Comparative Genomic Analysis of Rapid Evolution of an Extreme-Drug-Resistant *Acinetobacter baumannii* Clone

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Abstract

The emergence of extreme-drug-resistant (EDR) bacterial strains in hospital and nonhospital clinical settings is a big and growing public health threat. Understanding the antibiotic resistance mechanisms at the genomic levels can facilitate the development of next-generation agents. Here, comparative genomics has been employed to analyze the rapid evolution of an EDR *Acinetobacter baumannii* clone from the intensive care unit (ICU) of Rigshospitalet at Copenhagen. Two resistant *A. baumannii* strains, 48055 and 53264, were sequentially isolated from two individuals who had been admitted to ICU within a 1-month interval. Multilocus sequence typing indicates that these two isolates belonged to ST208. The *A. baumannii* 53264 strain gained colistin resistance compared with the 48055 strain and became an EDR strain. Genome sequencing indicates that *A. baumannii* 53264 and 48055 have almost identical genomes—61 single-nucleotide polymorphisms (SNPs) were found between them. The *A. baumannii* 53264 strain was assembled into 130 contigs, with a total length of 3,976,592 bp with 38.93% GC content. The *A. baumannii* 48055 strain was assembled into 135 contigs, with a total length of 4,049,562 bp with 39.00% GC content. Genome comparisons showed that this *A. baumannii* clone is classified as an International clone II strain and has 94% synteny with the *A. baumannii* ACICU strain. The ResFinder server identified a total of 14 antibiotic resistance genes in the *A. baumannii* clone. Proteomic analyses revealed that a putative porin protein was down-regulated when *A. baumannii* 53264 was exposed to antimicrobials, which may reduce the entry of antibiotics into the bacterial cell.

Key words: *Acinetobacter baumannii*, antibiotic resistance, comparative genomics, single-nucleotide polymorphism.

Introduction

Multidrug-resistant bacterial strains have emerged as the causes of nosocomial infections worldwide (Nikaido 2009). Recently, pandrug-resistant (PDR) bacterial strains, which are resistant to all antimicrobial agents except tigecycline and the polymyxins, and extreme-drug-resistant (EDR) bacterial strains, which are resistant to all antimicrobial agents, were isolated from hospital-acquired infections (Paterson and Doi 2007). There is a huge risk of these "superbugs" extending into the community and threatening public health.

The Gram-negative, nonmotile, aerobic bacterium *Acinetobacter baumannii* is an example of a fast-evolving organism, which causes healthcare-associated infections (Garnacho-Montero and Amaya-Villar 2010). *Acinetobacter baumannii* was sensitive to most antibiotics in the 1970s, but now it is resistant to virtually all antibacterial drugs (Howard et al. 2012). *Acinetobacter baumannii* is responsible for approximately 2–10% of all Gram-negative infections in intensive care units (ICUs) and significantly increased mortality of infected patients (Poirel et al. 2003; Lockhart et al. 2007). Ribotyping and amplified fragment length polymorphism genomic fingerprinting approaches have identified three international groups of epidemic *A. baumannii* strains: clone I, clone II, and clone III (Dijkshoorn et al. 1996; van Dessel et al. 2004). However, the fingerprinting-based methods can only provide very limited phylogenetic information, and their results cannot identify genetic distinctness within the same clones and among different clones. Thus, whole-genome sequences are required for thorough epidemiological analysis and antibiotic resistance profiling of *A. baumannii*.

In this study, we have sequenced a colistin-sensitive PDR *A. baumannii* 48055 strain and an EDR *A. baumannii* 53264 strain from the ICU of Rigshospitalet in Copenhagen, Denmark. Both these strains have similar antimicrobial resistant profiles and were sequentially isolated from two individuals who had been admitted to ICU, within a 1-month interval. Through comparative genomic analysis, we identified the origin and phylogeny of this *A. baumannii* strains. We also profiled the drug resistance mechanisms present in the genomes of these *A. baumannii* strains. We also analyzed the single-nucleotide polymorphisms (SNPs) between the *A. baumannii* 53264 and 48055 strains to shed light on the mechanisms for the evolution of a PDR strain into an EDR one. Furthermore, we have employed 4-Plex isobaric tag for relative and absolute quantification (iTRAQ)-based quantitative proteomic analysis to investigate the resistance mechanisms of *A. baumannii* 53264 strain toward three different classes of antibiotics: tobramycin, colistin, and ceftazidime.

Materials and Methods

Bacterial Strains and Patient Background Information

The *A. baumannii* 48055 strain was isolated from a patient with 50% burn trauma, in the ICU of Rigshospitalet, Copenhagen, Denmark, on September 2010. This patient received antibiotic treatment with meropenem (2 g intravenously [i.v.], three times daily), ciprofloxacin (600 mg i.v., twice daily), and fucidin (500 mg i.v., three times daily) to prevent Gram-negative bacterial infections and treat *Staphylococcus aureus* bacteremia. After *A. baumannii* was found in the blood and airway, the treatment was changed to meropenem and colistin (6 million units i.v., once daily) and fucidin (500 mg i.v., three times daily).The treatment was later changed to meropenem (2 g i.v., three times daily), colistin (2 million units, three times daily), and vancomycin (1 g i.v., twice daily). After several operations to remove the necrosed tissues and skin transplantation, the patient recovered and was sent home.

A month later, the *A. baumannii* 53264 strain was isolated from a patient with 50% burn trauma, in the ICU of Rigshospitalet, Copenhagen, Denmark, on October 2010. This patient received antibiotic treatment with meropenem (1 g i.v., three times daily) and ciprofloxacin (400 mg i.v., twice daily) in the beginning, then with fucidin (500 mg orally, three times daily) due to *S. epidermidis* bacteremia. The treatment was changed to ceftazidime (1 g i.v., three times daily) with ciprofloxacin (400 mg i.v., twice daily) and vancomycin (1 g i.v., twice daily) to prevent Gram-negative bacterial infection and treat *S. haemolyticus* bacteremia. After *A. baumannii* was found in the blood and airway, the treatment was changed to meropenem (1 g i.v., three times daily), ciprofloxacin (400 mg i.v., twice daily), colistin (2 million units i.v., three times daily), and colistin inhalation (2 million units, twice daily). After several operations to remove the necrosed skin tissues and skin transplantation, the patient recovered very well and was sent back to the local hospital to continue treatment until complete recovery.

Antimicrobial Susceptibility Assay

Susceptibility of the two *A. baumannii* strains to 18 antimicrobial agents was tested by disc diffusion following the Clinical and Laboratory Standards Institute (CLSI) recommendations using the blood agar plate produced by Statens Serum Institut, Denmark. The *A. baumannii* 53264 and 48055 strains were resistant to all our tested antibiotics including ampicillin, aztreonam, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, colistin, gentamicin, imipenem, mecillinam, meropenem, penicillin, piperacillin/tazocin, sulfonamide, tigecycline, tobramycin, and trimethoprim.

To confirm this result, the minimum inhibitory concentration (MIC) was determined by microdilution for the A. *baumannii* 53264 strain, *A. baumannii* 48055 strain, and an antibiotic-sensitive *A. baumannii* strain 52082 to the following six representative antibiotics: tobramycin (aminoglycoside), colistin (antimicrobial peptide), ceftazidime (cephalosporin), tetracycline, ciprofloxacin (fluoroquinolone), and meropenem (carbapenem). Diluted overnight cultures of bacteria were seeded into wells containing serially diluted antibiotic stocks, from concentrations ranging from 0 to 1,024 μg/ml.

Genome Sequencing and Assembly

Whole-genome DNA of the *A. baumannii* strains were purified using Wizard genomic DNA purification kit (Promega) and sequenced by the Beijing Genomics Institute on an Illumina Hiseq2000 platform generating 90 bp long paired-end reads. Reads were mapped against the genome of *A. baumannii*

ACICU (Genbank accession number CP000863) using Novoalign (Novocraft Technologies) (Krawitz et al. 2010). The best assembly result was then assembled by SOAPdenovo (http://soap.genomics.org.cn/; version 1.05) with filtered data. Average insert sizes were 468 nucleotides, and the average genomic coverage depths were 112–116-fold. Pileups of the read alignments were produced by SAMtools release 0.1.7 (Li et al. 2009).

Genome Comparison

The *A. baumannii* 53264 and *A. baumannii* 48055 genomes were first compared with the genomes of 11 *A. baumannii* strains available from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A pair-wise genome content distance matrix was computed using Progressive Mauve (Darling et al. 2010), followed by whole-genome alignment. The distance matrix was converted to a heat map using the R heatmap function clustering package (http://www.r-project.org/). Progressive Mauve was then used to compare the genomes of these two strains with the genome sequences of 42 other *A. baumannii* strains (their genome sequences were downloaded from National Center for Biotechnology Information [NCBI] FTP site). First, the Mauve software computed a genome content distance matrix for all 44 *A. baumannii* strains, after which a neighbor-joining algorithm was used to produce a phylogenetic guide tree. Phylogenetic tree diagrams were prepared using the software FigTree ver 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Genome Annotation

For genome annotation, the *A. baumannii* 53264 and *A. baumannii* 48055 sequence files were submitted to the Rapid Annotations using Subsystem Technology (RAST) Server (Aziz et al. 2008) for bacterial genome annotation. Default settings were used. The RAST-annotated *A. baumannii* 53264 and *A. baumannii* 48055 genomes are accessible from the RAST server by logging in with the guest account (userID: guest, password: guest) at the web addresses: http://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=470.131 (last accessed April 16, 2013) and http://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=470.130 (last accessed April 16, 2013), respectively.

SNP Comparison and Analysis

The identification of SNPs between the *A. baumannii* 53264 strain, the *A. baumannii* 48055 strain, and the annotated *A. baumannii* ACICU strain (NC_010611) was performed using DNASTAR SeqManNGen and analyzed using DNASTAR SeqMan Pro software version 10.1.1 (DNASTAR, Inc., Madison, WI). Paired-end reads in FASTQ format were mapped against the respective annotated Genbank template file.

Prediction of Antibiotic Resistance Genes

The *A. baumannii* 53264 and *A. baumannii* 48055 sequences were also submitted to the Antibiotic Resistance Genes Database (ARDB; Liu and Pop 2009) and the recently described ResFinder database (Zankari et al. 2012), with a 98% threshold for identification of genes involved in antibiotic resistance.

Nucleotide Sequence Accession Numbers

The Whole Genome Shotgun bioproject for *A. baumannii* 53264 has been deposited at DDBJ/EMBL/GenBank under the accession ALPW00000000. The version described in this article is the first version, ALPW01000000. The Whole Genome Shotgun bioproject for *A. baumannii* 48055 has been deposited at DDBJ/EMBL/GenBank under the accession AOSP00000000. The version described in this article is the first version, AOSP01000000.

iTRAQ-Based Proteomics Analysis

iTRAQ-based proteomic analysis was used to study the changes in protein expression of the *A. baumannii* 53264 strain in response to three antibiotics: colistin, tobramycin, and ceftazidime. Proteomics experiments were performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore. A full description of the materials and methods is included as supplementary material, Supplementary Material online.

Results

General Characteristics of *A. baumannii* 53264 and *A. baumannii* 48055 Genomes

The clinical isolates *A. baumannii* 53264 and *A. baumannii* 48055 were isolated from the ICU of Rigshospitalet at Copenhagen, Denmark. *Acinetobacter baumannii* 53264 exhibited high resistance to all the 18 tested antibiotics, including tigecycline and the polymyxin, colistin; thus, *A. baumannii* 53264 is classified as an EDR strain. In comparison, *A. baumannii* 48055 is a colistin-sensitive PDR strain that was isolated 1 month before *A. baumannii* 53264. Both *A. baumannii* strains belong to multilocus sequence type ST208, a molecular type previously reported in European clone II (Runnegar et al. 2010).

The general characteristics of the *A. baumannii* 53264 and *A. baumannii* 48055 strains obtained from the RAST server (Aziz et al. 2008) are presented in table 1. For *A. baumannii* 53264, we obtained 130 contigs with a total length of 3,976,592 bp and 3,791 predicted coding sequences. For *A. baumannii* 48055, we obtained 135 contigs with a total length of 4,049,562 bp and 3,858 predicted coding sequences. The average GC% of *A. baumannii* 53264 and *A. baumannii* 48055 is 38.93 and 39.00, respectively.

Acinetobacter Synteny and Phylogeny

The *A. baumannii* 53264 and *A. baumannii* 48055 genomes were first compared with the genomes of 11 *A. baumannii* strains available from the KEGG database. A pair-wise genome content distance matrix was computed using Progressive Mauve (Darling et al. 2010), followed by whole-genome alignment. The distance matrix was converted to a heat map (fig. 1) using the R statistical package and revealed that the *A. baumannii* 53264 and *A. baumannii* 48055 genomes had the greatest amount of similarity to each other. The average nucleotide identity between these two *A. baumannii* strains and another multidrug resistant strain *A. baumannii* ACICU (Iacono et al. 2008) from European clone II is 94% as determined by Progressive Mauve (Darling et al. 2010).

To find out which clonal group the *A. baumannii* 53264 and *A. baumannii* 48055 genomes belonged to, Progressive Mauve was then used to compare the genomes of these two strains with the genome sequences of 42 other *A. baumannii* strains (their genome sequences were downloaded from NCBI FTP site). The phylogenetic tree based on the neighbor-joining algorithm shows that the *A. baumannii* 53264 and *A. baumannii* 48055 strains belong to the group of International Clone II *A. baumannii* strains (fig. 2).

Table 1

General Characteristics of the *Acinetobacter baumannii* 53264 and *A. baumannii* 48055 Genomes as Obtained from the RAST Annotation Server (Aziz et al. 2008)

Characteristic	Value	
	***A. baumannii* 53264**	***A. baumannii* 48055**
Genome		
Size (bp)	3,976,592	4,049,562
No. of contigs	130	135
G + C content (%)	38.93	39.00
No. of coding sequences	3,791	3,858
No. of subsystems	440	440
No. of RNAs	62	63

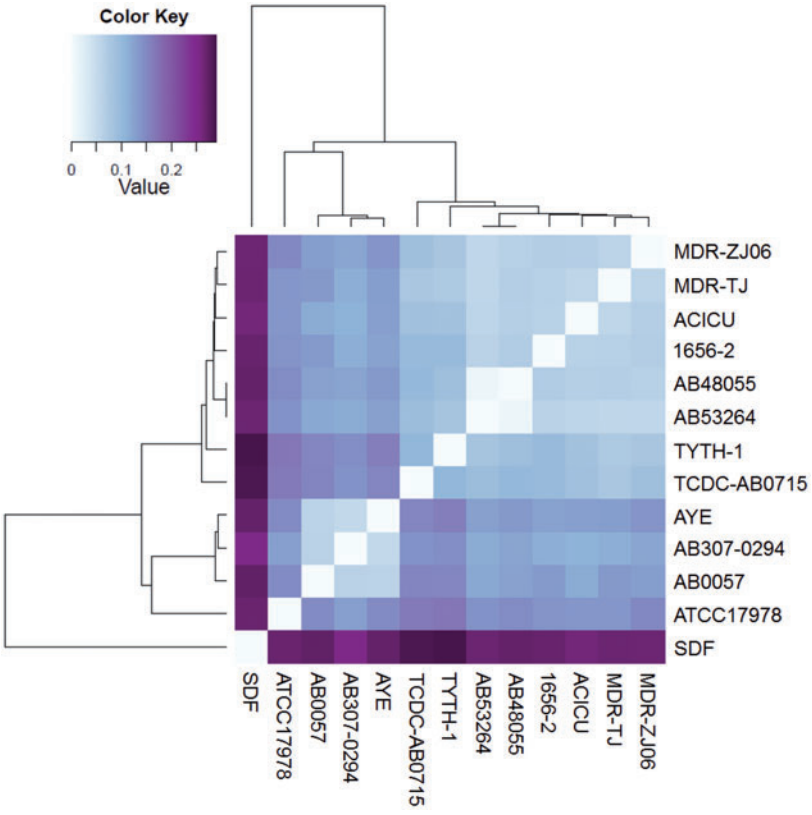

Fig. 1.—Heat map based on a pair-wise distance matrix of whole-genome alignment as computed by Progressive Mauve. Pair-wise genome alignments were performed using the genomes of *Acinetobacter baumannii* 53264, *A. baumannii* 48055, and 11 *A. baumannii* clones whose complete sequences were available in the KEGG database. This heat map was created using the R statistical program (http://www.r-project.org/) with heatmap clustering methods. Dendrograms across the top and left of the diagram indicate the relatedness of the genomes based on genome conservation, while strain names are listed to the right of the heatmap. Distance values range from 0.0 to 0.3 and correspond to a gradient of color steps ranging from light blue (lowest distance value) to dark purple (highest distance value).

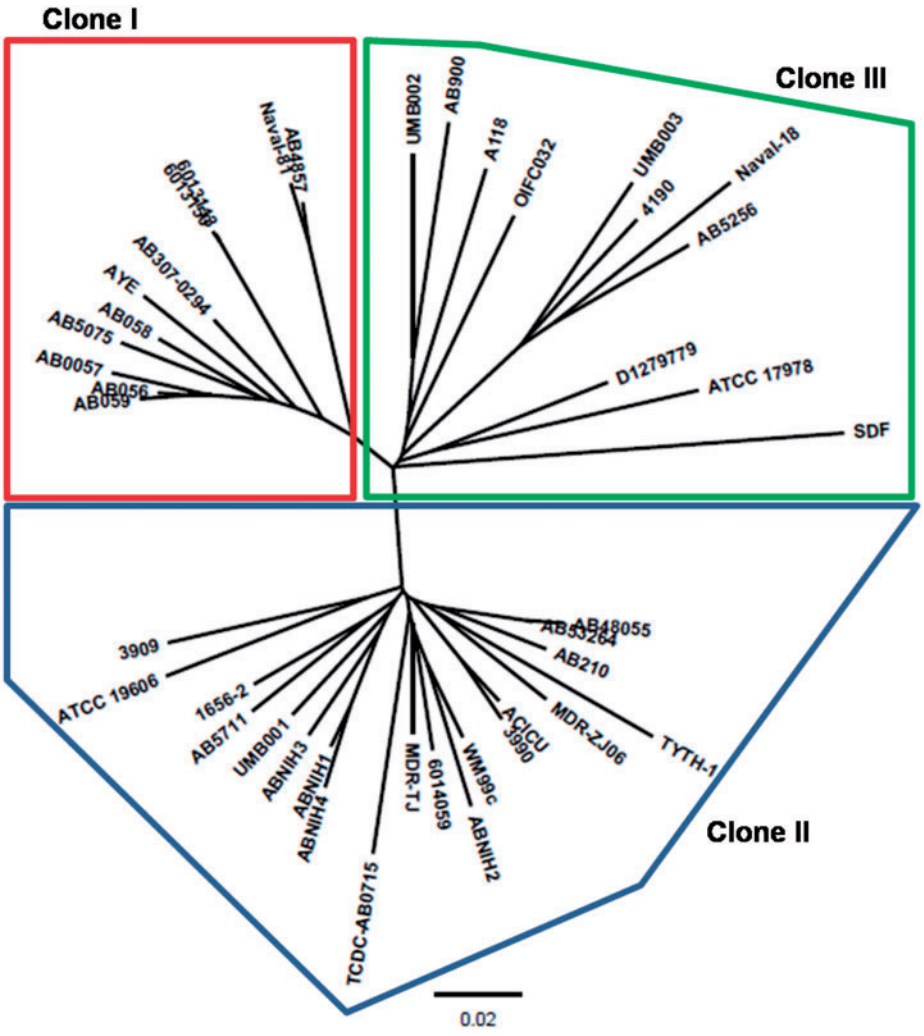

Fig. 2.—An unrooted phylogenetic tree showing the *Acinetobacter baumannii* 53264 and *A. baumannii* 48055 strains in relation to 42 other *A. baumannii* strains. The clonal groups are as follows: International Clone I (red box), Clone II (blue box), and Clone III (green box). Genome sequences of these 42 sequences were downloaded from NCBI FTP site. This phylogenetic tree was produced by pair-wise genome comparisons by Progressive Mauve. The *A. baumannii* 53264 and *A. baumannii* 48055 strains belong to the group of International Clone II *A. baumannii* strains.

Antibiotic Resistance Profile of the *A. baumannii* 53264 and 48055 Strains

Using disc diffusion antimicrobial susceptibility testing, the *A. baumannii* 53264 and 48055 strains were resistant to all 18 tested antibiotics, except for *A. baumannii* 48055's sensitivity to colistin. To confirm this result, the minimum inhibitory concentration was determined for A. *baumannii* 53264, *A. baumannii* 48055, and an antibiotic-sensitive *A. baumannii* 52082 strain to the following six representative antibiotics: tobramycin (aminoglycoside), colistin (antimicrobial peptide), ceftazidime (cephalosporin), tetracycline, ciprofloxacin (fluoroquinolone), and meropenem (carbapenem); the antibiogram is presented in table 2. The *A. baumannii* 52082 strain was sensitive to all six antibiotics (MIC: 1–2 μg/ml). The MIC profiles of the *A. baumannii* 53264 and 48055 strains were

Table 2

Minimum Inhibitory Concentrations (μg/ml) for the EDR *Acinetobacter baumannii* 53264 and PDR *A. baumannii* 48055 Strains, and the Antibiotic-Sensitive *A. baumannii* 52082 Strain toward the Following Six Antibiotics: Tobramycin, Colistin, Ceftazidime, Tetracycline, Ciprofloxacin, and Meropenem

Antibiotic Tested	Minimum Inhibitory Concentrations (μg/ml)		
	***A. baumannii* Strain 53264 (EDR)**	***A. baumannii* Strain 48055 (PDR)**	***A. baumannii* Strain 52082 (Sensitive)**
Tobramycin	256	256	2
Colistin	128	2	2
Ceftazidime	32	64	2
Tetracycline	>1,024	>1,024	1
Ciprofloxacin	64	64	1
Meropenem	16	64	1

Table 3

Antibiotic Resistance Profiles of *Acinetobacter baumannii* 53264 and *A. baumannii* 48055 Strains

Antibiotic Class	Resistance Gene[a]	NCBI DNA Accession	Description of Gene	Description of Gene Product[b]	Resistance Conferred[b]	Source
Aminoglycosides	*aac(3)-Ia*	X15852	Plasmid R1033 (Tn1696) *aacC1* gene for gentamicin acetyltransferase-3-I (AAC(3)-I).	Aminoglycoside N-acetyltransferase, which modifies aminoglycosides by acetylation.	Astromicin Gentamicin Sisomicin	Plasmid R1033, from *Pseudomonas aeruginosa*
	aac(6′)-Iaf	AB462903	*P. aeruginosa* DNA, class 1 integron In123, complete sequence.		Amikacin Dibekacin Isepamicin Netilmicin Sisomicin Tobramyci	*P. aeruginosa*
	aac(6′)-Il	Z54241	*Citrobacter freundii* int and aac(6′)-I genes. 6′-N-aminoglycoside acetyltransferase.			*C. freundii*
	aph(3′)-Ia	V00359	Transposon Tn903.	Aminoglycoside O-phosphotransferase, which modifies aminoglycosides by phosphorylation.	Gentamicin Kanamycin Lividomycin Neomycin Paromomycin Ribostamycin	*Escherichia coli*
	aph(3′)-Ic	X62115	*Klebsiella pneumoniae* plasmid pBWH77 *aphA7* gene for neomycin phosphotransferase.			*K. pneumoniae*
	Aph(3′)-VIa	X07753	*Acinetobacter baumanniiaph A-6* gene.		Amikacin Butirosin Gentamicin Isepamicin Kanamycin Neomycin Paromomycin Ribostamycin	*A. baumannii*
	strAlaph(3″)-Ib	M96392	*Erwinia amylovora* plasmid pEa34 transposon Tn5393 streptomycin phosphotransferase (*strA*) and streptomycin phosphotransferase (*strB*) genes.		Streptomycin	*E. amylovora*
	strBlaph(6)-Id					
Beta-lactam	bla_{OXA-23}	HQ700358	*A. baumannii* isolate AB210 AbaR4-type multiple antibiotic resistance island, complete sequence.	Class D beta-lactamase.	Carbapenems	*A. baumannii*
	blaTEM-1IRblaTEM-1	AF188200	*E. coli* beta-lactamase variant *TEM-1D* (*blaTEM-1D*) gene.	Class A beta-lactamase. This enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properties.	Cephalosporin Penicillin	*E. coli*

(continued)

Table 3 Continued

Antibiotic Class	Resistance Gene[a]	NCBI DNA Accession	Description of Gene	Description of Gene Product[b]	Resistance Conferred[b]	Source
Fluoroquinolones	No resistance genes found.					
Fosfomycin	No resistance genes found.					
Fusidic Acid	No resistance genes found.					
MLS—Macrolide—Lincosamide—Streptogramin B	No resistance genes found.					
Phenicol	No resistance genes found.					
Rifampicin	No resistance genes found.					
Sulfonamide	*sul1*	AY224185	*Escherichia coli* isolate Ec1484R sulphonamide resistance protein (*sulI*) gene.	Sulfonamide-resistant dihydropteroate synthase, which cannot be inhibited by sulfonamide.	Sulfonamide	*Escherichia coli*
	sul2	FM179941	*Pasteurella multocida* pCCK1900 plasmid, isolate 1900.			*P. multocida*
	sul3	AB281182	*P. aeruginosasul3* gene for dihydropteroatesynthetase.			*P. aeruginosa*
Tetracycline	*tet(B)*	AP000342	*Shigella flexneri* 2b plasmid R100 DNA, complete sequence.	Major facilitator superfamily transporter, tetracycline efflux pump.	Tetracycline	Plasmid R100 from *S. flexneri*
Trimethoprim	No resistance genes found.					
Glycopeptide	No resistance genes found.					

[a]Resistance genes identified with greater than 98.00% sequence identity by ResFinder (Zankari et al. 2012).
[b]Antibiotic resistance conferred is based on ARDB (Liu and Pop 2009).

Table 4

Nonsynonymous SNPs Observed in the *ampC*, *gyrB,* and *parC* Genes of *A. baumannii* 53264 and *A. baumannii* 48055 Strains in Reference to the *A. baumannii* ACICU Strain

	AB 53264 vs. ACICU		AB 48055 vs. ACICU	
	DNA Change	Protein Change	DNA Change	Protein Change
SNPs in *ampC* gene	c.31->T	S11fs		
	c.217C>A	R73S	c.217C>A	R73S
	c.427C>A	Q143K	c.427C>A	Q143K
	c.827T>C	F276S	c.827T>C	F276S
	c.828->G	F276fs	c.828->G	F276fs
	c.911G>A	S304N	c.911G>A	S304N
	c.1001A>C	N334T	c.1001A>C	N334T
	c.1114G>A	D372N	c.1114G>A	D372N
SNPs in *gyrB* gene	c.1738A>G	Y580H	c.1738A>G	Y580H
SNPs in *parC* gene	c.251C>T	S84L	c.251C>T	S84L
	c.623G>A	G208E	c.623G>A	G208E
	c.1982T>C	V661A	c.1982T>C	V661A

NOTE.—fs, frameshift.

similar, differing only in their sensitivity to colistin (MIC: 128 vs. 2 μg/ml).

The *A. baumannii* 53264 and *A. baumannii* 48055 sequences were submitted to both the recently described ResFinder database (Zankari et al. 2012) and the older ARDB (Liu and Pop 2009) to identify genes involved in antibiotic resistance. Table 3 lists the genes involved in the resistance of these EDR *A. baumannii* strains to aminoglycosides, beta-lactams, sulphonamides, and tetracyclines.

Antibiotic resistance genes present in the *A. baumannii* 53264 and 48055 genomes are similar to those found in a wide variety of other bacteria. *Acinetobacter baumannii* acquires its multiantibiotic resistance phenotype through the acquisition of mobile genetic elements, for example, plasmids and transposons (Fournier, Richet, et al. 2006). From table 3, we note that our *A. baumannii* strains possess the antibiotic resistance genes, *aac(6')-Iaf* and *sul3*, which are similar to genes present in *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* and *A. baumannii* are the two most prevalent nonfermentative bacteria isolated from hospital patients (Karlowsky et al. 2003) and can be assumed to be in close contact with each other, for example, in an infection site, thus allowing gene transfer. A striking example of this is transfer of an extended-spectrum β-lactamase integron (bla_{VEB-1}) from *P. aeruginosa* to *A. baumannii* in a hospital setting (Poirel et al. 2003).

Regarding beta-lactam resistance genes, our EDR *A. baumannii* strains carry the bla_{OXA-23} and the bla_{TEM-1} gene. The bla_{OXA-23} gene that confers imipenem resistance was first observed in Scotland (Scaife et al. 1995) but was later observed even in China (Zhou et al. 2007), in Bulgaria (Stoeva et al. 2008), in Brazil (Carvalho et al. 2009), and eventually world-wide (Mugnier et al. 2010). The bla_{OXA-23} gene encodes a Class D beta-lactamase that confers resistance against carbapenems (e.g., imipenem and meropenem) and ceftazidime (Mugnier et al. 2010). The bla_{TEM-1} gene encodes a Class A beta-lactamase that is found in 90% of ampicillin-resistant *Escherichia coli* strains (Livermore 1995). Because the bla_{TEM-1} gene is plasmid borne and utilizes transposon-mediated transfer, this gene spreads easily among bacteria and has been observed in *Enterobacteriaceae*, *P. aeruginosa*, and *Haemophilus influenza* (Bradford 2001).

Comparison of SNPs across *A. baumannii* Strains

We then studied the nonsynonymous (coding change) SNPs between the *A. baumannii* ACICU strain (Iacono et al. 2008) and our EDR *A. baumannii* 53264 and PDR *A. baumannii* 48055 strains. Table 4 presents the SNPs in three particular genes: *ampC, gyrB*, and *parC*.

Overproduction of the AmpC cephalosporinase by *A. baumannii* isolates has been shown to be important in conferring high levels of resistance to beta-lactam antibiotics (i.e., ceftazidime) (Corvec et al. 2003). Point mutations within the *ampC* gene promoter and attenuator regions have been shown to result in the hyperproduction of AmpC in *E. coli* strains (Nelson and Elisha 1999). In our case, the SNPs detected were within the *ampC* gene coding sequence. Molecular evolution of beta-lactamases confers extended substrate specificity, thus improving bacterial inactivation of a wider range of antibiotics (Nukaga et al. 1995). In *P. aeruginosa*, point mutations within the *ampC* gene resulted in increased beta-lactamase activity of AmpC, which resulted in increased resistance to ceftazidime (Tam et al. 2007). The *parC* gene encodes subunit A of topoisomerase IV, whereas the *gyrB* gene encodes DNA gyrase B. These products are the target for inhibition by quinolone-based antibiotics, and mutations within the *parC* and *gyrB* genes confer resistance to quinolones (Yoshida et al. 1991; Vila et al. 1997; Eaves et al. 2004).

The SNPs detected in the two *A. baumannii* strains are identical, except for an additional thymine inserted at position 31 of the *ampC* gene of *A. baumannii* strain 53264. The *A. baumannii* 53264 strain was isolated about a month after the *A. baumannii* 48055 strain was isolated, which would explain the additional time for mutation. This also lends support to the fact that the EDR *A. baumannii* 53264 strain has rapidly evolved from the PDR *A. baumannii* 48055 strain in as short as a 1-month period.

We then analyzed the SNP differences between the EDR *A. baumannii* 53264 and PDR *A. baumannii* 48055 strain to find out the reasons behind the evolution of colistin resistance. There were 61 nonsynonymous SNPs detected between the two strains (table 5). From table 5, we noticed two SNPs within the histidine kinase sensor *qseC* gene; QseC is a highly conserved regulator of virulence that responds to

Table 5
List of SNP Differences between the PDR *A. baumannii* 48055 and the EDR *A. baumannii* 53264 Strains

No.	Feature Name	DNA Change	Protein Change
1	LSU ribosomal protein L34p	c.136A>-	45fs
2	Biosynthetic aromatic amino acid	c.536G>T	P179H
3	Transcriptional regulator, LysR family	c.[847G>T]+[847G>G]	Q283K, Q283Q
4	FIG000988: Predicted permease	c.122T>G	V41G
5	FIG000906: Predicted permease	c.[866G>T]+[866G>G]	C289C, C289F
6	FIG000906: Predicted permease	c.[869C>T]+[869C>C]	S290S, S290F
7	FIG000906: Predicted permease	c.[874A>T]+[874A>A]	I292I, I292F
8	Acetoacetyl-CoA synthetase (EC	c.[1213C>C]+[1213C>A]	G405G, G405C
9	FIG00350520: hypothetical protein	c.266T>A	F89Y
10	FIG022199: FAD-binding protein	c.1206C>A	N402K
11	Transcriptional regulator, LysR family	c.762C>A	F254L
12	FIG00350303: hypothetical protein	c.[1502T>T]+[1502T>G]	V501G, V501V
13	L-carnitinedehydratase/bile acid-inducible	c.[530T>T]+[530T>G]	V177G, V177V
14	FIG00350277: hypothetical protein	c.2311A>T	F771I
15	CmaU	c.[27T>T]+[27T>G]	F9L, F9F
16	CmaU	c.32T>C	V11A
17	CmaU	c.34C>A	P12T
18	4Fe-4S ferredoxin, iron-sulfur binding	c.62C>A	A21D
19	FIG00350535: hypothetical protein	c.[56T>T]+[56T>G]	V19G, V19V
20	Cell surface protein	c.[571A>G]+[571A>A]	T191T, T191A
21	FIG00352920: hypothetical protein	c.[163G>G]+[163G>A]	E55K, E55E
22	FIG00352920: hypothetical protein	c.[176T>T]+[176T>C]	V59A, V59V
23	FIG00351830: hypothetical protein	c.[278G>G]+[278G>C]	G93A, G93G
24	putative hemolysin	c.[50G>T]+[50G>G]	C17C, C17F
25	Phenylacetic acid degradation protein	c.1004T>G	N335T
26	Two-component hybrid sensor and regulator	c.1561C>T	H521Y
27	5′-nucleotidase (EC 3.1.3.5)	c.[526A>T]+[526A>A]	F176I, F176F
28	Transcriptional regulator, AraC family	c.322A>C	F108V
29	Transcriptional regulator, AraC family	c.313A>C	F105V
30	Xylonatedehydratase (EC 4.2.1.82)	c.419G>C	G140A
31	Phenylalanine-specific permease	c.[203C>C]+[203C>A]	C68C, C68F
32	Alcohol dehydrogenase (EC 1.1.1.1)	c.440G>A	G147E
33	Alcohol dehydrogenase (EC 1.1.1.1)	c.443G>T	G148V
34	RNA polymerase sigma factor RpoH	c.107G>A	G36E
35	Sulfatepermease	c.[829A>C]+[829A>A]	C277G, C277C
36	FIG00351986: hypothetical protein	c.4G>T	P2T
37	FIG00349989: hypothetical protein	c.[10C>T]+[10C>C]	V4M, V4V
38	RarD protein	c.689T>G	E230A
39	RarD protein	c.677A>C	F226C
40	RarD protein	c.674A>C	V225G
41	Glycerate kinase (EC 2.7.1.31)	c.571C>G	P191A
42	Transcriptional regulator, TetR family	c.3C>A	L1F
43	Long-chain-fatty-acid–CoA ligase (EC 6.2.1.3)	c.1325C>A	G442V
44	FIG00352445: hypothetical protein	c.[1485T>T]+[1485T>A]	Q495Q, Q495H
45	FIG00352445: hypothetical protein	c.[1200T>T]+[1200T>A]	Q400Q, Q400H
46	Cell division protein FtsJ/ribosomal	c.473G>A	A158V
47	putative hemagglutinin/hemolysin-related protein	c.[592T>T]+[592T>C]	T198T, T198A
48	Putative hemagglutinin/hemolysin-related protein	c.[589T>T]+[589T>G]	I197I, I197L
49	Putative hemagglutinin/hemolysin-related protein	c.[436C>T]+[436C>C]	V146I, V146V
50	Putative hemagglutinin/hemolysin-related protein	c.[205T>T]+[205T>C]	I69I, I69V
51	Sensory histidine kinase QseC	c.680A>G	V227A
52	Sensory histidine kinase QseC	c.623G>A	P208L
53	Putative stomatin/prohibitin-family	c.[172G>T]+[172G>G]	V58V, V58L
54	Hypothetical protein; putative signal peptide	c.407G>T	A136D

(continued)

Table 5 Continued

No.	Feature Name	DNA Change	Protein Change
55	FIG00351726: hypothetical protein	c.[947A>C]+[947A>A]	V316G, V316V
56	N-carbamoylputrescineamidase (3.5.1.53)	c.388->T	I130fs
57	FIG00350819: hypothetical protein	c.79C>A	A27S
58	Sodium-dependent transporter	c.618C>A	M206I
59	FIG00350872: hypothetical protein	c.713G>T	T238K
60	Histone acetyltransferase HPA2	c.[122T>T]+[122T>C]	Q41Q, Q41R
61	Histone acetyltransferase HPA2	c.[118T>T]+[118T>C]	T40T, T40A

NOTE.—The changes in DNA and protein that occur in *A. baumannii* 48055 strain, with reference to the *A. baumannii* 53264 genome sequence.

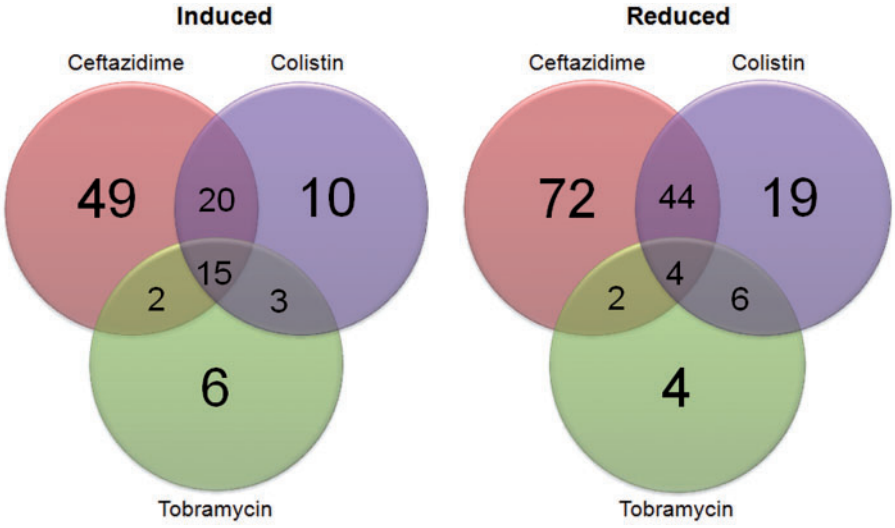

FIG. 3.—The Venn diagram on the left shows the number of proteins whose expression was induced in the presence of a specific antibiotic. The Venn diagram on the right shows the number of proteins whose expression was reduced in the presence of a specific antibiotic.

Table 6

Proteins Whose Expression Was Down-Regulated by All Three Antibiotics

Accession No.	Name	Function	No. of Matched Peptides (95%)	%Cov (95%)	Treatment with		
					Cef	Col	Tob
gi|183211243	Succinylargininedihydrolase	Arginine and proline metabolism	31	68.9	0.45	0.43	0.43
gi|183209017	Dihydroorotase	Pyrimidine biosynthesis	7	68.6	0.44	0.10	0.47
gi|183210937	Long-chain fatty acid transport protein	Membrane transport of long-chain fatty acids	19	60.04	0.35	0.37	0.32
gi|183211425	Putative porin	Membrane transport	7	62.35	0.30	0.17	0.49

NOTE.—Cef, ceftazidime; Col, colistin; Tob, tobramycin. Percent coverage (%Cov) refers to the percent of the residues in each protein sequence that has been identified at 95% confidence level. Numbers in the Cef, Col and Tob columns refer to the fold change of the protein abundance when compared with the control sample (without antibiotics).

both bacteria signals and host cell factors (Clarke et al. 2006). Inhibition of QseC markedly reduced the virulence of *Salmonella enterica* serovar Typhimurium (Rasko et al. 2008). Transcriptomic analysis revealed a role for QseC in the antimicrobial peptide (i.e., polymyxins) and oxidative stress resistance responses (Karavolos et al. 2008).

As these mutations occurred within a 1-month period, these 61 SNP changes indicate the fast evolution of the *A. baumannii* strains. Colistin resistance has been shown to result from a complete loss of lipopolysaccharide (LPS) production by deletion of LpxA, LpxB, and LpxD (Moffatt et al. 2010) or modifications of LPS through mutations in the pmrAB

two-component system (Beceiro et al. 2011). EDR strains are defined by their resistance to colistin; however, we were not able to detect any SNP changes that would confer resistance to colistin. Hence, we decided to use iTRAQ-based quantitative proteomics to study the proteome changes in the EDR *A. baumannii* 53264 strain in response to antibiotics.

Comparative Analysis of Antibiotic-Tolerance-Related Proteins of the EDR *A. baumannii* 53264 Strain Using iTRAQ

We then studied the stress response of the *A. baumannii* 53264 strain to treatment by three different classes of antibiotics (colistin, ceftazidime, and tobramycin) to understand the stress response of this EDR *A. baumannii* clone to various antibiotics. Figure 3 shows the number of proteins whose expression was induced or reduced in the presence of a specific antibiotic (ceftazidime, colistin, or tobramycin).

We defined induced proteins as those whose abundance was increased by at least 2-fold versus the control (without antibiotic addition). Conversely, reduced proteins were defined as those whose abundance was decreased by at least 2-fold versus the control. Overlapping regions of the Venn diagrams show the number of proteins whose expression was found to be commonly induced (or reduced) by one or more antibiotics.

There was more similarity in the genes induced (or reduced) in the presence of ceftazidime and colistin when compared with those induced (or reduced) by tobramycin. This indicates that the resistance mechanisms of *A. baumannii* 53264 strain to ceftazidime and colistin have more in common than its resistance mechanism to tobramycin. A different set of proteins may be required for its resistance to tobramycin. This could be due to the fact that ceftazidime and colistin target the bacteria cell wall (Hayes and Orr 1983) and cell membrane (Falagas et al. 2005), respectively, whereas tobramycin targets the 30S ribosomal subunit (Walter et al. 1999).

Next, we wanted to study the common set of genes that were induced or reduced by all three antimicrobials, as this would indicate a core set of genes essential to the stress response of EDR *A. baumannii* 53264 to antibiotics. Table 6 lists four proteins that were found to be commonly down-regulated (by at least 2-fold) by *A. baumannii* 53264 in all three antibiotic treatments. (The list of commonly up-regulated proteins is included as supplementary table S1, Supplementary Material online.)

From table 6, we note the decreased expression of a putative porin (gi|183211425). This outer membrane protein (OMP) was found to be at least 2-fold under expressed in all three antibiotic treatments, with the colistin treatment causing a more than 5-fold decrease in expression of this porin. OMPs are involved in the uptake of antibiotics into the bacterial cell. For example, the OMP OprD of *P. aeruginosa* has been shown to be important in the uptake of positively charged peptides and carbapenem antibiotics (Nikaido 2003). Also, the expression of the OMP OmpW of *A. baumannii* was found to be reduced in a colistin-resistant strain (Vila et al. 2007). Hence, down-regulation of porin expression may reduce colistin uptake by the *A. baumannii* 53264 strain and explain its resistance toward colistin.

Supplementary Material

Supplementary table S1 is available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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