cluster encoding an RND efflux pump system being flanked by two ISaba1 elements, which may modify the expression of the efflux pump genes, thus conferring resistance to a number of antimicrobial agents.

Potential virulence factors. Because our recent studies have shown that the LAC-4 strain is much more virulent than other A. baumannii strains in the mouse model of intranasal infection, the LAC-4 genome was searched for genes coding for potential virulence factors against entries from VFDB. A total of 615 gene hits were generated which may modify the identification, evaluation and validation of virulence factors in this strain and the species (see Supplementary Table S3 online). Potential virulence factor loci that are unique for LAC-4 include ABLAC_03200 (which may encode a fimbrial protein, PilE), ABLAC_37000 and ABLAC_37010 (two genes of K locus gene cluster) (see Supplementary Table S3 online). In addition, several other potential virulence factor loci are found in only 1 to 4 genomes of the 20 completely sequenced A. baumannii genomes: ABLAC_05370 (encoding a transcriptional activator protein CusR/CopR), ABLAC_05450 (encoding an Fe2+ transport system protein FeoB) and K locus gene cluster genes ABLAC_36940–39960 (encoding UDP-N-acetylgalactosamine 2-epimerase (FnuC), reductase (FnuB) and 4,6-dehydratase, 3-and 5-epimerase (FnaA)) (see Supplementary Table S3 online and Fig. 4). Additional genes of the K locus gene cluster (ABLAC_36850, and ABLAC_36870–ABLAC_36910) were also identified as potential virulence factor genes (see Supplementary Table S3 online). Recently, Russo and colleagues identified, within the K locus gene cluster of A. baumannii strain AB307-0294, two loci pil and epsA (encode a putative protein tyrosine kinase and a putative polysaccharide export outer membrane protein, respectively) that are required for capsule-positive phenotype, for survival in human serum and survival in a rat soft tissue infection model. These encoded proteins of these two genes shared 90% and 95% identity over the entire protein lengths with the products of loci ABLAC_37120 and ABLAC_37100, respectively, suggesting importance of K locus gene cluster genes to the virulence of A. baumannii. Figure 4 | LAC-4 K locus gene organization and functional assignment. GI12 harbors genes of K locus in LAC-4. Color coding scheme follows that of Hu et al. Two of the three-sugar repeating unit recently determined for LAC-4 surface polysaccharide can be produced via reactions catalyzed by gene products of the genes in the cluster (bottom). The product of wafP gene probably catalyzes the formation of the 1→3 glycosyl bond between α-D-FucNAc and α-D-GlcNAC. The wbpV gene (marked by *) is a pseudogene (with two separate ORFs) as a result of a point mutation.
| Antimicrobial agent class | Gene products/function | Gene name (if available) | LAC-4 protein locus tag |
|--------------------------|------------------------|-------------------------|------------------------|
| **β-lactams and**<br>cefalosporins | Class A β-lactamase | ABLAC_20980 | |
| | Class B β-lactamase (metallo-β-lactamase superfamily) | ABLAC_06190, ABLAC_08350, ABLAC_25540, ABLAC_27020, ABLAC_27490, ABLAC_34080 | |
| | Class C β-lactamase | ampC | ABLAC_10940, ABLAC_14670, ABLAC_34320 |
| | Class D β-lactamase | blaOXA-236, blaOXA-48, blaOXA-51-like | ABLAC_23600 |
| **Aminoglycosides** | Aminoglycoside 6-phosphotransferase | strB [aph(6)-Id] | ABLAC_01130 |
| | Aminoglycoside 3′-phosphotransferase | strA [aph(3′)-Ib] | ABLAC_01120 |
| | Aminoglycoside 2′-nucleotidytransferase | ant(2′)-Ia | ABLAC_00010 |
| **Phenicols** | Chloramphenicol acetyltransferase | ABLAC_08080 |
| **Fluoroquinolones** | Mutated GyrA [Ser-83-Leu], Mutated ParC [Glu-84-Lys] | ABLAC_08740, ABLAC_35720 |
| **Folate pathway inhibitors** | Dihydropteroate synthase | sul2 | ABLAC_01810 |
| | Dihydropteroate synthase type 2 | copD, copC, acp, capS, capR, capB, czcC-like, czcB-like, czcA-like | ABLAC_05320–ABLAC_05470 |
| **Heavy metals** | Copper resistance protein CopD, | copD, | ABLAC_00030 |
| | Copper resistance protein CopC, | capC, | ABLAC_00630 |
| | Copper-exporting ATPase, | capR, | ABLAC_13820 |
| | Transcriptional activator protein CapR, | capA, | ABLAC_06230 |
| | Copper-resistance protein CapB, | capB, | ABLAC_01810 |
| | Heavy metal RND efflux outer membrane protein CzcC-like, | czcC-like, | ABLAC_05320–ABLAC_05470 |
| | Copper binding periplasmic protein CzcB-like, | czcB-like, | ABLAC_05320–ABLAC_05470 |
| | Chromate transport protein, | chrA | ABLAC_22900 |
| | Chromate transporter | ABLAC_22910 |
| **Multiple agents** | MFS (major facilitator superfamily) efflux pump | ABLAC_00030, ABLAC_00610 |
| | Bcr/Cfr subfamily | bcr/cfrA | ABLAC_06830 |
| | MFS permease | ABLAC_31900 |
| | RND (resistance-nodulation-division) family efflux pump | ABLAC_00030 |
| | Membrane fusion protein | acrA-like | ABLAC_00030 |
| | RND pump protein | acrB-like | ABLAC_000320, ABLAC_06650, ABLAC_11930 |
| | Membrane fusion protein | adeA | ABLAC_26450 (partial gene) |
| | RND pump protein | adeB | ABLAC_26440 |
| | Outer membrane protein | adeC | ABLAC_26430 |
| | Membrane fusion protein | adeD | ABLAC_07530 |
| | RND pump protein | adeE | ABLAC_07520 |
| | Outer membrane protein | adeF | ABLAC_07510 |
| | Membrane fusion protein | adeG | ABLAC_11940 |
| | RND pump protein | adeH | ABLAC_11930 |
| | Outer membrane protein | adeI | ABLAC_11920 |
| | RNA pump protein | adeJ-like | ABLAC_08370 |
| | Co/Zn/Cd efflux system RND type | czcA | ABLAC_02870 |
| | | czcB | ABLAC_02860 |
| | | czcC | ABLAC_02860 |
| | Copper efflux system RND type | czcA-like | ABLAC_05430 |
| | | czcB-like | ABLAC_05420 |
| | | czcC-like | ABLAC_05410 |
| | MATE (multidrug and toxic compound extrusion) family efflux pump | norM | ABLAC_01030 |
| | | matE | ABLAC_00610 |
| | | qacE11-like | ABLAC_06380 |
| | SMR (small multidrug resistance) family protein | ABLAC_14500 |
| | ABC transporter | ABLAC_02880 |
| | Cadmium, cobalt and zinc/H(+)-K(+)- antiporter | ABLAC_14500 |
**Methods**

**Bacterial strains.** The 20 outbreak strains (LAC-1 to LAC-20) were collected from four apparent clinical nosocomial outbreaks from 3 hospitals in Los Angeles County between 1996 and 2004 and obtained from Los Angeles County Public Health Laboratory. The antimicrobial susceptibility and genetic profiles of these non-duplicate isolates were described in details previously. Specifically, strains LAC-1 through LAC-5 were isolated from an outbreak in Hospital A that lasted for several years (1996–1999), while LAC-6 to LAC-10 were from the 2001 outbreak in the same hospital. Separately, strains LAC-11 to LAC-15 were obtained from an outbreak in Hospital B during 2003–2004. Furthermore, there was an outbreak in Hospital C spanning 1997 and 1998 where LAC-16 to LAC-20 were archived.

**Clonal relationships and sequence typing.** Tri-locus multiplex PCR as described by Turton and colleagues was used to determine clonal relationships of these A. baumannii clinical isolates [Global Clones (GC) I, II or III; also known as International Clones I, II or III]. Multi-locus sequence typing (MLST) was performed based on methods of Diancourt et al. PCR amplification was performed in separate reactions of a final volume of 50 μl. After amplification, aliquots of the PCR reactions were used to tag agarose gel electrophoresis analysis. If successful, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced on an ABI 3730 automated fluorescent sequencer. Determination of the sequence type was carried out using details on the MLST scheme at www.pasteur.fr/mlst. To obtain MLST ST assignments for A. baumannii strains whose genomes are complete, genome FASTA sequences were retrieved from NCBI and uploaded in the “batch sequence query” mode of MLST (Pasteur) database on the Acinetobacter baumannii MLST Databases website (http://pubmlst.org/abbaumannii/), which will generate allelic profiles and STs.

**Genomic DNA sequencing, assembly and gap closing.** The LAC-4 strain was grown overnight at 37°C to stationary phase in LB medium and total DNA was isolated from harvested cells. The genome sequence of LAC-4 was first determined using Roche 454 FLX+ and assembled with the GS De Novo Assembler, resulting in sequences with 43.5-fold coverage. Then 105 large contigs (≥500 kb) were obtained by a combination of re-sequencing by Illumina HiSeq2000 (paired-end sequencing for 400-bp library, with 255.6-fold coverage) and Illumina Miseq (mate-pair sequencing for 3000-bp library, with 408-fold coverage). Finally, gaps between these contigs were closed by genomic PCR and sequencing of PCR products using conventional Sanger method (Applied Biosystems 3730 Genetic Analyzer). An integrity check that performs a BLASTp analysis on neighboring pairs of proteins identified 37 pairs of proteins whose genes that either have or have split into two open reading frames (ORFs) due to sequencing errors. Additional PCR and DNA sequencing analysis have either corrected sequencing mistakes (likely as a result of 454 sequencing errors around homopolymer nucleotides) or combined gene pairs into single pseudogenes.

**Genome annotation and comparative genomics analysis.** The replication origin (oriC) in LAC-4 genome was predicted by OriFinder. The assembled genome sequences were annotated by using programs Glimmer 3.02 for identification of protein-coding sequences (CDSs) with manual validation of predicted CDS on the
basis of annotations of the BJAB7015 genome2 and the CRMA database3, 4. RNAseq data for mRNA genes4 and, tRNAseq data for tRNA genes5. CDS functions were predicted using BLASTp searches6 of the NCBI non-redundant database followed by manual curation using the annotations of A. baumannii ATCC 17978 (NCBI accession no. NC_009083.1) and BJAB7015 (NC_021733.1) as references. The virulence factor genes were predicted with the BLASTp searches against the virulence factor database of the Joint Fleming Development (JFD)7. The mobile genetic elements in the LAC-4 genome sequences were detected by the following online tools and/or open-access database and manual examinations: MobilerFINDER for the tRNA/tmRNA gene-related genomic islands (GI)8, IslandViewer for the island-like regions9 and IS Finder for insertion sequence (IS) elements10. New IS names were provided by the curators of IS database10, while transposon (Tn) numbers were assigned by the Tn Number Registry11. The gene clusters carried by genomic islands (GIs) or involved in the heme utilization in the sequenced A. baumannii genomes were aligned by using the standalone package MultiGenes Blast12, an approach also known as “BLASTp searches + hit collocation.”

The genome sequence comparisons of LAC-4 with the other 20 completely sequenced A. baumannii genomes were performed with the rapid multiple genome alignment tool mGenomeSubtractor13. All the annotated LAC-4 protein-coding genes (as served query) were examined by mGenomeSubtractor-facilitated BLASTn searches, using an H-value cut-off > 0.42 for conserved genes, against the other A. baumannii genomes (as served subject). The H-value (0 < H-value ≤ 1.0) reflects the degree of similarity in terms of the length of match and the degree of identities at a nucleotide level between the matching gene in the subject genome and the query gene examined14.

GenBank accession number. The genome sequences of the LAC-4 chromosome and two plasmids pABLAC1 and pABLAC2 have been submitted to the GenBank under accession numbers CP007712, CP007713, and CP007714, respectively.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of the clinical isolates or strains were determined using the broth microdilution protocols of Clinical and Laboratory Standards Institute15 according to methods described previously16. Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) were used as quality control strains in the testing.

Molecular detection of hemO cluster genes. PCR primer pairs specific for each gene of the eight-gene hemO cluster17 were designed based on alignment of gene cluster sequences of six A. baumannii strains [strains ACICU7, SDF-2, MDR-ZJ061, TYYH-1, TDCC-AB07115, and AB005718], available as of May, 2013. The sequence alignment was performed by using Lasergene MegaAlign program from DNASTAR, Inc. (Madison, WI). Primers were chosen from regions of highly conserved sequences and are shown in Supplementary Table S5 online. The PCR amplification of the hemO gene cluster was performed for the clinical isolates using genomic DNA as a template. Individual PCR reactions involved a 30 μl reaction mixture containing 1 × 5 PRIME MasterMix, 200 μM of forward and reverse primers, and 1 μl of genomic DNA template. Amplification was carried out with 5 min at 95°C; 36 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and 10 min at 72°C. The resulting PCR products were visualized and imaged through agarose gel electrophoresis analysis.

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Author contributions
H.H.X., X.H., H.Y.O. and W.C. conceived the study, X.H., S.N.K., B.M.M. and P.J.E. performed experiments, H.Y.O. and H.H.X. performed genomics and bioinformatics analyses. H.H.X., H.Y.O., W.C., X.H., Z.D. and M.O. wrote the paper. All authors read and approved the final manuscript.

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