Transcriptome Remodeling of *Acinetobacter baumannii* during Infection and Treatment

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**ABSTRACT** *Acinetobacter baumannii* is an increasingly common multidrug-resistant pathogen in health care settings. Although the genetic basis of antibiotic resistance mechanisms has been extensively studied, much less is known about how genetic variation contributes to other aspects of successful infections. Genetic changes that occur during host infection and treatment have the potential to remodel gene expression patterns related to resistance and pathogenesis. Longitudinal sets of multidrug-resistant *A. baumannii* isolates from eight patients were analyzed by RNA sequencing (RNA-seq) to identify differentially expressed genes and link them to genetic changes contributing to transcriptional variation at both within-patient and population levels. The number of differentially expressed genes among isolates from the same patient ranged from 26 (patient 588) to 145 (patient 475). Multiple patients had isolates with differential gene expression patterns related to mutations in the *pmrAB* and *adeRS* two-component regulatory system genes, as well as significant differences in genes related to antibiotic resistance, iron acquisition, amino acid metabolism, and surface-associated proteins. Population level analysis revealed 39 genetic regions with clade-specific differentially expressed genes, for which 19, 8, and 3 of these could be explained by insertion sequence mobilization, recombination-driven sequence variation, and intergenic mutations, respectively. Multiple types of mutations that arise during infection can significantly remodel the expression of genes that are known to be important in pathogenesis.

**IMPORTANCE** Health care-associated multidrug-resistant *Acinetobacter baumannii* can cause persistent infections in patients, but bacterial cells must overcome host defenses and antibiotic therapies to do so. Genetic variation arises during host infection, and new mutations are often enriched in genes encoding transcriptional regulators, iron acquisition systems, and surface-associated structures. In this study, genetic variation was shown to result in transcriptome remodeling at the level of individual patients and across phylogenetic groups. Differentially expressed genes include those related to capsule modification, iron acquisition, type I pili, and antibiotic resistance. Population level transcriptional variation reflects genome dynamics over longer evolutionary time periods, and convergent transcriptional changes support the adaptive significance of these regions. Transcriptional changes can be attributed to multiple types of genomic change, but insertion sequence mobilization had a predominant effect. The transcriptional effects of mutations that arise during infection highlight the rapid adaptation of *A. baumannii* during host exposure.
demonstrated that pathogens mutate during persistent host infection, and many of
these mutations are likely pathoadaptive (2–10). By using high-resolution genomic
analysis of longitudinal series of Acinetobacter baumannii isolates, we recently found
that newly arising nonsynonymous nucleotide substitutions and insertion sequence
events were enriched in certain functional classes of genes, including two-component
regulatory systems (TCRS) and other transcriptional regulators, iron acquisition and
other transporters, and genes coding for surface-associated proteins like capsule
and pilus genes (11). Here we explore how these mutations impact the transcriptome
and whether there is evidence for convergence toward a common transcriptional
profile in clinical A. baumannii strains. Population level genomic analysis of A. bauman-
nii strains also reveals a dynamic genome modified by mutations, IS events, deletions,
and gene acquisitions (12, 13). Therefore, we also assessed how population level
genetic variation alters the transcriptome across major lineages. Understanding the
impact of these genetic changes can reveal potential new targets for intervention that
would impair the ability of A. baumannii to persist in the human host.

A. baumannii infections are increasingly difficult to treat because of high levels of
antibiotic resistance in a majority of strains causing health care-associated infections
(14). Previous studies have emphasized the importance of iron acquisition, transporters,
and biofilm formation for virulence (15–17), but studies of human clinical isolates are
limited. Transcriptional studies involving A. baumannii responses to iron or zinc limi-
tation, antibiotic exposure, planktonic or biofilm conditions, and growth phase differ-
ences highlighted transcriptional changes in genes coding for type 1 pili (csu genes),
biofilm formation, quorum sensing, and iron acquisition (18–21). Most of these studies
used the ATCC 17978 strain as a model. ATCC 17978 has an unusual gene regulatory
system mediated by plasmid-encoded transcriptional regulators where plasmid dynam-
ics regulate chromosomal genes (22). Therefore, the transcriptional responses of clinical
isolates with well-defined genetic variation remain largely unexplored.

In this study, we integrated high-resolution genomic variation with analysis of gene
expression across single-isolate and population levels to examine how mutations that
arise during infection and those that are maintained across distinct phylogenetic
lineages can contribute to the transcriptional variation upon which selection can act.

RESULTS
Differentially expressed genes were identified by using the defined thresholds in 24
A. baumannii isolates from eight patients and using comparisons among isolates from
each patient (intrapatient differences) and among phylogenetic clades (interclade
differences). Considering all eight intrapatient isolate sets, a total of 424 genes were
differentially expressed in at least one patient and 127 of those genes were differen-
tially expressed in more than one patient series. The number of differentially expressed
genes ranged from 26 (patient 588, two isolates over 26 days) to 145 (patient 475, three
isolates over 334 days) per patient (Table 1). In general, the expression patterns of
isolates within each patient set were more like each other than like those of isolates
from other patients on the basis of hierarchical clustering of the core gene transcrip-
tome distance matrix, with a few notable exceptions (e.g., ABUH475239, ABUH410103,
and ABUH66276) that are discussed below (Fig. 1A). Rank-based hierarchical clustering
also indicated a strong phylogenetic signal in the transcriptional data, where each clade
exhibited distinct sets of gene expression patterns (Fig. 1B). One exception was isolates
from patient 81, which clearly belong to clade A in the core single-nucleotide variant
(SNV) phylogeny but have several gene content differences from other clade A isolates
(11). In some cases, a single isolate contributed most of the differentially expressed
genes within a patient, for example, ABUH66726 in patient 66 and ABUH475239 in
patient 475, whereas in the other patient sets, the variation in gene expression was
distributed more evenly across all of the strains (Fig. 1C).

Detailed below are examples of transcriptional variations among isolates from the
same patient where a candidate within-patient genetic change could be hypothesized
to lead to transcriptional variation of adjacent genes among isolates from the same
| Patient Isolate Strain | Date       | Hospital | Ward    | Source       | Clade | No. of SNVs | No. of ISs | No. of differentially expressed genes | RNA-Seq SRA accession no. |
|------------------------|------------|----------|---------|--------------|-------|-------------|-----------|---------------------------------------|--------------------------|
| 66 241 ABUH66241       | 6/24/2008  | Main     | General | Miscellaneous | B     | 3           | 4         | 0                                     | 131 SRX1485325, SRX1485336 |
| 66 253 ABUH66253       | 7/16/2008  | Main     | General | Fluid        | B     | 8           | 0         | 0                                     | 103 SRX1485338, SRX1485339 |
| 66 268 ABUH66268       | 8/13/2008  | Main     | General | Blood        | B     | 0           | 0         | 0                                     | 135 SRX1485343, SRX1485341 |
| 66 271 ABUH66271       | 8/15/2008  | Main     | General | Wound        | B     | 0           | 0         | 0                                     | 136 SRX1485356, SRX1485365 |
| 66 276 ABUH66276       | 8/24/2008  | Main     | ICU     | Fluid        | B     | 12          | 3         | 0                                     | 137 SRX1485383, SRX1485385 |
| 81 366 ABUH81366       | 4/22/2009  | Main     | ICU     | Sputum       | A     | 3           | 5         | 0                                     | 41 SRX1562109, SRX1562113 |
| 81 389 ABUH81389       | 8/1/2009   | Main     | ICU     | Sputum       | A     | 2           | 0         | 1                                     | 102 SRX1562119, SRX1562120 |
| 81 452 ABUH81452       | 11/26/2009 | Main     | ICU     | Sputum       | A     | 1           | 0         | 0                                     | 103 SRX1562121, SRX1562122 |
| 280 81 ABUH28081       | 11/21/2007 | Community A | General | Bronch       | A     | 1           | 8         | 0                                     | 75 SRX1485405, SRX1485407 |
| 280 92 ABUH28092       | 12/4/2007  | Community A | ICU     | Bronch       | A     | 0           | 0         | 0                                     | 104 SRX1485408, SRX1485410 |
| 280 93 ABUH28093       | 12/5/2007  | Community A | ICU     | Sputum       | A     | 1           | 0         | 0                                     | 105 SRX1485413, SRX1485416 |
| 280 99 ABUH28099       | 12/20/2007 | LTCF     | General | Sputum       | A     | 2           | 0         | 0                                     | 106 SRX1485418, SRX1485419 |
| 315 100 ABUH315100     | 12/21/2007 | Main     | ICU     | Sputum       | A     | 2           | 0         | 0                                     | 33 SRX1485293, SRX1485291 |
| 315 101 ABUH315101     | 12/22/2007 | Main     | ICU     | Bronch       | A     | 2           | 0         | 0                                     | 107 SRX1485314, SRX1485304 |
| 348 13 ABUH34813       | 9/30/2007  | Main     | ICU     | Sputum       | D     | 4           | 0         | 2                                     | 66 SRX1562097, SRX1562100 |
| 348 27 ABUH34827       | 10/6/2007  | Main     | ICU     | Sputum       | D     | 3           | 3         | 0                                     | 108 SRX1562102, SRX1562107 |
| 410 96 ABUH41096       | 12/15/2007 | LTCF     | General | Sputum       | C     | 0           | 2         | 0                                     | 124 SRX1562075, SRX1562081 |
| 410 103 ABUH410103     | 12/23/2007 | LTCF     | General | Sputum       | C     | 0           | 3         | 0                                     | 109 SRX1562083, SRX1562082 |
| 410 128 ABUH41028      | 1/23/2008  | LTCF     | General | Trachea      | C     | 0           | 2         | 0                                     | 110 SRX1562089, SRX1562084 |
| 475 197 ABUH475197     | 5/9/2008   | Outpatient | General | Bronchus     | A     | 0           | 0         | 1                                     | 145 SRX1485319, SRX1485318 |
| 475 239 ABUH475239     | 6/12/2008  | Outpatient | Bronchus |              | A     | 1           | 2         | 0                                     | 111 SRX1485322, SRX1485324 |
| 475 361 ABUH475361     | 4/8/2009   | Main     | ICU     | Stool        | A     | 2           | 2         | 0                                     | 112 SRX1485401, SRX1485403 |
| 588 656 ABUH588656     | 7/4/2013   | Main     | ICU     | Sputum       | C     | 4           | 0         | 2                                     | 26 SRX1562123, SRX1562124 |
| 588 663 ABUH588663     | 7/30/2013  | Main     | ICU     | Sputum       | C     | 3           | 3         | 0                                     | 113 SRX1562126, SRX1562127 |

aICU, intensive care unit.
bLTCF, long-term care facility.
FIG 1 (A) Hierarchical clustering of VST count data from core clusters (n = 3,167 genes). (B) Hierarchical clustering of Spearman rank-based clustering from core clusters. (C) Clustering of VST data for all within-patient shared genes for each patient.
patient, illustrating how genetic variation that arises during infection contributes to transcriptional remodeling among very closely related isolates. Several common themes were observed, with mutations in independent patients leading to transcriptional variation in a shared set of genes, suggesting convergent evolution of functional variation. This is followed by information on genetic mechanisms that underlie inter-patient and population level transcriptional variations across more evolutionarily divergent isolates.

**Within-patient expression.** Putative genetic changes associated with transcriptional variation were found in the following patients.

**Patient 66.** Five strains isolated over 61 days had 27 SNVs and three IS events among them, and 132 differentially expressed genes were identified (Table 1). Isolate ABUH66276, the last isolate in the series, accounted for most of the differentially expressed genes (see Table S1 in the supplemental material), and many of these genes were adjacent to IS and mutation events (see Tables S2 and S3). An ISaba12S insertion upstream from ACICU_00087 likely resulted in decreased transcription of ACICU_00087 to ACICU_00091, genes involved in capsular polysaccharide (cps) biosynthesis (Fig. 2C). An ISaba1 insertion in ACICU_01765, a transcriptional regulator, was associated with significantly decreased expression of ACICU_01762, a predicted aspartate ammonia lyase, in ABUH66276. ABUH66276 also had ISaba1 inserted upstream from ACICU_01814, which likely causes the overexpression of ACICU_01812 and ACICU_01813, involved in pilus assembly, a region also disrupted by patient-restricted ISaba1 in patient 41, upstream of ACICU_01813. ISaba1 has a strong outward-facing promoter that can result in overexpression of the upstream flanking gene when oriented in the opposite direction. A predicted EamA-like transporter, ACICU_03323, is also more highly transcribed in ABUH66276, though sequences within patient 66 isolates are identical across 5 kb of sequence flanking the gene. ABUH66276 has two other transporters with patient-specific SNVs (ACICU_03495 and ACICU_01674) and an ISaba1 insertion in a gene encoding another membrane protein (ACICU_03345). ABUH66276 has a nonsynonymous mutation in ACICU_03477, a predicted 4-aminobutyrate transaminase, and the expression of adjacent ACICU_03476 to ACICU_03479, part of the 4-aminobutyrate catabolic pathway, is significantly increased. IS-associated deletion of ACICU_01775 to ACICU_01779 was present in ABUH66276, flanked downstream by an ISaba1 insertion in ACICU_01780 common to all clade C isolates and upstream by an additional isolate-specific ISaba1 insertion at ACICU_01774, in a region encoding a transcriptional regulator, transporters, and dehydrogenases of unannotated specificity. Point mutations in ACICU_03495 and ACICU_03498 (a TCRS hybrid sensor histidine kinase/response regulator) in ABUH66276 are associated with decreased transcription of genes in this cluster, including a predicted membrane transport system. ACICU_01781 (predicted to be part of a phenol degradation pathway) is more highly expressed in patient 66 isolates than in the other isolates likely because of the ISaba1 insertion in ACICU_01780 (a flavin reductase gene).

TCRS mutations were also present in isolates from this patient: pmrB (ABUH66253) and two independent adeS mutations (ABUH66253 and ABUH66276) discussed in further detail below.

**Patient 81.** In patient 81, three isolates differed by six SNVs and one insertion sequence event that occurred during the 218 days of patient infection, with 41 differentially expressed genes among them (Table 1; see Table S1). ABUH81366 had mutations in two efflux systems, adeR (encoding the response regulator) of the AdeABC efflux pump and early termination of adeJ of the AdeIJK RND family efflux pump, while the other two isolates from this patient had a 5-bp deletion upstream of adeA (ACICU_01825) (see Table S2). The adeABC genes have ~25-fold lower expression in ABUH81389 and ABUH81452 than in other isolates with the wild-type genotype (Fig. 3B), while ABUH81366 did not differ in transcription across this region. The predicted early termination of adeJ in ABUH81366 likely led to the significantly lower expression of the downstream gene adeK. A pmrB mutation (T114I) is present in
ABUH81389 and ABUH81452, and the pmrCAB operon has ~4× higher expression in these strains than in ABUH81366 (Fig. 3C). An insertion-sequence-associated mutation in ACICU_00266, and several genes related to iron acquisition were differentially expressed. A gene duplication of the siderophore region (ACICU_01683) in ABUH41096 led to significantly higher expression of those genes. An ISAb125 insertion upstream from ACICU_00087 in ABUH66276 led to significantly lower expression of cps synthesis genes. Color intensity is scaled within each panel, with darker shades indicating higher expression; see Table S7 for values. Gray shading indicates genes absent from those isolates.

Patient 280. Four isolates spanning 29 days had 74 differentially expressed genes among them, with 12 SNVs and no IS events occurring during this time (Table 1). The
first isolate, ABUH28081, was the most divergent, but its transcriptional profile was more like that of the closely related patient 475 isolates (Fig. 1B). Each isolate had mutations in \textit{pmrB}, with a gradient of transcriptional effects, with ABUH28099 having the greatest increase in \textit{pmrCAB} gene expression (Fig. 3C; see Tables S1 and S2). ABUH28081 had an isolate-specific \textit{pmrB} SNV, while the other three isolates shared a different \textit{pmrB} mutation and ABUH28099 had an additional isolate-specific SNV in \textit{pmrB}.

**Patient 315.** Two isolates obtained 1 day apart had 33 differentially expressed genes and four SNVs between them (Table 1) and gene content differences, including partial loss of the Tn548-like transposon encoding the aminoglycoside resistance gene \textit{armA} in ABUH315100. Increased expression of ACICU\textunderscore 01471 to ACICU\textunderscore 01475 (ubiquinol oxidase genes) was observed in ABUH315101, with a mutation in the adjacent gene encoding a LysR family transcriptional regulator (ACICU\textunderscore 01470). Phage-like genes in
ABUH315101 were also more highly transcribed. Other genetic variation between the two isolates included a mutation in ACICU_03157, encoding a histidine kinase transcriptional regulator, a gene also mutated in ABUH475239, but there was no observed transcriptional variation in flanking genes in either isolate.

**Patient 348.** We identified 66 differentially expressed genes between two isolates obtained 6 days apart from patient 348 (Table 1). Both isolates have an SNV in pmrB in common with all clade D isolates and showed elevated transcription of Pmr regulon genes (Fig. 3C). ABUH34813 has an additional mutation in a predicted lipopolysaccharide export permease (ACICU_00253), while ABUH34827 has a nonsynonymous mutation in a predicted lipid A export permease (ACICU_01602) (see Table S2), which may explain the lower expression across the Pmr regulon genes (Fig. 3C) relative to ABUH34827. ABUH34813 had an additional mutation in the ferrous iron transporter gene feoB (ACICU_00266), and the transcription of iron acquisition genes, including multiple siderophore genes, was significantly elevated in this isolate (Fig. 2B). ABUH34813 had an ISAb125 interruption of ACICU_00745, where transcription of the adjacent ACICU_00746 and ACICU_00747 genes was significantly lower. Isolate ABUH34827 had an ISAb12-associated deletion in the csu region (ACICU_02420 and the 5’ end of ACICU_02421) (see Table S3), and transcript levels across this region (ACICU_02414 to ACICU_02421) were significantly lower (Fig. 3A). Both isolates have an ISAb12 insertion within ACICU_01721, md, encoding RNase D (also ISAb125 in ABUH475239). ABUH34827 has an additional ISAb12 insertion in ACICU_01726 that likely led to the deletion of ACICU_01721 to ACICU_01726, encoding hypothetical proteins and a transcriptional regulator, in ABUH34827.

**Patient 410.** Three isolates spanning 39 days had 123 differentially expressed genes among them, with the intermediate isolate (ABUH410103, day 8) having the more divergent transcriptome than the other two isolates (Table 1; Fig. 1C). Duplication of a siderophore gene region in ABUH41096 likely resulted in significantly higher expression of these genes (Fig. 2B). Genomic read mapping reveals >2× coverage of ACICU_01673 to ACICU_01685 (340× for ABUH41096 versus 130× for the other two patient 410 genomes across this region). There was a frameshift (1-bp deletion) in a gene encoding a component of the shikimate pathway (ACICU_03387) in the ABUH410103 genome, which also had significantly elevated transcription of the phenylacetic acid (paa) pathway (ACICU_01135 to ACICU_01345). These transcriptional changes may represent a response to aromatic amino acid limitation. An intergenic ISAb1 between ACICU_01103 and ACICU_01104 in ABUH410103 (see Table S3) was associated with lower expression of the adjacent genes, a predicted dihydroorotase (ACICU_01103) and RNase T (ACICU_01102). ABUH410103 also had an ISAb12 insertion within ACICU_02538, encoding an RNA methyltransferase. ABUH410128 had an ISAb1 upstream of ACICU_02420, csuA, likely leading to higher expression of the chaperone usher system genes (Fig. 3A). The mutations in RNA methyltransferase and RNase T may, in part, contribute to the divergent transcriptome in ABUH410103.

**Patient 475.** Three isolates collected over 334 days had 145 differentially expressed genes among them, with three SNVs and five insertion sequence mobilization events distinguishing them (Table 1). ABUH475239 (day 34) had the most divergent transcriptome (Fig. 1C). An ISAb125 insertion near the C terminus of RNase D (md; ACICU_01720) at amino acid (aa) 360 was identified in this isolate (see Table S3), and expression of the adjacent gene’s hypothetical protein, ACICU_01721, was lower. An ISAb125 insertion in ACICU_02613, a LysR transcriptional regulator, had no apparent effect on the adjacent predicted choline transporters, but the expression of ACICU_00888 to ACICU_00891 was significantly elevated. This region also contains genes predicted to be involved in choline transport and betaine biosynthesis. ABUH475239 also had an intergenic ISAb125 insertion between ACICU_00288 and ACICU_00289, and the transcription of the adjacent gene, ACICU_00289, a putative DNA binding protein upstream from a type II secretion system region, was significantly higher. There is also an example of intergenic sequence length variation likely associated with transcriptional variation,
where ABUH475239 is missing 76 bp between ACICU_02427 (AraC-type DNA binding domain protein) and ACICU_02428 (predicted threonine or homoserine lactone transporter), adjacent to blsA, encoding a blue light sensor domain (BLUF) protein (ACICU_02430) (23). Many genes were differentially expressed in ABUH475239 relative to the other two isolates (Fig. 3A), but no single mutations could be identified as likely causes of these differences. It is possible that the RNase D mutation altered tRNA processing or that a potential phase change around the ACICU_02427 region is linked to the expression differences.

**Patient 588.** Two isolates obtained 26 days apart had 26 differentially expressed genes and were distinguished by seven SNVs and five IS locations (Table 1). The two pmrB mutations in ABUH588663 (see Table S2) likely contributed to many of the differentially expressed genes. ABUH588663 also had a nonsynonymous mutation in a predicted metal transporter (ACICU_00170). This isolate had 6× greater expression of ACICU_03142, encoding a TonB receptor domain protein, although no mutations were identified in the flanking region that might explain the expression difference. ABUH588565 had an ISaba27 insertion in carO, ACICU_02813 (see Table S3), encoding an outer membrane protein, and the expression of that gene was significantly lower. CarO disruption, including by insertion sequences, has been shown to result in increased carbapenem resistance (24–27).

**Population level genetic and transcriptomic variations.** To further explore how genome-wide genetic changes can alter expression at the population level, the most variably expressed genes were identified by using two statistical models (i) where each isolate was independent and (ii) where isolates were assigned to a clade on the basis of the core SNV phylogeny in reference 11. Clade level differences in gene expression were clustered in 42 colocated blocks comprising 68 differentially expressed genes. Of those 42 blocks, a rationale for the causative mutation could be inferred on the basis of genetic differences in cis for 30 of the loci: 19 with insertion sequence events, 8 with nonsynonymous coding changes, and 3 with intergenic changes. Of the 130 most variable genes across all of the isolates, there were 78 blocks of gene clusters with consistent transcriptional patterns. Of these blocks, 21 were linked to insertion sequence events, 11 were linked to sequence divergence, and 9 were linked to single-base nonsynonymous mutations. There were 32 genes identified as both highly variable across all of the isolates and having clade-differentiated expression levels.

**Convergent changes.** (i) *pmrAB* mutations: defining the Pmr regulon in *A. baumannii*. Nine isolates from five patients had mutations in genes encoding TCRS PmrAB that encode a known mechanism conferring resistance to colistin in *A. baumannii* through lipid A modification. All four isolates from patient 280 had *pmrB* mutations: ABUH28081 had an isolate-specific SNV, ABUH28092 and ABUH28093 shared a second *pmrB* mutation, and ABUH28099 shared this site and had an additional mutation (see Table S2). Differential expression along this gradient indicated that the V31F mutation conferred an ~5× higher level of transcription of *pmrC*, the gene encoding lipid A phosphoethanolamine transferase, in ABUH28092 and ABUH28093, while the additional L257I mutation in ABUH28099 resulted in 30× higher expression of *pmrC* (Fig. 3C). All of the isolates had the same colistin MIC of 0.5 μg/ml, as tested in a broth microdilution assay. Transcriptome analysis indicated that isolates carrying *pmr* mutations had 21 differentially expressed genes in common that help define the Pmr regulon in *A. baumannii* (Fig. 3C). These genes have considerable overlap with the colistin-regulated genes described by Cheah et al. (28). Interestingly, none of the isolates with *pmr* mutations had *in vitro* colistin MICs of >1 μg/ml and were classified as phenotypically susceptible.

(ii) *adeRS* mutation: contrasting transcriptional patterns. Mutations in the TCRS genes *adeRS* and in the *adeABC* efflux pump genes, which are associated with tigecycline resistance (29–32), were associated with both elevated and reduced expression (Fig. 3B). Clade A isolates from both patients 280 and 475 had an ISaba22 insertion in *adeA* (ACICU_01825) that likely rendered it nonfunctional, and expression of the *adeABC* operon was significantly lower. Clade A isolates from patient 81 also had
mutations in this region, including a patient-specific mutation in adeS (ACICU_01827). There was an isolate-specific mutation in ABUH81366 in adeR (A80P), while the two later isolates (ABUH81389 and ABUH81452) have a 5-base deletion —40 bp upstream from adeA, with adeABC expression significantly higher in ABUH81366 than in the other isolates from patient 81. Phenotypically, the tigecycline MIC for ABUH81366 was 16 μg/ml, while the MIC for both ABUH81389 and ABUH8155 was 4 μg/ml. A patient-specific adeR mutation in patient 588 was associated with ~2× greater transcription of adeABC. Patient 66 had two isolates with independent adeS mutations, with ABUH66253 (D167N) having ~6× greater adeABC expression than ABUH66276 (R312S), yet the transcription of this region was significantly higher in both of these isolates than in the other three isolates from patient 66. The tigecycline MIC for ABUH66253 was 16 μg/ml, compared to 8 μg/ml for ABUH66276. No additional gene expression effects of adeRS mutations were apparent.

(iii) Type I pilus alteration: csu region. The genome region with the greatest variability in gene expression was a 37-kb region containing the csu genes (ACICU_02399 to ACICU_02436) (see Table S7). The Csu proteins make up a type I pilus chaperone usher secretion system that is important for biofilm formation and adhesion (33, 34). The csu region was interrupted by deletions, IS events, and SNVs (see Tables S2 and S3). The comparison of isolates within clade C from patients 588 and 410 highlights this. An ISAb27 interruption of csuB (ACICU_02417) is unique to patient 588. There is an isolate-specific ISaba1 insertion between ACICU_2420 and ACICU_2421 in ABUH410128 associated with increased expression of ACICU_02414 to ACICU_02421 relative to that in the other two isolates from patient 410 (Fig. 3A). Isolates from both patients have IS interruptions within ACICU_02430 (blue light sensor domain protein). The ACICU_02430 insertions involve unique locations in this gene and involve different IS elements (ISaba125 in patient 410 and ISaba27 in patient 588). Isolates from both patients have an ISaba1 insertion between ACICU_02428 and ACICU_02429 in common, but the upstream flanking sequence adjacent to the ISaba1 element, including ACICU_02421, is deleted from the patient 588 strains. The transcription of this region reflects the influence of these elements, as isolates from patients 410 and 588 have elevated transcription relative to the other isolates from ACICU_02430 (patient 410 isolates) or ACICU_02431 (patient 588 isolates) through ACICU_02436 (Fig. 3A). This region contains additional genes that may be involved in the structure of the cell surface, including a predicted glycosyltransferase and a gene with a conserved domain associated with glycosyl-phosphatidylinositol anchor biosynthesis.

In clade D patient 348, there is an isolate-specific ISaba12 insertion within ACICU_02421, a transcriptional regulator, in ABUH34827, with decreased expression of the csu operon in this isolate relative to that in ABUH34813. Patient 66 (clade B) is missing ACICU_02401 to ACICU_02421, while patient 81 (clade A) is missing ACICU_02398 to ACICU_02423, because of IS26-mediated deletions. ABUH315100 (clade A) has an isolate-specific IS26-associated deletion starting at ACICU_02398 (major facilitator superfamily transporter) through ACICU_02414 (csuE gene). Patient 315 also has a patient-specific mutation in csuE with two amino acid differences from other clade A isolates. Genomic and transcriptomic analyses reveal a dynamic remodeling of this region with a consistent trend toward loss or diminished function of the csu region.

(iv) Type 1 pilus alteration, take 2: ACICU_01812 to ACICU_01815. Additional convergent mutations occurred in another set of genes encoding components of the type I pilus system. All of the isolates in clades A to C have an ISaba1 insertion (at aa 6) within ACICU_01812, a pilus assembly protein. An isolate-specific mutation in ACICU_01814 in ABUH66276 is associated with the overexpression of adjacent ACICU_01813, also a pilus assembly protein. Isolates from clade D have an intergenic deletion of 417 bp between ACICU_01813 and ACICU_01814. Since all of the isolates have mutations relative to ACICU, understanding what wild-type expression of this region looks like is not possible, but these genes differ significantly from each other in expression. Other isolates from the UHHS population have ISaba1
insertions in this region as well: isolate-specific IS\textit{Aba}1 in ABUH35559 and patient-restricted IS\textit{Aba}1 in isolates from patient 41.

(v) Convergent mutation and IS-mediated alterations of ACICU\textit{01919} to ACICU\textit{01923}. Clade C isolates from patient 588 have two IS insertions in the ACICU\textit{01919}-to-ACICU\textit{01923} region: IS\textit{Aba}27 in ACICU\textit{01919} (a predicted amidase) and IS\textit{Aba}1 in ACICU\textit{01921} (transcriptional regulator). ACICU\textit{01919} and ACICU\textit{01920} transcription is significantly lower in patient 588 isolates, but ACICU\textit{01921} transcription is increased. However, the insertions are within the coding region, so ACICU\textit{01919} and ACICU\textit{01920} are not likely functional in these isolates. Clade B isolates from patient 66 have a 1-bp deletion in ACICU\textit{01920}, a predicted quinol monooxygenase, that causes a frameshift at aa 56, but transcriptional effects of this are equivocal. The clade D isolates from patient 348 isolates are missing over 70 kb of sequence (ACICU\textit{01890} to ACICU\textit{01950}), including this region. The deletion was possibly due to a recombination between IS\textit{Aba}1 elements because one edge of the deleted region is marked by an IS\textit{Aba}1 insertion in ACICU\textit{01890}, annotated as hemolysin D.

IS-mediated mobilization of osmC and \textit{crp} genes. A subset of strains from clades A to C have an IS\textit{Aba}1 insertion near the end of ACICU\textit{01161}, predicted to be \textit{crp}, encoding the cyclic AMP receptor protein, a transcription factor. Clade A strains have an additional IS\textit{Aba}1 downstream of ACICU\textit{01162}, an osmC-like gene predicted to be involved in the osmotic stress response. A composite transposon composed of IS\textit{Aba}1, \textit{crp}, osmC, and IS\textit{Aba}1 appears to be readily mobilized and was found in several strains in distinct locations. Clade A strains have a second copy in ACICU\textit{01570} (hypothetical periplasmic protein), and a third copy was found in ACICU\textit{03149} (a hypothetical protein in the outer core locus for lipooligosaccharide synthesis) of the clade A patient 280 and 475 strains. In the latter strains, the expression of ACICU\textit{01161} and ACICU\textit{01162} was significantly higher, while clade D strains without duplications of these genes had the lowest expression (see Table S6). The PacBio assembly of ABUH28099 (AYOH01000000) (13) contains three copies of this transposon and confirms this mobilization.

Other IS effects at the patient and clade levels. Patient 348 isolates share a clade D-specific IS\textit{Aba}1 near the beginning of pgaA (ACICU\textit{02365} at aa 39), encoding the poly-\(\beta\text{-1,6}\) N-acetyl-\(\alpha\)-glucosaminie export protein, which is important for biofilm formation (35), and significantly increased transcription of the adjacent ACICU\textit{02361}-to-ACICU\textit{02365} genes relative to the other isolates (Fig. 2A; see Table S7). Patient 410 has patient-specific IS\textit{Aba}1 in ACICU\textit{01189} at aa 206 (out of 261 aa), and the expression of both that gene and the adjacent gene for ACICU\textit{01188}, annotated as a benzoate transporter, is increased. An example of convergence in insertion sequence events affects \textit{rnd}, encoding RNase D (ACICU\textit{01720}): an IS\textit{Aba}12 insertion is present in all clade D isolates, and an isolate-specific IS\textit{Aba}125 event was found near the C terminus in clade A isolate ABUH475239.

Intergenic mutations. Intergenic mutations can change expression by altering promoter or DNA binding sites without changing gene function. A mutation 4 bp upstream of the start codon for ACICU\textit{00684} (a predicted membrane protein) in clade C isolates, is associated with significantly higher expression of this gene, though there is unexplained variation in the expression of this gene among the clade A isolates as well. Of additional note, the ACICU genome has IS\textit{Aba}125 inserted upstream of this gene. Clade B isolates all have a mutation –40 bp upstream from the start codon of ACICU\textit{00708}, encoding a diguanylate cyclase domain protein, and expression of this gene is significantly higher in clade B strains than in the others.

Sequence variation through recombination. Clade level variation in expression of genes not explained by insertion sequence or single-base differences can also be attributed to sequence variation or regions of elevated sequence divergence indicative of historical recombination events (see Table S5). Because the strains investigated here are all part of multilocus sequence type 2 (ST2) or the global clone II lineage, the average nucleotide identity in core genes is often \(>99.9\%\). However,
some genomic regions have elevated SNV density (13, 15, 36). One of these regions starts around 3.51 Mbp (36) and encompasses several genes identified as differentially expressed at the clade level: ACICU_03220 to ACICU_03627 (see Table S5), encoding proteins involved in pilus assembly, amino acid metabolism, and environmental sensing and response proteins. For example, in ACICU_03431 and ACICU_03432, clade D isolates are identical to ACICU, missing 11 aa within a membrane protein (ACICU_03432), where clade A to C sequences are identical to TYTH-1 (M3Q_3669). Transcription is significantly lower in the clade D isolates. A 314-bp region is variably present among A. baumannii genomes where these extra bases start at ACICU coordinate 3650446 in the transmembrane protein ACICU_03437 of clade A to C isolates (as well as clade E isolates in reference 13) and identical to the AC29 finished genome. This 314-bp sequence is absent from clade D isolates, which are identical to ACICU here. Clade B isolates also have an ISAba1 insertion that interrupts ACICU_03437. Transcription of this region is significantly higher in patient 348 clade D isolates, while the flanking Eam-like transporter domain ACICU_03436 and cold shock protein domain ACICU_03438 are also variably expressed. A third location of expression variance in this region is around ACICU_03471. Isolates in clades A to C are identical across this 2,538-bp region, while clade D isolates are instead all 2,534 bp long, with frameshifts present in ACICU_03471, a predicted S-adenosylmethionine-dependent methyltransferase. Sequence conservation is low here, however, where clades A to C have 36 single-nucleotide polymorphisms (SNPs) over this ~2.5-kb region relative to ACICU as well. An isolate-specific ISAba125 insertion is also present in ABUH410103 in ACICU_03472 (a NAD-flavin adenine dinucleotide binding domain protein) and is associated with decreased expression of ACICU_03471.

**DISCUSSION**

Bacteria face selective pressures from host defenses and antibiotic therapies during colonization and infection. We previously identified mutations that arose during infection, finding enrichment in certain classes of genes, including transcriptional regulators, transporters, and iron acquisition- and surface-associated structures (11). In this study, we examined how mutations can contribute to transcriptional variation within and among patients and across phylogenetically distinct lineages. Transcriptional changes among isolates could be linked to a combination of single base substitutions (intergenic and intragenic), insertion sequence mobilization, IS-associated deletions, duplications, and recombination events. We found that closely related isolates can have very different transcriptional profiles and that transcriptional changes can arise over the time frame of patient infection. Additionally, there is convergence at the transcriptome level, with independent genetic changes causing shared patterns of transcriptional remodeling of regions associated with antibiotic resistance, iron acquisition, biofilm formation, surface structures like pili and cps architecture, and amino acid metabolism. The clade level comparison identified expression differences that reflect evolutionary divergence, and many of these differences can be attributed to insertion sequence events and recombination-associated sequence variation in the same genes with altered expression in the within-patient series.

Genetic analysis indicated an enrichment in TCRS mutations during host infection (11). Regulatory networks are complex, and mutations in one system can have effects through cross-talk among regulatory networks (37–40). Multiple independent occurrences of pmrAB mutations allowed us to more rigorously establish the Pmr regulon in clinical A. baumannii isolates, which identified 18 additional genes whose transcription was altered in the mutant strains, including several hypothetical proteins and sugar transferases, including those that may be involved in galactosamine modification of lipid A (41). Most isolates with pmr mutations originated from patients treated with colistin (11), supporting the inference that these mutations arose in response to colistin selective pressure. However, the pmrB mutation common to all clade D isolates represents a lineage-specific variation that may confer reduced susceptibility to colistin.

A second TCRS mutated in multiple patients is adeRS. The contrasting transcriptional
patterns of mutations around the adeRS and adeABC loci highlight the potential adaptive trade-off surrounding antibiotic resistance and fitness. Yoon et al. (42) quantified the fitness cost of increased production of A. baumannii efflux pumps, as a strain with increased expression of adeABC demonstrated less in vitro and in vivo fitness than the wild-type strain. Previous genomic surveys also indicated substantial genetic variation in adeRS (30), which is not always associated with a tigecycline resistance phenotype, highlighting that this region is likely experiencing multiple sources of selection. A recent study examined the transcriptional response of A. baumannii in adeRS deletion strains (20). Several other operons were found to be differentially expressed, a pleiotropic effect not observed in this study, though this may be because deletion mutants were investigated in that study. Feugeas et al. (43) demonstrated that genetic diversity correlates with transcriptional variation under in vitro growth conditions for Escherichia coli cells, including an enrichment in TCRS, illustrating the significant impact mutations these genes can have on transcription and fitness.

Multiple independent genetic events in the same region are evidence of the potential adaptive significance of these regions. For example, the csu region has been altered multiple independent times in A. baumannii (13, 15) and we observed both increased and decreased expression relative to that in isogenic isolates from several patients. In an analysis of IS locations in over 1,000 A. baumannii genomes, the 5-kbp region containing the csu genes had over 90 independent insertion events, a density approximately three times higher than that in any other 5-kb region of the genome (12). This provides compelling evidence that alteration of this region is under selection. The csu region is known to be critical for biofilm formation and adhesion (34) and therefore thought to be important for virulence, but the prevalence of deletions and transcriptional downregulation also suggests that under certain conditions, expression of the Csu proteins might be selected against or that this region may be under diversifying pressure. A second type I pilus system also experienced convergent genetic and transcriptional changes. Mutations in type 1 fimbriae have been described in Shigella spp. that led to loss of function, and yet fimbrial production increased epithelial cell invasion success (44), suggesting a complex interplay of selective pressures acting on these loci.

Iron acquisition is also critical for within-host A. baumannii survival (45, 46). Two isolates had mutations in iron acquisition: ABUH41096 (duplication across the siderophore synthesis region) and ABUH34813 (point mutation in the ferrous iron transporter FeoB). Both isolates had significantly increased expression of the siderophore region, ACICU_01672 to ACICU_01683, while ABUH41096 had increased expression across an additional 36 iron acquisition-related genes (Fig. 2B), suggesting iron limitation in this isolate even during growth in rich laboratory medium. Free ferrous iron is likely limited during host infection (47), so the in vivo fitness implications of this mutation remains to be tested, though the two subsequent isolates (ABUH410103 and ABUH410128) from the patient did not have this mutation.

Though convergence was observed among patients, within-patient analysis indicated that transcription in most isolates varied only in a limited number of genes. One exception is ABUH66276 from patient 66, which had many more differentially expressed genes (Fig. 1C). This isolate also had the most isolate-specific mutations: 12 SNVs and three insertion sequence events. A potential explanation for this divergence of expression patterns involves the cumulative effects of recA, rpoB, and rne RNase (E/G) mutations that likely impact the transcription of DNA damage-inducible proteins and RNA processing (48). This example highlights that an isolate can experience rapid transcriptome remodeling through a relatively small number of genetic changes.

Many of the gene expression differences observed in isogenic sets of isolates from individual patients could be associated with specific mutations in adjacent genomic regions. A larger number of expression differences affected genes without obvious candidate mutations. There are at least two explanations for this. First, regulatory networks are complex and pathway interactions are incompletely characterized in A. baumannii, especially for nonmodel strains. We also lacked the power to detect
trans-acting mutations. Second, although biological replicate data sets were highly similar for each isolate (Fig. 1C; see Table S7), subtle growth phase effects could have influenced transcriptional profiles. For example, 11 of the most variable genes among isolates involved the phenylacetic acid (paa) pathway, a pathway whose expression is known to be tightly coupled to the growth phase (49). Furthermore, transcriptional effects of some mutations were likely not detected under the in vitro growth conditions used here, where additional expression differences may be detected in vivo. Finally, expression data do not directly equate to whether a gene has lost its function or changed a phenotype, and additional phenotypic characterization of these isolates would yield important information regarding the pathoadaptive significance of these mutations. For example, clade D isolates share genetic and transcriptional alterations of the biofilm-associated pga region and the Pmr regulon such that the surface properties of these cells are likely to be very different from those of other A. baumannii strains.

This analysis highlights how genetic changes can significantly alter the transcription of phenotypically relevant genes, including those associated with antibiotic resistance, iron acquisition, and surface-associated properties, among highly similar isolates. That multiple mechanisms of genetic change contributed to this variation underscores the importance of analyzing all types of genetic variation, not only SNVs, to understand how bacterial genomes reflect the selective pressures that cells experience.

**MATERIALS AND METHODS**

**Isolates and patient data.** This research study was reviewed and approved by the Institutional Review Board of the University Hospitals Case Medical Center and conformed to the Helsinki Declaration. A subset of isogenic isolates from the longitudinal series from the University Hospitals Health System (UHHS) in Ohio, described in reference 11, was selected for RNA sequencing (RNA-Seq) to represent a range and type of mutational events, as well as to have at least one representative series from each primary clade in references 11 and 13 (Table 1). All of the isolates belong to ST2. These 24 isolates originated from eight patients, and duplicates of each from single colonies were grown overnight in LB broth, transferred to fresh medium, and harvested at mid-log growth phase (optical density at 600 nm of 0.5). Cells were then pelleted (2 min at 13,000 × g), the supernatant was removed, and the pellet was flash frozen in liquid nitrogen. Cell pellets were stored at −80°C before RNA was extracted with the MagJET RNA purification kit (Thermo Fisher) with an additional DNase treatment. rRNA was depleted with RiboZero (Illumina). Paired-end RNA-Seq libraries were made with PrepX kits on the Wafergen Apollo liquid handling system and sequenced on the Illumina NextSeq platform.

**RNA-Seq methods.** RNA-Seq reads were mapped with CLC Genomic Workbench (Qiagen) to each isolate’s respective genome assembly and to the A. baumannii ACICU complete reference genome (CP000863.1) to minimize the effect of assembly differences in the draft genomes. Each isolate had >99.7% average nucleotide sequence identity to ACICU. By using the option to “Map to gene regions only,” each read pair (or RNA fragment) was counted as aligning to the single gene with the longest alignment. Orthologs among isolates were identified with PanOCT (50), and core genes among these isolates were operationally defined as those present in ACICU and at least 23 of the study isolates (n = 3,167 genes). Raw fragment counts for each gene were output from CLC Genomic Workbench for statistical analysis in DESeq2. Genes were identified as differentially expressed if their adjusted P value was <0.05 and the log$_{2}$-fold change was greater than |1.5| (i.e., a >3-fold change) to minimize artifacts associated with multiple-comparison testing with statistical models comparing isolates within a patient or among phylogenetic clades. To additionally identify gene expression variability among individual isolates, the most variable core genes were defined as those that had >1 standard deviation from the mean variance-stabilizing transformed (VST) DESeq2 count data. This equates to 130 genes or ~4% of the core ACICU predicted coding regions.

**Genetic variation detection.** SNVs were identified as described in reference 11. Briefly, genomic reads were mapped to the first isolate from the series and SNVs were called with FreeBayes (arXiv: 1207.3907v2). Insertion sequence events were identified with ISseeker (12). Clade level genetic differences were identified by assembly-based SNV calling in kSNP (51) and verified manually to confirm that variants were isolate, patient, or clade specific by using blast queries with all of the available A. baumannii genomes in the UHHS population (11, 13).

**Accession number(s).** The RNA-Seq reads and genome assemblies obtained in this study are publicly available from the NCBI under BioProject no. PRJNA262565.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02193-16.

**TABLE S1,** PDF file, 0.3 MB.

**TABLE S2,** PDF file, 0.05 MB.

**TABLE S3,** PDF file, 0.01 MB.
TABLE S4, PDF file, 0.03 MB.
TABLE S5, PDF file, 0.04 MB.
TABLE S6, PDF file, 0.02 MB.
TABLE S7, XLSX file, 0.9 MB.

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