Comparative Genomics of Multidrug Resistance in *Acinetobacter baumannii*

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Acinetobacter baumannii is a species of nonfermentative gram-negative bacteria commonly found in water and soil. This organism was susceptible to most antibiotics in the 1970s. It has now become a major cause of hospital-acquired infections worldwide due to its remarkable propensity to rapidly acquire resistance determinants to a wide range of antibacterial agents. Here we use a comparative genomic approach to identify the complete repertoire of resistance genes exhibited by the multidrug-resistant A. baumannii strain AYE, which is epidemic in France, as well as to investigate the mechanisms of their acquisition by comparison with the fully susceptible A. baumannii strain SDF, which is associated with human body lice. The assembly of the whole shotgun genome sequences of the strains AYE and SDF gave an estimated size of 3.9 and 3.2 Mb, respectively. A. baumannii strain AYE exhibits an 86-kb genomic region termed a resistance island—the largest identified to date—in which 45 resistance genes are clustered. At the homologous location, the SDF strain exhibits a 20 kb-genomic island flanked by transposases but devoid of resistance markers. Such a switching genomic structure might be a hotspot that could explain the rapid acquisition of resistance markers under antimicrobial pressure. Sequence similarity and phylogenetic analyses confirm that most of the resistance genes found in the A. baumannii strain AYE have been recently acquired from bacteria of the genera Pseudomonas, Salmonella, or Escherichia. This study also resulted in the discovery of 19 new putative resistance genes. Whole-genome sequencing appears to be a fast and efficient approach to the exhaustive identification of resistance genes in epidemic infectious agents of clinical significance.

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Introduction

The prevalence of nosocomial infections in hospital intensive care units due to Acinetobacter baumannii currently ranges from 2% to 10% of all gram-negative bacterial infections in Europe [1] and account for about 2.5% of them in the United States [2]. A. baumannii exhibits a remarkable ability to rapidly develop antibiotic resistance that led to multidrug resistance (MDR) within a few decades [3]. To date, some strains of A. baumannii have become resistant to almost all currently available antibacterial agents [4], mostly through the acquisition of plasmids [5], transposons [6], or integrons [7,8] carrying clusters of genes encoding resistance to several antibiotic families [6-8] at once. With the emergence of increasingly resistant strains, the management of A. baumannii infections has become a public health problem in many countries. Recently, a high incidence of MDR A. baumannii bloodstream infections in US Army service members injured during Afghanistan and Iraq/Kuwait military operations was reported [9]. To date, no study has been designed to investigate at once the various resistance mechanisms involved in the acquisition of an MDR phenotype by a given A. baumannii strain. We had the opportunity to cultivate the MDR A. baumannii strain AYE, which is resistant to β-lactams (except imipenem, piperacillin-tazobactam, and ticarcillinclavulanate), aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, and rifampin; is epidemic in 54 healthcare facilities in eight French administrative regions; and is associated with a mortality of 26% of infected patients [8]. Simultaneously, we also cultured the remarkably susceptible strain SDF, which is associated with human body lice [10]. We used a whole-genome sequencing approach to compare the gene content of *A. baumannii* AYE and SDF strains, with a special emphasis on gene categories related to antibacterial resistance. The parallel genome sequence annotation of both strains allowed us to identify all genes associated with previously known antibiotic resistance, and, unexpectedly, to discover that most of these genes were clustered in an 86-kb region, or "island," of the *A. baumannii* strain AYE genome. To our knowledge, this is the largest resistance island described to date. In addition, we discovered 19 putative resistance genes not previously described in *A*.

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Abbreviations: MDR, multidrug resistance (-resistant); ORF, open reading frame

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Synopsis

The bacterial species Acinetobacter baumannii is a major cause of hospital-acquired infection throughout the world, and it is an increasing public health concern due to its increasing resistance to antibiotic treatment. Coincidently, a high incidence of multidrugresistant A. baumannii bloodstream infections was recently reported in US Army service members injured during Afghanistan and Iraq/ Kuwait military operations. A. baumannii exhibits a remarkable ability to rapidly develop antibiotic resistance, which led from fully susceptible to multidrug-resistant strains within three decades. The authors used whole-genome sequencing and bioinformatic analyses to identify the complete repertoire of resistance genes exhibited by the multidrug-resistant A. baumannii strain AYE, which is epidemic in France, and to investigate the mechanisms of their acquisition by comparison with the fully susceptible A. baumannii strain SDF, which is associated with human body lice. This study led to the discovery in the AYE genome of an 86-kb region called a resistance "island"the largest identified to date-that contains a cluster of 45 resistance genes. The homologous location in the susceptible strain, curiously, exhibited a 20-kb genomic island that is devoid of resistance markers. This ability to "switch" its genomic structure probably explains the unmatched speed at which A. baumannii captures resistance markers when under antibacterial pressure, such as is found in hospital intensive care units.

baumannii. The sequence analysis of *A. baumannii* AYE antibacterial resistance genes indicates that frequent genetic exchanges take place with *Pseudomonas* spp., *Salmonella* spp., or *Escherichia* spp.

Results

Shotgun Genome Sequences of *A. baumannii* Strains AYE and SDF

A preliminary assembly of the whole shotgun genome sequences of the strains AYE and SDF, as described below in Materials and Methods, led to a size estimate of 3.9 Mb and 3.2 Mb, respectively, with G + C contents of 38.8% and 38.2%.

In addition to their main chromosomes, the AYE and SDF strains harbor three plasmids (5, 9, and 94 kb) and two plasmids (6 and 25 kb), respectively. Surprisingly, none of the plasmids carry any known resistance marker. A detailed analysis of these two genome sequences will be published elsewhere; the present report focuses on the genes predicted to be involved in antibacterial resistance that, as expected, were found to account for the main difference in gene complement between *A. baumannii* strains AYE and SDF. These genes, together with the type of antibacterial resistance they display, are listed in Tables 1–3.

An 86-kb Resistance Island in A. baumannii Strain AYE

The AYE strain genome encoded 52 genes predicted to be associated with resistance to antimicrobial drugs, while only seven were identified in the SDF strain genome. Interestingly, 45 (86.5%) of the 52 AYE resistance genes are tightly clustered in an 86,190-bp genomic region disrupting a putative ATPase open reading frame (ORF) (Figures 1 and 2). The insertion site was identical in the two strains (Figure 1) and the insert was flanked at both extremities by a fivenucleotide direct repeat (ACCGC). This repetition is likely due to a duplication, which suggests a transposition mechanism for the insertion. However, no inverted repeats characteristic of transposons were found at the extremities of the island. The 86-kb region was classified as a genomic island (and designated AbaR1) on the basis of its size (>10 kb), marked G + C content difference from the rest of the chromosomes (52.8% versus 38.8%), the presence of genes associated with genome instability such as integrases, transposases, and insertion sequences, and the diverse phylogenetic origin of the associated ORFs [11]. At the same position, flanked by identical nucleotide sequences and within the homologous ATPase ORF, the A. baumannii strain SDF genome sequence exhibits a genomic island (designated AbaG1), albeit only 19,632 bp in length, with a G + C content of 31.3% (Figure 1). This shorter genomic island encodes 25 putative ORFs, 12 of which had a match in databases, but only ten of which were assigned a function: four transposases, a transposition helper, a thymidylate synthase, anthranilate synthase components 1 and 2, a putative Δ -aminolevulinic acid dehydratase, and a mutT/NUDIX hydrolase (Figure 3). Detailed sequence comparison of the two islands failed to reveal any significant similarity, including among the transposases found at their extremities.

Genomic islands containing resistance markers are referred to as resistance islands [12]. Resistance islands have been described mainly in y-proteobacteria, including Shigella flexneri, Salmonella enterica, and Vibrio cholerae, but also in Staphylococcus aureus [11]. Their sizes range from 20 to 60 kb [11]. The AbaR1 resistance island specific to the A. baumannii AYE strain is thus, at 86 kb, to our knowledge the largest described to date, encoding 88 predicted ORFs, 82 of which could be assigned a predicted function (Figure 2, Tables 1–3). According to amino acid sequence similarities greater than 90% in most cases, 39 genes (44%) are likely to have originated from Pseudomonas spp., 30 (34%) from Salmonella spp., 15 (17%) from *Escherichia* spp., and four (4%) from other microorganisms. Of the 45 resistance genes, 25 are associated with various classes of antibiotics. These include genes that had not been previously described in Acinetobacter species: strA, strB, aphA1, and aac6' (resistance to aminoglycosides); putative tetracycline-resistance genes *tet*A (tetracycline efflux pump) and tetR (repressor); dfrX (resistance to cotrimoxazole); and the chloramphenicol-resistance gene cmlA (chloramphenicol efflux pump). In addition, genes previously found in Acinetobacter species were identified, including those that encode the β-lactamases VEB-1 and OXA-10; the aminoglycoside acetyl transferase gene aac3 and the aminoglycoside adenylyltransferases aadA1/DA1/B; the cotrimoxazole resistance-associated dfrI; tetA and tetR; cmlA5 and one copy of the chloramphenicol acetyl-transferase cat; the rifampin ADP-ribosyltransferase gene arr-2; and five copies of the sulfonamide-resistance gene sull encoding dihydropteroate synthetase, a component of class 1 integrons. The annotated sequences of the AbaR1 and AbaG1 islands, which include the genes discussed above, have been deposited in the EMBL database.

Antiseptic Resistance-Associated Genes in *A. baumannii* Strain AYE

The AbaR1 also encodes two complete operons, one associated with arsenic resistance [13] and the second with mercury resistance (Figure 2) [14]. The latter is widely distributed both in clinical strains of gram-negative bacteria and in environmental bacterial strains [15]. The arsenic-

Table 1. A. baumannii AYE Strain ORFs Putatively Associated with Resistance to Antibiotics

Antibiotic	Gene Name	Predicted Specificity	AbaR1 Location ^a	Also Present in	GenBank Match	Best Match If Not in
				SDF (% Amino Acid Identity)	(% Amino Acid Identity)	(% Amino Acid Identity)
ß-lactams	blayEB-1 (clace D) (1 163)	All hla evcent carb	Vec (CT075832)		4 haumannii (100%) [8]	P derucionsa (100%) [42]
	Putative class A B-lactamase (2, 314)	Unknown	No (CT025947)	Yes (97%) (AM086638)		Thermus thermophilus (36%) [43]
	<i>amp</i> C (class C) (27_169)	All bla except ctx, caz. fep	No (CT025798)	No	Acinetobacter genomosp. 3 (98%) [33]	
	^{bla} OXA-10 (class D) (1_176)	All bla except esc, carb	Yes (CT025832)	No	A. baumannii (100%) [8]	P. aeruginosa (100%) [44]
	^{bla} OXA-69 (class C) (1_131)	Unknown	No (AY859527)	Yes (96%) (AM086637)	A.baumannii (100%) [45]	
Aminoglycosides	aac3 (acetyl-transferase) (1_339)	Gen	Yes (CT025832)	No	A. baumannii (100%) [46]	E. coli (100%) [47]
	aac6' (acetyl-transferase) (1_126)	All amg except gen	Yes (CT025832)	No	No	Nostoc punctiforme (46%) (ZP_00109305)
	aadA1 (adenylyltransferase) (1_179)	Stre, spe	Yes (CT025832)	No	A. baumannii (99%) [20]	P. aeruginosa (100%) [42]
	aadDA1 (adenylyltransferase) (1_348)	Stre, spe	Yes (CT025832)	No	A. baumannii (100%) [48]	E. coli (100%) (Zienkiewicz, Kern-Zdanowicz, Golebiewski, and Cerdowski, unu hiliched data)
	aadB (adenvlvltransferase) (1 165)	Gen. kan. tob	Yes (CT025832)	No	A. <i>baumannii</i> (100%) [8]	B. aprilationsa (100%) [42]
	Ditative adenvivitualisticase (41 208)		No (CT025824)	Vec (00%) (AM086636)		F coli (41%) (Norskov-1 auriteen
	rutauve auenyiyittansielase (+1_200)		(1000000) ON	(00000011114) (04.66) 531	2	and Sandvang, unpublished data)
	aphA1 (phosphotransferase) (1_522)	Amikacin	Yes (CT025832)	No	No	E. coli (100%) [49]
	strA (phosphotransferase) (1_59)	Stre	Yes (CT025832)	No	No	S. typhi (98%) [50]
	strB (phosphotransferase) (1_62)	Stre	Yes (CT025832)	No	No	S. typhi (98%) [50]
Fluoroquinolones ^t	^b Mutation at position 80 in <i>par</i> C: Ser \rightarrow Leu (42_46)	All flu	No (CT025948)	No	A. baumannii [51] 100%	
	Mutation at position 83 in gyrA: Ser \rightarrow Leu (25_18)	All flu	No (CT025946)	No	A.baumannii [51] 100%	
Tetracyclines	tetA (efflux pump) (1_103)	All tet	Yes (CT025832)	No	No	S. typhimurium (100%) [52]
	tetR (tetracycline repressor) (1_748)	All tet	Yes (CT025832)	No	No	S .typhimurium (100%) [52]
	tetA (efflux pump) (1_258)	All tet	Yes (CT025832)	9	A. baumannii (99%) [53]	 Styphimurium (100%) (Pasquali, Kehrenberg, Manfreda, and Schwarz, unnuhlished data)
		11 ÷ - +				
	tetk (tetracycline repressor) (1_598)	All tet	Yes (L1025832)	ON	A. baumannu (100%) [53]	 Syphimurum (100%) (Pasquali, Kehrenberg, Manfreda, and Schwarz, unpublished data)
	Putative tetA (efflux pump) (12_578)	Unknown	No (CT025784)	Yes (96%) (AM086635)	No	A. tumefaciens (43%) [54]
Trimethoprim	dhfri (1_73)	Tri	Yes (CT025832)	No	Yes (100%) [17]	A. baumannii (100%) [17], S. albany (100%) [55]
	dhfrX (1_196)	Hi I	Yes (CT025832)	No	No	S. agona (100%) [56]
Chloramphenicol	cm/A (efflux pump, MFS family) (1_88)	Clo	Yes (CT025832)	No	No	S. typhimurium (90%) [25]
	cm/A5 (efflux pump, MFS family) (1_173)	Clo	Yes (CT025832)	No	A. baumannii (100%) [8]	P. aeruginosa (100%) [57]
	cat (acetyltransferase) (1_569)	Clo	Yes (CT025832)	No	A. calcoaceticus (99%) [23]	E. coli (99%) (AAT37967)
Rifampin	arr-2 (1_166)	Rifampin	Yes (CT025832)	No	A. baumannii (100%) [8]	P. aeruginosa (100%) [42]
Sulfonamides	sull (1_81)	All sulfonamides	Yes (CT025832)	No	A. baumannii (100%) [58]	P. aeruginosa (90%) [59]
	<i>sul</i> l (3 identical copies) ¹ (1_187, 1_203, 1_3	356) All sulfonamides	Yes (CT025832)	No	A. baumannii (100%) [58]	P. aeruginosa (100%) [42]
	sull (1_442)	All sulfonamides	Yes (CT025832)	No ::	Acinetobacter spp. ADP1 (72%) [32]	Pseudomonas spp. (78%) [60]
	sull (32_1034)	All sulfonamides	Yes (AM086633)	No	Acinetobacter spp. ADP1 (70%) [32]	Pseudomonas spp. (76%) [60]
Putative new A. baumann	nii resistance associated genes identified in this study are indicat	ted in bold red. ORF sequences exhibiting	ess than 70% identical residue	is with their closest homologs in <i>A. baumannii</i> were	considered "new." The same similarity threshold was a	pplied to assess the presence of orthologs in the SDF

⁴Genbank accession numbers of the corresponding sequence are indicated in parenthesis. ^{Nessistance to fluoroquinolones results from mutations in the QBDR region of the constitutive genes *parC* and *gyA* and not from the acquisition of genes. ^TIncreade genes, Genbank accession numbers are indicated in parenthesis. The increasion numbers are indicated in parenthesis. Distributions May, any applicitlin; max, amondalin; bia, beta-hactams; cab, catbapenems; caz, cefazidine; cef, cefoxitin; clo, choramphenicol; ctx, ceftriaxone; esc, extended-spectrum cephalosporines; ety, erythromycin; fep, cefepime; flu, fluoroquinolones; gen, gentamicin; kan, kanamycin; spe, spectromotions; its, streptomycin; its, treptomycin; tet, utimethoprim; tzp, piperacillin-tazobactam. DDI: 10.1371/journal.pgen0.0200074001}

Antiseptic Class	Gene Name	Predicted Specificity	AbaR1 Location	Also Present	GenBank Match	Best Match If Not in
				in A. baumannii SDF (% Amino Acid Identity)	with Acinetobacter spp. (% Amino Acid Identity)	Acinetobacter (% Amino Acid Identity)
Heavy metals (arsenic, mercury)	Arsenic resistance operon	Arsenic, antimony				
	arsB (1_816) ^a		Yes (CT025832)	No	No	B. cereus (60%) [61]
	arsC (1_814) ^a		Yes (CT025832)	No	No	P. aeruginosa (52%) [62]
	arsC (1_812) ^a		Yes (CT025832)	No	No	E. coli (65%) [63]
	arsH (1_817)		Yes (CT025832)	No	No	P. putida (72%) [64]
	arsR (1_813)		Yes (CT025832)	No	No	P. aeruginosa (67%) [62]
	Mercury resistance operon	Mercury				
	merA (1_621)		Yes (CT025832)	No	A. calcoaceticus (100%) (93%) [24]	S. typhi (100%) [50]
	merC (1_615)		Yes (CT025832)	No	A. calcoaceticus (100%) (100%) [24]	S. typhi (100%) [50]
	merD (1_622)		Yes (CT025832)	No	A. calcoaceticus (99%) [24]	Salmonella typhi (100%) [50]
	merE (1_609)		Yes (CT025832)	No	A. calcoaceticus (100%) [24]	P. aeruginosa (100%) [65]
	merP (1_612)		Yes (CT025832)	No	A. calcoaceticus (96%) [24]	S. typhi (100%) [50]
	merR (1_244)		Yes (CT025832)	No	A. calcoaceticus (100%) [24]	S. typhi (99%) [50]
	merT (1_609)		Yes (CT025832)	No	A. calcoaceticus (90%) [24]	S. typhi (100%) [50]
Other heavy metals	<i>pbr</i> (2 identical copies) (1 808, 1 465)	Lead	Yes (AM086632)	No	No	P. putida (93%) [21]
	Heavy metal (Co/Zn/Cd)	Cobalt, zinc, cadmium	Yes (CT025832)	No	No	P. putida (95%) [21]
	efflux pump (2 identical copies)					
	czcD (Co/Zn/Cd efflux system) (5_1363)	Cobalt, zinc, cadmium	No (CT025826)	Yes (99%) (AM086634)	No	Azotobacter vinelandii (62%) (ZP_00088783)
Ammonium	<i>gacEA</i> 1(3 identical copies) (efflux pump, SMR family) (1_78, 1_183, 1_352)	Quat, quaternary ammonium compounds	Yes (CT025832)	No	A. baumannii (100%) [17]	P. aeruginosa (100%) [66]
	<i>qacE</i> 11 (efflux pump, SMR family) (1_199)	Quat, quaternary ammonium compounds	Yes (CT025832)	No	A. baumannii (99%) [17]	S. typhi (100%) [55]
	qacEA1 (efflux pump, SMR family)	Quat, quaternary	No (CT025799)	No	No	B. bronchiseptica (67%) [67]
	(29_139)	ammonium compounds				

"The ar8b and ar5C genes were detected by probe hybridization only in *Acinetobacter* genomospecies 15 [13]. DOI: 10.1371/journal.pgen.0020007.4002

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Cation/multidrug efflux pump Unknown No (CT025801) Yes (99%) (29 167)	No	B. bronchiseptica (64%) [6
	No	Dechloromonas aromatica 00152407)
24 224 224 224 224 224 224 224 224 224		Novosnhingohium (53%) (1

Table 3. Contir	nued.					
Transporter Gene Family	Gene Name	Predicted Specificity	AbaR1 Location	Also Present in A. baumannii SDF (% Amino Acid Identity)	GenBank Match with <i>Acinetobacter</i> spp. (% Amino Acid Identity)	Best Match If Not in <i>Acinetobacter</i> (% Amino Acid Identity)
	17_38	Unknown	No (CT025945)	Yes (99%)	Acinetobacter sp. ADP1 (80%) [32]	
	Cation/multidrug efflux pump (36_440)		No (CT025820)	Yes (99%)	Acinetobacter sp. ADP1 (83%) [32]	
MATE family	36_503	Unknown	No (CT025821)	Yes (94%)	No	B. bronchiseptica (37%) [67]
	36_127	Unknown	No (CT025818)	Yes (99%)	Acinetobacter sp. ADP1 (83%) [32]	
SMR family	6_165	Unknown	No (CT025829)	Yes (99%)	Acinetobacter sp. ADP1 (83%) [32]	
ABC superfamily	2_694	Unknown	No (CT025804)	Yes (98%)	Acinetobacter sp. ADP1 (82%) [32]	
DMT family	Permease (31_349)	Unknown	No (CT025806)	Yes (99%)	No	P. fluorescens (64%) (ZP_00267472)
APC family	D-serine/D-alanine/glycine transport protein (41_138)	Amino acids	No (CT025822)	Yes (94%)	Acinetobacter sp. ADP1 (87%) [32]	
	D-serine/D-alanine/glycine transport protein (41_141)	Amino acids	No (CT025823)	Yes (92%)	Acinetobacter sp. ADP1 (82%) [32]	
Abbreviations: Amg, amino; DOI: 10.1371/iournal.poen.0	gycosides; clo, chloramphenicol; eny, erythromycin. 1020007.t003	; EthBr, Ethidium bromide; flu, fluoroquina	olones; tet, tetracyclines; tri, trimeth	oprim.		

A. baumannii Multidrug Resistance Genes

resistance operon lacks the *ars*A and *ars*D genes [16] associated with high levels of resistance to both arsenic and antimony, but it includes *ars*H and *ars*R, which have not been found previously in *Acinetobacter* species. In addition, two genes encoding heavy metal efflux pumps, and four *qacEA1* genes encoding small multidrug resistance (SMR)-family efflux pumps, known to confer a low level of resistance to ammonium antiseptics, are scattered throughout the AbaR1 resistance island. The *qacEA1* genes were probably not acquired independently, as they are parts of the structure of class 1 integrons.

Fine Structure of the AbaR1 Resistance Island

Fourteen of the antibiotic resistance genes could be mapped to three class 1 integrons (Figure 2). One integron carries *dfr*I [17]. A second integron is a composite made of a complete class 1 integron identified in *P. aeruginosa* [8], which carries ^{bla}VEB-1, ^{bla}OXA-10, *arr-2*, *cmlA*, *aad*A1 [18], and a duplication of the 3'-conserved segment region that includes orf513 and *dfr*X cassettes. Such additional genes have been observed in *In*6-like class 1 integrons [19]. The third integron encodes *aac*3 and *aad*DA1 [20]. No other integron was detected outside of AbaR1 in the AYE strain genome, nor in the SDF strain genome.

In addition to integrons, a record 22 ORFs encoding transposases or other mobility-associated proteins were identified in the AbaR1 resistance island, suggesting that transposons are central to the island dynamic and the rapid acquisition of foreign resistance genes by the A. baumannii AYE strain. For instance, the island exhibits a near perfect duplication of a cluster of four genes including *pbr*R (a heavy metal-associated cation efflux transporter-encoding gene), lspA (lipoprotein signal peptidase gene), and a gene encoding a tnpA-like transposase (Figure 2). Such a gene cluster was previously encountered in a *Pseudomonas putida* transposable element (ISPpu12) [21]. Another four transposons, previously described in Acinetobacter spp. or other bacteria, were found within AbR1. These included a truncated Tn5393 transposon made of strA, strB, and tnpM [22]; a truncated Tn1721 transposon comprising tetR, tetA, pecM, and tnpA [22]; an IS1like transposable element including ybjA, cat, insA, and insB [23]; and a Tn21-like transposon made of tnpA, urf2y, and the mercury-resistance operon [24]. Another five insertion sequences from the IS15 and IS26 classes were found in AbR1, including two IS26 that flanked the aphA1 gene. In addition, the remaining five genes involved in antibiotic resistance were located near a transposase gene, thus suggesting a possible role of transposition in their acquisition.

Genomic islands are unstable regions and hotspots for the successive integration of resistance determinants in *Salmonella* spp., *Escherichia coli*, or *Streptococcus thermophilus* [12]. We can safely assume that the mosaic-like structure of the AbaR1 island is the result of successive acquisitions of DNA fragments from different hosts [12], mainly *Pseudomonas* spp., *Salmonella* spp., and *E. coli* (Figure 2). We also identified a cluster of genes similar to a fragment of the *Salmonella* genomic island 1 that comprises *cmlA*, *tetR*, *tetA*, *lysR*, a transposase-like gene, and a *GroEL*-integrase fusion protein-encoding gene [25]. However, no obvious mechanism for these integrations is suggested by the structure of the *A. baumannii* genome. Although AbaR1 contains many genes



Figure 1. Comparison of Insertion Sites of the AbaR1 and AbaG1 Islands in an ATPase ORF

Insertion sites of the AbaR1 (A) and AbaG1 (B) islands in an interrupted ATPase-encoding ORF are compared. ORFs 1_837 and 1_434 in strain AYE, and 1_126 and 1_87 in strain SDF, retain a strong similarity to the 3' and 5' fragments, respectively, of the ATPase sequence encoded by *Acinetobacter* strain ADP1 [32]. The 5-bp direct repeats flanking the islands are underlined. DOI: 10.1371/journal.pgen.0020007.g001



Figure 2. Layout of the Complete AbaR1 Inserted into the AYE Strain ATPase-Encoding Gene

The nomenclature of each ORF is indicated. Each ORF is identified according to EMBL entry CT025832. Colors are used to indicate ORF categories: resistance to antibiotics in red, resistance to heavy metals or antiseptics in blue, transposases in brown, integrases in yellow, and other functions in white. Genes exhibiting a best matching homolog in *Pseudomonas* are underlined in green, in yellow for *Salmonella*, in light blue for *E. coli*, and in turquoise for other bacteria. Complete integrons are indicated by red dashed lines. Transposons are indicated by blue dashed lines. Black dashed lines indicate a gene cluster found in *Salmonella* genomic island 1. DOI: 10.1371/journal.pgen.0020007.g002



Figure 3. Layout of the Complete AbaG1 Inserted into the SDF Strain ATPase-Encoding Gene

The nomenclature of each ORF is indicated. Each ORF is identified according to EMBL entry CT025833. ORF categories are indicated by colors: transpositions in brown and other functions in white. The phylogenetic origin of genes is highlighted by color markers. Dashed lines indicate genes found in the same order in other bacteria. DOI: 10.1371/journal.pgen.0020007.g003

found on plasmids in other bacteria, it is devoid of plasmid markers. It has been proposed that a strain of *A. calcoaceticus* could stably integrate antibiotic resistance genes carried by an R plasmid into its chromosome, and then discard the plasmid [6]. Such a mechanism could have occurred in the *A. baumannii* AYE strain. Another possible acquisition mechanism might involve natural transformation [26]. Transformation has been demonstrated in *Acinetobacter* spp. [27–31]. As a matter of fact, the first genome of an *Acinetobacter* spp. to be sequenced was that of the highly transformable *Acinetobacter* spp. strain ADP1 [32]. The close evolutionary proximity of *Acinetobacter* spp. and *Pseudomonas* spp. may facilitate direct gene exchanges between them.

Antibacterial Resistance Genes outside of the Genomic Islands

In addition to the two β -lactamase genes located in AbaR1, three others were identified in AYE: the previously described *ampC* [33,34], surprisingly absent from the SDF strain, and two new putative β -lactamases (Table 1), both of which had orthologs in the SDF strain. One of them, a class D-type oxacillinase named ^{bla}OXA-69, was demonstrated to have a narrow-spectrum hydrolysis profile including, at low level, imipenem and meropenem [35]. We also identified an ORF encoding a 43-kDa porin that is homologous to the *Acinetobacter* heat-modifiable protein A [36]. The reduction in the expression of this porin, in concordance with the presence of an OXA-24 oxacillinase, is thought to play a role in carbapenem resistance in *A. baumannii* [37]. However, no ortholog to OXA-24 has been found in the AYE strain, and the level of expression of the putative porin is unknown.

Additional ORFs encoding a putative aminoglycoside adenylyltransferase and a tetracycline efflux pump *tet*A were also found in both *A. baumannii* strains. As these putative resistance genes did not confer a resistant phenotype to the SDF strain, their exact function is not known. In contrast, only the AYE strain exhibited a Ser \rightarrow Leu mutation at positions 83 and 80 of the gyrA and parC genes, respectively, that confer resistance to fluoroquinolones [38].

An additional 46 ORFs putatively associated with resistance to antimicrobials were identified in the AYE strain. These ORFs encoded putative efflux pumps from the resistancenodulation-cell division (RND) family (32 ORFs), major facilitator superfamily (MFS) family (seven ORFs), multidrug and toxic efflux (MATE) family (two ORFs), SMR family (one ORF), ATP binding cassette (ABC) superfamily (one gene), drug/metabolite transporter (DMT) family (one gene), and amino acids, polyamines, organic cations (APC) transporter family (two genes) (Table 3). Among the RND gene family, the AYE strain, but not the SDF strain, exhibited the AdeABC efflux pump-encoding genes (adeA, adeB, and adeC) and their regulating genes adeR and adeS. However, the latter two genes did not exhibit the Thr153 \rightarrow Met or Pro116 \rightarrow Leu mutations previously associated with an MDR phenotype [39]. Finally, both strains AYE and SDF possess the adeIJK efflux pump (encoded by adeI, adeI, and adeK).

Most of the genes associated with competence in *Acineto-bacter* strain ADP1 [31,32,40] were identified in the sequences of the AYE and SDF strains (to be described elsewhere). However, as we have no direct evidence of natural transformation in these isolates, and as a single missing gene may kill the function, it is not yet certain that these genes play a significant role in the acquisition of new resistance genes.

Discussion

This study demonstrates the usefulness of comparative genome sequencing for a rapid survey of all putative resistance mechanisms in *A. baumannii*. The determination and detailed comparison of the genome sequences of the MDR strain AYE, and the strain SDF, which is free from most resistance acquired by *A. baumannii* over recent decades, allowed the identification of many genes associated with antibacterial resistance at once. We identified 52 genes associated with resistance in the AYE strain, including 17 genes not, to our knowledge, previously described in *A. baumannii*. The clustering of 45 (86.5%) of them within an 86-kb AbaR1 resistance island was unexpected.

The detailed sequence analysis of the AbaR1 island, the largest genomic island identified to date, showed that it was built through the recursive insertion of broad host-range mobile genetic elements (transposons, gene cassettes from class 1 integrons), with gene cassettes (sometimes chimeric) mostly originating from the genera *Pseudomonas, Salmonella,* and *Escherichia.* Together with *A. baumannii,* many members of these genera are commonly found in aqueous environments of healthcare facilities, where, under antimicrobial pressure in these settings, genetic exchange among them may be promoted.

Another unexpected finding was the presence of a similar structure in the genome of susceptible strain SDF, identically inserted in the homologous ATPase-like ORF. This genomic island was found in an "empty" state, exhibiting mobility-associated genes but no resistance markers. This coincidental genetic insertion in the two strains strongly suggests that this ATPase ORF constitutes a specific hotspot of genomic instability in the A. baumannii genome. This prompted us to investigate whether this feature was common to all A. baumannii strains. Using a polymerase chain reaction assay based on the conserved ATPase ORF flanking sequences (Protocol S1), 17 (77%) out of the 22 clinical A. baumannii isolates were found to exhibit an intact ATPase ORF. These 17 isolates included 11 isolates resistant to several antibiotic families, including β -lactams, and six susceptible to β -lactams. Among the five isolates exhibiting an interrupted ATPase ORF, four were resistant to most β lactams except imipenem, including some that were also resistant to other antibiotic families, and one was susceptible to β -lactams but resistant to cotrimoxazole and rifampin (Protocol S1). The presence of a genomic island within the ATPase ORF is thus not a conserved feature among isolates, and its absence at this location is not predictive of the observed pattern of antibiotic susceptibility. This suggests a particular flexibility of the A. baumannii genome, in line with its exceptional ability in gathering foreign genetic material. Whole-genome sequencing of additional A. baumannii strains will be needed to assess the full range of mechanisms through which clinical isolates can so efficiently acquire new resistance genes.

Finally, our last surprise was the identification of several putative resistance genes in a strain not exhibiting the associated phenotype. This was the case for two putative β lactamases (present in the AYE and SDF), including the class D β-lactamase ^{bla}OXA-69. The expression of OXA-69 in *E. coli* resulted in low levels of carbapenem resistance [35], and its sequence is 97% identical to the OXA-51 carbapenemase, found associated with full carbapenem resistance [41]. However, the presence of ^{bla}OXA-69 in the susceptible AYE strain suggests that it might only be a step away from acquiring this new resistance through subtle changes in expression level or a specific mutation or series of mutations that alter the substrate profile and enhance catalytic activity. Besides the direct acquisition of genetic material from resistant bacterial species, the maintenance of spare copies of "ready-to-optimize" resistance genes in the genome of A. baumannii, perhaps selected by exposure to subinhibitory levels of the drug in the environment, might also play a role in its rapid adaptation to new derivatives of the major antibiotic classes.

Materials and Methods

A. baumannii strains. Strains AYE and SDF were identified as A. baumannii using both phenotypic (API 20NE system; BioMerieux, Marcy l'Etoile, France) and genotypic methods [8,10]. Both strains were grown on trypticase-soy agar (BioMerieux).

Sequencing. Genomic DNA of AYE and SDF strains was mechanically sheared and fragments of 6 kb and 14 kb were cloned into two plasmid vectors, pNAV (A) and pCNS (B) (pcDNA2.1- and pSU18derived, respectively). Plasmid DNAs were purified and endsequenced by dye-terminator chemistry on ABI3730 sequencers (Applied Biosystems, Foster City, California, United States). The PHRED/PHRAP/CONSED software package was used for sequence assemblies. Gap closure and quality assessment were made for AbaR1 and AbaG1 islands.

Annotation. Initially, a preliminary set of putative ORFs of 300 nucleotides or more of both shotgun genome sequences was obtained. These included a proportion of hypothetical ORFs. The ORF nomenclature used in this study refers to this initial step. Then, the coding potential of ORFs was evaluated using the SelfID program, and systematic sequence similarity search of ORFs was conducted against the NCBI nonredundant protein database using the gapped BLASTP program. Gene identities were obtained using a reciprocal best match strategy. ORFs with neither coding potential nor similarity were no longer considered. Tentative ORF functions were assigned on the basis of sequence similarity against protein sequence databases (KEGG, COG, SWISSPROT, and the nonredundant protein database) and domain/ motif databases (Pfam and PROSITE). Orthologous versus paralogous relationships were identified with multiple sequence alignments and neighbor-joining trees constructed by ClustalW. The functional classification was based on the scheme provided by the Acinetobacter spp. ADP1 genome project [32] as well as that provided by KEGG.

Sources of software and databases. The PHRED/PHRAP/CONSED software package is available at http://www.phrap.com. The SelfID program is available at http://igs-server.cnrs-mrs.fr/~audic/selfid.tgz. The gapped BLAST program is available at http://www.cbi.nlm.nih.gov/BLAST. KEGG is available at http://www.genome.jp/kegg. COG is available at http://www.genome.jp/kegg. COG is available at http://www.cbi.nlm.nih.gov/COG. SWISSPROT is available at http://pfam. wustl.edu. PROSITE is available at http://www.expasy.org/prosite.

Supporting Information

Protocol S1. Molecular Detection of Genomic Islands in Clinical Isolates of *A. baumannii*

Found at DOI: 10.1371/journal.pgen.0020007.sd001 (25 KB DOC).

Accession Numbers

The EMBL (http://www.ebi.ac.uk/embl) accession numbers for the resistance islands in the two *A. baumannii* strains are, for AYE, AbaR1 (CT025832); and for SDF, AbaG1 (CT025833). Other genes discussed, also in the EMBL database, are *adeA* (CT025811), *adeB* (CT025812), *adeC* (CT025813), *adeI* (CT02577), *adeJ* (CT025788), *adeK* (CT025790), *adeR* (CT025814), *adeS* (CT025815), aminoglycoside adenylyltransferase (CT025824), *ampC* (CT025798), a putative β-lactamase (CT025947), *gyrA* (CT025946), *parC* (CT025948), *tetA* (AM086635). The GenBank (http:// www.ncbi.nlm.nih.gov) accession number of ^{bla}OXA-69 is AY859527.

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Author contributions. PEF conceived and designed the experiments. VB, SA, CR, SM, and JW performed the experiments. PEF, DV, SA, HO, LP, HR, CA, DR, and JMC analyzed the data. PEF, SA, HO, HR, PN, JW, DR, and JMC contributed reagents/materials/analysis tools. PEF and JMC wrote the paper.

Competing interests. The authors have declared that no competing interests exist.

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