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Class 1 integrons in *Acinetobacter baumannii*: a weak expression of gene cassettes to counterbalance the lack of LexA-driven integrase repression

Elodie Couvé-Deacon[a], Thomas Jové[a], Pamela Afouda[b], Olivier Barraud[a], Valentin Tilloy[a], Erwan Scaon[c], Bastien Hervé[c], Christophe Burucoa[d], Marie Kempf[e], Javier Yugueros Marcos[f], Marie-Cécile Ploy[a,*], Fabien Garnier[a,*]

[a] *Univ. Limoges, INSERM, CHU Limoges, RESINFIT, U1092, F-87000 Limoges, France*
[b] *Aix Marseille Univ, IRD, APHM, MEPHI, IHU-Méditerranée Infection, Marseille, France*
[c] *Université de Limoges, cellule bioinformatique, plateforme biologie intégrative santé chimie environnement, 87000 Limoges, France*
[d] *CHU de Poitiers, Laboratoire de Bactériologie, Hôpital La Milétrie, Poitiers, France*
[e] *CRCINA, Inserm, Université de Nantes, Université d'Angers, Angers, France*
[f] *Medical Diagnostic Discovery Department, bioMérieux S.A., Grenoble*

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ABSTRACT

Integrons recruit resistance genes through integrase-driven recombination events that are regulated by the bacterial SOS response and require the repressor LexA. Class 1 integrons genes are expressed from a common promoter, Pc, of which at least 5 predominant variants, classified from weak to strong, have been described. In *Escherichia coli*, there is an intertwined regulation between gene cassette expression and integrase activity: the stronger the promoter, the weaker the integrase. Class 1 integrons have been frequently described in *Acinetobacter baumannii*. However, *Acinetobacter* spp. lack the LexA repressor, suggesting that the integrase is constitutively expressed. We characterized the integron content of 83 clinical and environmental *A. baumannii* strains. We found a predominance of Pc variants described as strong in *E. coli*. The Pc expression level was 2- to 4-fold lower in *A. baumannii* than in *E. coli*, and the diversity of the gene cassette array was low. In *A. baumannii*, integrons with a PcS promoter might have been selected to enable sufficient resistance while avoiding the toxicity of a highly active integrase. Furthermore, a transcriptional interference between PcS and PintI1 (as shown in *E. coli*) may limit the expression of the integrase and thus counterbalance the lack of LexA-driven integrase repression to prevent the cost of the integrase.

1. Background

The genus *Acinetobacter*, and principally *Acinetobacter baumannii-calcoaceticus* complex, has emerged over the past two decades as a cause of both nosocomial and community-acquired infections. The World Health Organization (WHO) lists *A. baumannii* among critical antibiotic-resistant "priority pathogens" (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). Treatment of *Acinetobacter* infections is increasingly difficult because these bacteria are intrinsically resistant to multiple antimicrobial agents and adapt rapidly to environmental selection pressure, acquiring new resistance determinants through multiple mechanisms [1]. *A. baumannii* isolates that are resistant to all commonly used antimicrobials cause near-intractable infections [2]. Most clinical isolates belong to two main clones, GC1 and GC2, which are distributed worldwide [3,4]. Clones GC1 and GC2 are often multidrug-resistant [3,5]. They carry genes that encode resistance to antibiotics or heavy metals and are located in genomic resistance islands [6] named AbaR in GC1 and AbGRI1, AbGRI2 and AbGRI3 in GC2. AbaR and AbGRI1 are inserted in the *comM* gene encoding an ATPase [7]. AbGRI1, AbGRI2 and AbGRI3 have different structures and contain different resistance genes than AbaR. AbGRI2 and AbGRI3 are bounded by two copies of IS*26* [8]. AbaR contains a 16.3-kb backbone transposon (Tn6019) interrupted by a large composite transposon that

* Address correspondence to Fabien Garnier or Marie-Cécile Ploy, CHU Limoges, Laboratory of Bacteriology-Virology-Hygiene, 2 avenue Martin Luther King, 87042 Limoges cedex, France. Phone: +33 5 55 05 61 66; Fax: +33 5 55 05 67 22.
E-mail addresses: marie-cecile.ploy@unilim.fr (M.-C. Ploy), fabien.garnier@unilim.fr (F. Garnier).

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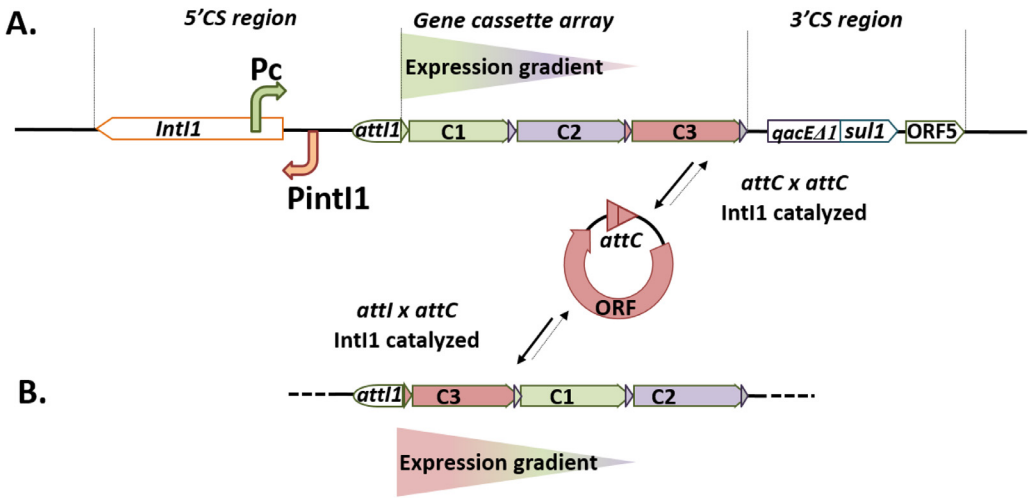

Figure 1. Organization of class 1 integrons. A. Structure of a class 1 integron, the gene cassette array consists of expressed cassette with a gradient of expression from the Pc, indicated by the fading fill color. Cassettes are an open reading frame (ORF) generally without promoter and flanked by two *att*C recombination sites. Cassettes can be excised through *attC* × *attC* recombination from any position in the array, catalyzed by the integrase. Pc, cassette promoter; Pint, integrase promoter; *intI1*, class 1 integron integrase gene; *attI1*, $attC_n$ and $attC_{n-1}$, recombination sites; C1, C2 and C3, gene cassettes. The integron functional platform, 5'-conserved region, contains 3 key elements i) the *intI* gene, encoding an integrase, ii) a specific recombination site, *attI*, and iii) a promoter, Pc, driving gene cassette expression. Most clinical class 1 integrons comprise a 3'-conserved region that contains the genes *qacEΔ1* and *sul1* conferring resistance to quaternary ammonium compounds and sulfonamides, respectively [40]. **B.** The excision intermediate can thus be integrated by the integrase, preferentially at the recombination site *attI*. Exogenous intermediates can also be integrated because of the low specificity of the integrase activity, rendering the system prone to horizontal transfer.

contains a variable region comprising antibiotic resistance genes, including a class 1 integron, bounded by copies of Tn6018 [9].

Integrons play a major role in the dissemination of multidrug resistance among Gram-negative bacteria through capture and expression of resistance genes embedded within cassettes [10]. A wide variety of gene cassettes encoding resistance to nearly all antibiotic families has been described [11]. Integration and excision of gene cassettes are driven by the integron integrase, through site-specific recombination (Figure 1). Moreover, the IntI1 integrase is regulated by the SOS response, which is a regulatory network controlled by the transcriptional repressor LexA [12]. Several classes of integron have been described, based on the integrase amino acid sequence. Class 1 integrons predominate in clinical settings. Gene cassettes are composed of an open reading frame and a recombination site *att*C. They are usually promoterless and are expressed from a common promoter, Pc, located within the integrase gene sequence. Several Pc promoter variants of different strengths have been identified in class 1 integrons. The five most frequent are the strong variants PcS, PcH2 and PcW_{TGN-10}, and the weak variants PcH1 and PcW [13]. A predominance of weak Pc variants has been reported in clinical *Escherichia coli* isolates [14,15], poultry-meat *E. coli* strains [16] and wastewater *Enterobacteriaceae* [17].

Multidrug resistance in *A. baumannii* has been associated with class 1 integrons [18–21] but no data are available on the Pc distribution in this species. Furthermore, *Acinetobacter* lacks the LexA protein [22,23], and consequently the LexA repression system, which indicates IntI1 might be constitutively expressed.

We thus examined the distribution and strength of class 1 integron Pc promoters in class 1 integron-containing *A. baumannii*, based on in silico and experimental analysis of clinical and environmental isolates. We characterized the integron content of the isolates and found a predominance of strong Pc variants. Interestingly, we showed that the Pc expression level was 2- to 4-times lower in *A. baumannii* than in *E. coli*, and that the diversity of the gene cassette array was low.

2. Materials and methods

2.1. Strains

We studied a collection of 83 class 1 integron-containing *A. baumannii* isolates of clinical (n=75) or environmental (n=8) origin (Table 1). We had access to either the strains (from French hospitals and Cameroonian hospital effluents) or total DNA (bioMerieux DNA collection, 3 hospital strains and 3 hospital effluent strains).

2.2. Plasmid construction

To study the strength of Pc variants in *A. baumannii*, we constructed plasmids derived from those used by Jové et al. [13], into which we cloned an origin of replication enabling plasmid replication in both *A. baumannii* and *E. coli* (Table 1). We used the reporter plasmid pSU38Δtot*lacZ* and three derived plasmids in which the Pc promoter was inserted in transcriptional fusion with the reporter gene *lacZ*. We inserted the replicon RSF1010, obtained by *Eco*R1 restriction of the pAL4000 vector [24], in the *Eco*R1 site of the different plasmids containing Pc variants, namely pPcS, pPcW and $pPcW_{TGN-10}$, to obtain pSU38Δtot*lacZ*RSF1010, pPcSRSF1010, pPcWRSF1010 and $pPcW_{TGN-10}$RSF1010 (Table 1). *A. baumannii* clinical strain CM148 and *E. coli* MG1656 were transformed with the recombinant plasmid.

The primers used in this study are listed in Table S1 (supplementary data).

2.3. DNA extraction

DNA was extracted for polymerase chain reaction (PCR) and Sanger sequencing using the QIAamp® DNA Mini Kit (QIAGEN), following the manufacturer's instructions. DNA for next-generation sequencing (NGS) was extracted using the SaMag™ Bacterial DNA extraction kit and the SaMag™-12 system (Sacace biotechnologies, Como, Italy).

2.4. Plasmid copy number quantification

Real-time PCR quantification was used to determine the number of plasmid copies per strain directly from a bacteria suspension. For each sample, we quantified the *lacZ* target gene and the 16S rRNA gene. Real-time PCR was performed on a Mx3005P (Stratagene) device in a final volume of 25 µL using PerfeCTa® SYBR® Green SuperMix (Quanta Biosciences) and suitable primers (for *lacZ*: lacZ LC4 / lacZ LC5 at a final concentration of 0.4 µM,

Table 1
Bacterial strains and plasmids used in the study.

Strain or plasmid	Description	Reference or source
Strains		
A. baumannii Collection	75 clinical strains and 8 environmental strains	This study See table 2
MG1656	Derivative of *E. coli* K12 MG1655 with *lacZ* deletion	[12]
CM148	Clinical competent *A. baumannii* strain	Kind gift from Thierry Lambert
Plasmids		
pAL4000	Contains an RSF1010 replicon active in *E. coli* and *Acinetobacter*	Greener et al. 1992
pSU38Δtot*lacZ*	pSU38 derivate carrying *lacZ* coding sequence with no translation initiation region or promoter	[13]
pPcW	pSU38Δtot*lacZ* derivate carrying PcW in transcriptional fusion with *lacZ*	[13]
pPcS	pSU38Δtot*lacZ* derivate carrying PcS in transcriptional fusion with *lacZ*	[13]
$pPcW_{TGN-10}$	pSU38Δtot*lacZ* derivate carrying PcW_{TGN-10} in transcriptional fusion with *lacZ*	[13]
pSU38Δtot*lacZ*RSF1010	Insertion of RSF1010 in *EcoR*1 in pSU38Δtot*lacZ*	This study
pPcWRSF1010	Insertion of RSF1010 in *EcoR*1 in pPcW	This study
pPcSRSF1010	Insertion of RSF1010 in *EcoR*1 in pPcS	This study
$pPcW_{TGN-10}$RSF1010	Insertion of RSF1010 in *EcoR*1 in $pPcW_{TGN-10}$	This study

for 16S rRNA: 518R and 331F at a final concentration of 0.2 µM). The mixture was subjected to denaturation for 10 min at 95°C, followed by 40 cycles: 30 s at 95°C and 1 min at 60°C. Data Analysis software automatically calculated the concentrations of *lacZ* target gene and 16S rRNA reference gene. We deduced the plasmid copy number from the target gene:16S rRNA gene ratio.

2.5. Amplification methods

PCRs for Pc variant detection, gene cassette characterization and plasmid construction were performed using One Taq® Quick-load (Biolabs) in a final volume of 25 µL, with a 0.9 µM final concentration of each primer, 12.5 µL of One Taq buffer and 1 µL of DNA extract. Amplification took place in the following conditions: 30 s at 95°C for initial denaturation, 30 cycles of 30 s at 95°C for denaturation, 30 s at 55°C for annealing, 30 s per amplified 500 bp at 68°C for extension, and a final 7-min extension step at 68°C.

2.6. Purification of PCR products

PCR products were analysed by electrophoresis in 0.8% agarose gel in 0.5 × TBE-containing Midori Green Advance DNA stain® (Nippon Europe Genetics GmbH, Düren, Germany). A UVP gel documentation system was used for DNA band visualization. Fragments of interest were purified using Wizard® SV Gel and the PCR Clean Up System (Promega, Lyon, France).

2.7. AbaR detection

First, the *resX* gene belonging to the AbaR genomic island was sought by amplifying a specific 425-bp fragment with ResX-F and ResX-R. Then, to identify the location of the integron within AbaR, the junction between the integron and *resX* was sought by using a forward primer located in the last integron gene cassette and ResX-R. To confirm the AbaR location of the integron, we performed an amplification using this forward primer and Tn6018R, a reverse primer located in Tn*6018*, downstream of the integron (Figure S1) [7].

2.8. Sequencing

Sanger sequencing PCRs were done using BigDye™ sequencing buffer (Applied Biosystems, Foster City, California, United States), according to the manufacturer's recommendations. PCR products were purified by gel filtration on Sephadex® G50 Superfine (Sigma-Aldrich). Sanger sequencing was performed on an ABI PRISM 3100 (Applied Biosystems). The sequences obtained were read with Chromas Lite software. Gene cassette sequences were compared to available GenBank sequences using BLAST (http://blast.ncbi.nlm.nih.gov/). The Pc variant was identified by comparing the obtained sequences to reference sequences reported by Jové et al. [13].

NGS was performed with Ion Proton™ technology (Thermo Fisher Scientific, Villebon sur Yvette, France) according to the manufacturer's instructions. Reads were assembled with mimicking intelligent read assembly (MIRA) and SPAdes-3.5.0, using raw reads and reads after removal of human reads. Then, the set of contigs was queried using blastn (version 2.5.0+) for the presence of known 5′ and 3′ conserved sequences. The extremities of these elements were then used as queries (blastn, version 2.5.0+) against custom databases (sequences were retrieved from the NCBI nucleotide database with the Entrez search engine): *A. baumannii* chromosome, *A. baumannii* plasmid. Blastn results were filtered and compiled with a custom script (blastn2spreadsheet.py).

2.9. Strain typing

Strains were compared by means of random amplified polymorphic DNA (RAPD) using primers VL1 and AP4 (Table S1). PCRs were carried out in 25-µL reaction mixes comprising 12.5 µL One Taq® Quick-load (Biolabs), 0.9 µM RAPD primer, and 2 µL DNA extract. The RAPD-PCR configuration was as follows: (i) initial denaturation (94°C for 5 min), followed by initial annealing for 1 min at 32°C for VL1 and 40°C for AP4, (ii) 36 cycles of extension (68°C for 3 min), denaturation (94°C for 1 min), and annealing (temperature adapted to each primer pair, for 1 min), and (iii) a final extension step (68°C for 1 min). The RAPD-PCR reactions were performed in a 2720 thermal cycler (Applied Biosystems, Foster City, California, United States). Bands were interpreted visually.

Multilocus sequence typing (MLST) was performed using the Pasteur scheme as described in http://pubmlst.org/abaumannii/. Each strain whole genome sequencing was uploaded to http://pubmlst.org/abaumannii/ to identify the MLST alleles and the sequence types (ST). The clonal complex was then determined using the ST type.

2.10. β-Galactosidase assay

β-Galactosidase assay was performed with 0.5-mL aliquots of exponential-phase cultures (optical density at 600 nm [OD_{600}] 0.6 to 0.8) as previously described [22]. Experiments were done at least five times for each strain. One-way analysis of variance (ANOVA) followed by post hoc Tukey HSD statistics were used to determine the significance of differences in expression levels ($P < 0.01$).

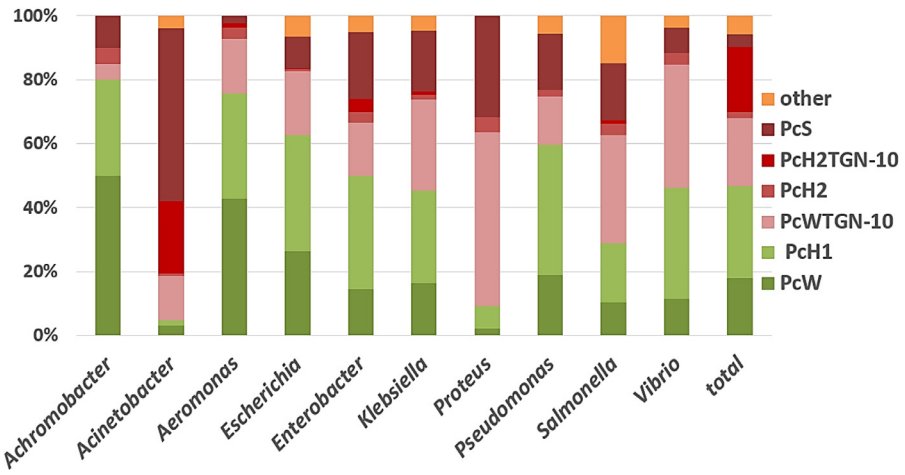

Figure 2. Distribution of Pc variants according to the host. Each bar represents the Pc variant distribution in the different class 1 integrons with a Pc sequence available (analysis performed on 13 October 2016) in the 10 most common mobile class 1 integron hosts: total of the different integron sequences (n=1604), *Acinetobacter* (n=124), *Pseudomonas* (n=340), *Escherichia* (n=435), *Klebsiella* (n=263), *Salmonella* (n=174), *Enterobacter* (n=96), *Aeromonas* (n=82), *Achromobacter* (n=20), *Proteus* (n=44) and *Vibrio* (n=26).

3. Results

3.1. In silico Pc variant distribution

Among 3747 class 1 integron sequences we recovered from NCBI on 13 October 2016, 1604 displayed a complete gene cassette array with an available Pc variant sequence. As previously shown by Jové et al. [13], the overall distribution was similar for the main Pc variants (PcS, PcH2, PcW_{TGN-10}, PcH1 and PcW). When sorted according to the bacterial genus, we noted that integrons from *Acinetobacter* mainly harbored Pc known as "strong" in *E. coli*, notably PcS and PcW_{TNG-10} (Figure 2). The rare integrons with other Pc variants were present in non-*baumannii Acinetobacter*. As the class 1 integrase is regulated by the SOS response, which is controlled by the transcriptional repressor LexA, we analysed LexA homologs and IntI1 sequences in the sequences recovered from NCBI. No LexA homologs were found in *Acinetobacter* genus, confirming the lack of that protein in this genus [22,23]. Interestingly, *Acinetobacter* presented the highest proportion, 43.3%, of truncated IntI1 after *Escherichia* (35.7%) (Table 3).

3.2. In vivo Pc variant distribution

We used a collection of 83 class 1 integron-containing clinical or environmental isolates from various geographical regions. We obtained 52 RAPD patterns, reflecting the genetic diversity of the isolates (Table 2). The diversity of the environmental isolates (8 profiles for 8 isolates) was higher than that of the clinical isolates (45 profiles for 75 isolates). All 83 strains had previously been screened for integrons with qPCR-targeting class 1, 2 and 3 integrases, as described previously [25]. All strains carried at least one class 1 integron (personal data). We characterized the entire integron content of each isolate by PCR mapping with primer pair 5'CS/3'CS. We detected 108 class 1 integrons based on the sizes of the PCR products, representing between 1 and 3 integrons per isolate. Four isolates contained 3 integrons, 17 harbored 2 integrons, and 59 strains harbored 1 integron (Table 2). Three isolates (see Table 2) yielded no amplification product, which indicates they harbored variations in the 3'-conserved region [26] or in the 5'-conserved region, although less common.

The Pc variant distribution among the 108 class 1 integrons was analysed by PCR and sequencing of the Pc promoter region. Amplification was performed with primers Int4b and DORF11 for the isolates containing 1 integron and with Int4b and a primer located in the first cassette of the network for the isolates with 2 or 3 integrons. We found that 98.2% of the integrons (106 of 108) contained a strong Pc variant, namely PcS (85.2%, n=92), PcW_{TGN-10} (7.4%, n=8), $PcH2_{TGN-10}$ (4.6%, n=5) or PcH2 (0.9%, n=1), and only 1.8% (n=2) of the integrons contained the weak PcW. The proportion of "strong" Pc variants in *A. baumannii* was even higher in vivo (98.2%) than in silico (73.3%). However, the predominance of these Pc variants was not linked to predominant clones (Table 2).

3.3. Evaluation of Pc strength in A. baumannii

The predominance of PcS variants, which are known to be strong promoter variants in *E.* coli, led us to evaluate their strength in *A. baumannii*. We constructed 4 plasmids that replicate in both *E.coli* and *A. baumannii*: 3 with different Pc variants in transcriptional fusion with the reporter gene *lacZ,* and a control plasmid without the Pc region (Table 1). Surprisingly, expression levels of all the Pc variants were 2- to 4-times weaker in *A. baumannii* than in *E. coli* ($P<0.01$; Figure 3). This difference in expression levels was not due to a difference in growth rates or plasmid copy numbers. Indeed, plasmid copy numbers were similar in the two species, without significant differences, whatever the plasmid construct (Table 4). The so-called "strong" Pc variants are thus weakly expressed in *A. baumannii.*

3.4. Gene cassette contents

Sequencing of the PCR products obtained with primer pair 5'CS/3'CS showed that all the gene cassettes we detected had already been described in Genbank and were composed of 11 different resistance genes and 18 different gene cassette arrays (Table 2). The majority (90.5%) of the gene cassettes coded for aminoglycoside resistance, and at least 1 or 2 of these gene cassettes were found in 15 (83.3%) of the 18 arrays. The arrays often comprised 2 gene cassettes encoding proteins of unknown function (gcuP and gcuQ). The most frequent arrays were *aacC1+orfP+orfQ+aadA1* and *aacC1*. We identified 5 arrays that had not previously been described in *A. baumannii* (*aacA4+aadA1, aacA4+catB8+orfP+orfQ+aadA1, aacA4+orfP+orfQ+aadA1, aadA12, dfrA1*). The array *aacA4+aadA1* had previously been described in *Pseudomonas aeruginosa* (Genbank accession number FN823039). The *aadA12* gene cassette had previously been found alone in

Table 2
Summary of our study results.

Type of isolates	Material	RAPD profile	Pc variant	Gene cassette array[a]	Localization[b]	Global clone	ST
clinical	strain	M	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
			PcS	*aacC1*			
clinical	strain	M	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcS	*aacC1*	Chromosomal		
clinical	strain	M	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
			PcS	*aacC1*			
clinical	strain	M	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
			PcS	*aacC1*			
clinical	strain	M	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
			PcS	*aacC1*			
clinical	total DNA	A05	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	-		
clinical	total DNA	A06	PcS	*aacC1+orfP+orfQ+aadA1*	-		
clinical	total DNA	E	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	E	PcS	*aacC1+orfP+orfQ+aadA1*			
clinical	total DNA	E	PcS	*aacC1+orfP+orfQ+aadA1*			
clinical	total DNA	A07	PcS	*aadB+blaOXA-21*	AbaR	GC1	
clinical	total DNA	F	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	G	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	F	PcS	*aacC1+orfP+orfQ+aadA1*			
clinical	total DNA	G	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
clinical	total DNA	H	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	H	PcS	*aacC1+orfP+orfP+orfQ+aadA1*			
clinical	total DNA	A08	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	F	PcS	*aacC1+orfP+orfQ+aadA1*			
clinical	total DNA	F	PcS	*aacC1+orfP+orfQ+aadA1*			
clinical	total DNA	I	PcS	*aacA4*	AbaR	GC1	
clinical	total DNA	I	PcS	*aacA4*			
clinical	total DNA	I	PcS	*aacA4*			
clinical	total DNA	A40	PcS	*aadB*	AbaR	GC1	
clinical	total DNA	A09	PcS	*aacA4*	-		
clinical	total DNA	A10	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	J	PcS	*aadB*	AbaR	GC1	
clinical	total DNA	K	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A11	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	J	PcS	*aadB*			
clinical	total DNA	J	PcS	*aadB*			
clinical	total DNA	J	PcS	*aadB*			
clinical	total DNA	J	PcS	*aadB*			
clinical	total DNA	J	PcS	*aadB*			
clinical	total DNA	A12	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A13	PcS	*aacC1+orfP+orfQ+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A14	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A15	PcS	*aacA4*	AbaR	GC1	
clinical	total DNA	A16	PcS	*aacC1+orfP+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A17	PcS	-	-		
clinical	total DNA	A18	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A19	PcS	-	-		
clinical	total DNA	A20	PcW_{TGN-10}	*aacA4*	AbaR	GC1	
clinical	total DNA	A21	$PcH2_{TGN-10}$	*aacA4+aadA1*	AbaR	GC1	
clinical	total DNA	A22	$PcH2_{TGN-10}$	*aacA4+catB8+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	K	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	
clinical	total DNA	A23	PcS	-	-		
clinical	total DNA	A24	PcS	*aadA1*	-		
environmental	strain	A25	PcS	*aacC1+orfP+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcH2	*aacA4+catB8+aadA1*	Chromosomal		
environmental	strain	A26	PcW_{TGN-10}	*aacC1+orfP+orfQ+aadA1*	Chromosomal	GC2	ST2
environmental	total DNA	A27	PcW_{TGN-10}	*dfrA5*	-		
environmental	total DNA	A38	PcS	*aacC1+orfP+orfQ+aadA1*	-		
environmental	strain	A29	PcS	*aacC1+orfP+orfQ+aadA1*	AbaR	GC1	ST1
environmental	total DNA	A30	PcS	*aacC1+orfP+orfQ+aadA1*	-		
environmental	strain	A31	$PcH2_{TGN-10}$	*aacA4+orfP+orfQ+aadA1*	Chromosomal	GC2	ST2
			$PcH2_{TGN-10}$	*aacA4+catB8+aadA1*	Chromosomal		
			PcS	*aacC1*	Chromosomal		
environmental	strain	A32	PcW_{TGN-10}	*dfrA1*	Chromosomal		ST499
Clinical	strain	B	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcS	*aacC1*	Chromosomal		
Clinical	strain	B	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcS	*dfrA1-orfP*	Chromosomal		
			PcS	*aadA12*	Chromosomal		
Clinical	strain	B	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			

(*continued on next page*)

Table 2 (*continued*)

Type of isolates	Material	RAPD profile	Pc variant	Gene cassette array[a]	Localization[b]	Global clone	ST
Clinical	strain	C	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcS	*aacC1*	Chromosomal		
Clinical	strain	D	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*	Chromosomal	GC2	ST2
			PcS	*aacC1*	Chromosomal		
Clinical	strain	D	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	D	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	D	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	B	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	C	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	D	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	C	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*			
			PcS	*aacC1*			
Clinical	strain	A01	PcS	*aacA4*	AbaR	GC1	
Clinical	total DNA	A02	PcS	*aacC1+orfP+orfQ+orfQ+aadA1*	-		
Clinical	total DNA	A03	PcW + P2	*blaIMP-10+aacA31+aadA1*	-		
Clinical	total DNA	A04	PcW	*blaIMP-10+aacA31+aadA1*	-		
Clinical	strain	A33	PcS	*aacC1*	Chromosomal	-	-
Clinical	strain	A34	PcS	*aacA4*	AbaR	GC1	
Clinical	strain	A35	PcS	*aadA1*	Chromosomal	GC1	ST1
			PcW_{TGN-10}	*dfrA1+orfP*	Chromosomal		
			PcS	*aacC1*	Chromosomal		
Clinical	strain	A36	PcW_{TGN-10}	*dfrA1+orfP*	Chromosomal	GC1	ST1
Clinical	strain	A37	PcS	*aacC1*	Chromosomal	GC2	ST2
Clinical	strain	A38	PcS	*aacA4*	AbaR	GC1	
Clinical	strain	A39	PcS	*aadA1*	Chromosomal	GC1	ST1
			PcW_{TGN-10}	*dfrA1+orfP*	AbaR		
			PcS	*aacC1*	Chromosomal		
Clinical	strain	L	PcS	*aacC1*	Chromosomal	GC2	ST2
Clinical	strain	L	PcS	*aacC1*			
Clinical	strain	L	PcS	*aacC1*			
Clinical	strain	L	PcS	*aacC1*			

[a] PCR of the gene cassette array using 5'CS/3'CS primers pair, « - » means negative PCR.
[b] Localization was determined by specific PCR (AbaR) or NGS (Chromosomal), « - » means AbaR-specific PCR was negative but there was not enough DNA to perform the NGS analysis.

an integron of *E. coli* (Genbank accession number FJ381668), *Yersinia enterocolitica* (Genbank accession number AY940491), and *Salmonella typhimurium* (Genbank accession number FJ460240). The *dfrA1* gene cassette had previously been found alone in *A. baumannii*, but only in a class 2 integron (Genbank accession number FJ785526). The gene cassette arrays *aacA4+catB8+orfP+orfQ+aadA1* and *aacA4+orfP+orfQ+aadA1* had not previously been described. Eight combinations of gene cassettes were identified only once (Table 2).

3.5. Genetic environment of the integrons

As multidrug resistance in *A. baumannii* is often linked to chromosomal multiresistance genomic islands frequently containing class 1 integrons, we characterized the genetic environment of the class 1 integrons. We focused on one strain per RAPD profile, except for profile B, for which we selected two isolates as we found two different gene cassette array combinations. A total of 53 isolates and 66 integrons were analysed. With an amplification strategy targeting the *resX* gene (belonging to the AbaR genomic island) and the junction sequence (see Materials and Methods), we found that 27 integrons from 27 strains were embedded within an AbaR island (Table 2), indicating that these strains belonged to the clone GC1.

Regarding the integrons that were not detected with the specific PCR, NGS analysis showed that 26 integrons were also located on the chromosome. However, a large number of repeated sequences limited the length of the contigs we obtained with Ion Proton™ technology, and the short contigs were too small to determine the whole resistance island harboring these integrons. Nevertheless, we could determine the strain MLST typing: 4 isolates were ST1 belonging to the GC1, 10 were ST2 belonging to GC2, and 1 was ST499. The low sequence quality of one isolate (A33 RAPD profile) prevented determination of the ST type. There was insufficient DNA for 13 integrons so we were unable to perform NGS. Overall, a chromosomal location was found for all the integrons analysed (n=53/66). As the other strains belonged to the same RAPD profile and/or had the same integron, we can hypothesize that all integrons in our strain selection are located on the chromosome.

4. Discussion

Acinetobacter baumannii resistance has previously been linked to class 1 integrons [18–21]. However, when we began this study, no data were available on the Pc variant distribution in clinical and environmental strains. Interestingly, using both in silico and in vivo data, we found a large proportion of so-called strong Pc variants in the class 1 integrons of *A. baumannii*.

Specific features of PcS and *A. baumannii* could explain these findings. First, we found that all these Pc variants were weaker in *A. baumannii* than in *E. coli*. Integrons with PcS might have been selected to allow *A. baumannii* to express antibiotic resistance at a sufficient level. A lower expression has already been reported in *A. baumannii* (or *A. calcoaceticus*, according to the former taxonomy) compared with *E. coli*. Expression of the *catA* gene under

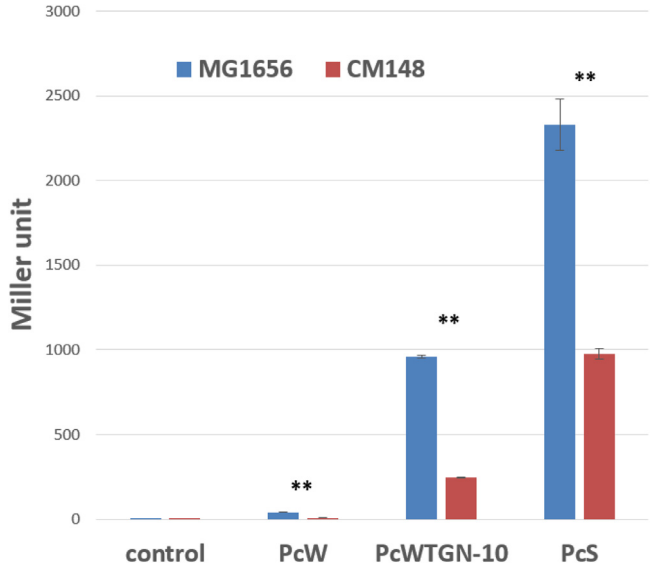

Figure 3. Expression of Pc variants. ß-galactosidase activity was measured in *E. coli* MG1656 (in blue) and *A. baumannii* CM148 (in red). Control: pSU38Δtot*lacZ*RSF1010, PcW: pPcWRSF1010, PcWTGN-10: pPcW$_{TGN-10}$RSF1010 and PcS: pPcSRSF1010. ** indicates significant statistical difference with $P<0.01$.

the control of a *lac* promoter, was two-fold lower in *A. calcoaceticus* than in *E. coli* [27]. Likewise, the *pca* gene cluster expression was 10- to 30-fold lower in *A. calcoaceticus* than in *E. coli* [28]. More recently, Girlich et al. reported that the transcription level of the *bla*$_{KPC}$ gene (encoding resistance to beta-lactams) was 10- to 100-times lower in *A. baumannii* than in *E. coli* but, interestingly, the resistance phenotypes did not correlate with the transcription level [29]. There is some evidence that inter-species differences in Pc expression level could be due to differences in transcriptional machinery. *A. baumannii* RNA polymerase has a similar structure [30] and 50% to 70% amino acid sequence homology compared with its *E. coli* counterpart, with good conservation of functional regions [23]. Despite this, the two RNA polymerases behave differently in vitro, with the *A. baumannii* enzyme less efficient than its *E. coli* counterpart [30]. Moreover, the *A. baumannii* and *E. coli* transcriptional σ^{70} factor amino acid sequences are very similar, with good conservation of functional regions [23,31]. Nevertheless, alternative σ factors are missing in *A. baumannii*, which indicates different regulation of transcription relative to *E. coli* [23]. Furthermore, to our knowledge, there is no -35 and -10 sequence consensus of *A. baumannii* but some data indicate variations from the promoter sequence consensus of *E. coli*, notably in the spacer region [32,33]. Such a different promoter consensus could influence both the transcription level and the resistance phenotype, as reported by Girlich et al. [29].

Second, the selection of PcS in *A. baumannii* could be associated with lower biological cost to avoid integrase toxicity. In *E. coli*, the class 1 integron integrase is regulated via the SOS response, which involves the LexA repressor, via a LexA binding site overlapping the *intI1* promoter PintI1 [12]. Moreover, the Pc promoter is located within the *intI1* sequence in *E.coli*, different Pc variants actually correlate with amino acid changes in the IntI1 sequence and the integrase variant IntI1$_{R32_N39}$ corresponding to the PcS promoter has the lowest efficiency [13]. We recently showed in *E. coli* that repression of the SOS regulon prevents expression of costly integrases for which cost is activity dependent. The lowest cost was observed with the integron encoding IntI1$_{R32_N39}$ integrase (associated with PcS) [34]. In *Acinetobacter baylyi* harboring an integron with a PcS variant, frameshift mutations rapidly emerge to inactivate the IntI1 protein and restore host fitness [35]. In silico we found a higher proportion (43%) of truncated integrases in *A. baumannii* compared with other integron hosts (Table 3). This indicates that selection of PcS in this species could only partially suppress integrase activity and, therefore, inactivation could be associated to prevent its toxicity. However, these in silico results should be confirmed in human, animal and environmental strains of different bacterial integron hosts.

Third, another mechanism regulating integrase expression in *E. coli* depends on transcriptional interference between PcS and PintI1, PcS preventing *intI1* expression [36]. *Acinetobacter* spp. lack the LexA repressor [22,23], which indicates the integrase is constitutively expressed. If this transcriptional interference also occurs in *A. baumannii*, the observed predominance of PcS may limit the expression of the integrase via this transcriptional interference and thus counterbalance the lack of LexA-driven integrase repression to prevent the cost of the integrase.

The predominance of a poorly efficient integrase in *A. baumannii* (IntI1$_{R32_N39}$ associated with PcS) would limit movement of gene cassettes and thus confer higher stability of gene cassettes within the array. This is in line with what we observed in the integron gene cassette arrays of our *A. baumannii* collection. Indeed, we found a redundancy of gene cassette arrays, with 5 arrays representing 75.2% (n=79) of all characterized integrons (n=105). This redundancy of gene cassette arrays was not due to clonal distribution of our isolates, as there were no identical RAPD profiles for strains with identical arrays (Table 2). All the environmental isolates in our collection had different RAPD profiles, which were also all different from those of the clinical isolates. The

Table 3
Proportion of truncated IntI1 and features of LexA homologue according to the host.

Genus	Online sequences with a complete *intI1*			LexA homologue Accession Number (% identity/e-value[a])
	Total	Functional IntI1	Truncated IntI1 (%)	
Achromobacter	14	10	4 (**29**)	WP_088596507 (60% / 7e-82)
Acinetobacter	90	51	39 (**43**)	None[b]
Aeromonas	28	23	5 (**18**)	WP_108537109 (73% / 5e-109)
Escherichia	224	144	80 (**36**)	NP_418467 (used as reference)
Enterobacter	78	66	12 (**15**)	WP_090464447 (94% / 1e-138)
Klebsiella	220	177	43 (**20**)	WP_ 110247500 (95% / 9e-140)
Proteus	40	35	5 (**13**)	WP_023583074 (87% / 5e-129)
Pseudomonas	174	138	36 (**21**)	WP_121784911 (84% / 1e-117)
Salmonella	140	96	44 (**31**)	WP_000646079 (97% / 3e-142)
Vibrio	13	12	1 (**8**)	WP_073583963 (76% / 8e-108)

The number of truncated IntI1 was analysed within available sequences (10 November 2018) with the complete sequence of its *intI1* gene. The LexA homologues were recovered on the same date using the NCBI BlastP alignment against the refseq_protein database.

[a] e-value obtained from NCBI BlastP alignment against the refseq_protein database. The lower the e-value, the more significant the score and the alignment.

[b] The closest match shares 38% identity (e-value = 2e-17).

Table 4
Plasmid copy number in *E. coli* and *A. baumannii*.

	Plasmid copy number/bacterial genome	
	E. coli MG1656 mean (std deviation of mean)	*A. baumannii* CM148 mean (std deviation of mean)
pSU38Δtot*lacZ*RSF1010	58.2 (±15.3)	52.5 (±8.8)
pPcWRSF1010	60.7 (±21.8)	62.4 (±11.1)
$pPcW_{TGN-10}$RSF1010	60.6 (±15.3)	56.0 (±8.1)
pPcSRSF1010	56.8 (±18.5)	54.1 (±16.9)

environmental and clinical isolates shared the two most frequent gene cassette arrays (*aacC1*+*orfP*+*orfQ*+*aadA1 and aacC1*), which accounted for 54.6% (n=6/11) of the environmental integrons and 60.6% (n=57/94) of the clinical integrons (Table 2). The environmental isolates were obtained from hospital wastewater and thus were influenced by antibiotic selection pressure. Aminoglycoside-modifying enzymes have been linked to class 1 integrons in *A. baumannii* [5,37]. Moreover, most of the gene cassette arrays (94.3%) in our study harbored at least one aminoglycoside resistance gene cassette (*aacC1,* 66.7%, *aadA1,* 50.5% and *aacA4,* 14.3%) (Table 2). The array containing two aminoglycoside resistance genes (*aacC1-orfP-(orfP-Q)orfQ-aadA1*) is frequently found in *A. baumannii* class 1 integrons [5,9,38]. Many strains contain either *aacC1* or *aacA4* associated with the *aadA1* gene cassette, encoding the resistance to 4-6 deoxystreptamine aminoglycosides (mainly used in clinical settings) and streptomycin. This high frequency of aminoglycoside-resistance gene cassettes has been previously described in *A. baumannii* [6]. However, predominance of gene cassettes encoding resistance to aminoglycosides is not specific to *Acinetobacter* as these gene cassettes are the most frequently found in class 1 integrons, whatever the host [11]. In *A. baumannii*, integrons are often found on the chromosome, usually within resistance islands [8,37,39]. All the studied integrons in our collection were also found on the chromosome and at least 27 were in an AbaR island.

5. Conclusion

Our results highlight that gene cassette PcS variants are predominant in *A. baumannii*. Integrons with a PcS promoter might have been selected to enable sufficient resistance while avoiding the toxicity of a highly active integrase. Altogether, our data obtained in *E. coli* [13,15,37] and *Acinetobacter* indicate that integrons need to be tightly regulated for an adaptive response designed to lower their cost and favor their maintenance.

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Declarations

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Competing Interests

The authors declare no conflict of interest regarding this work

Ethical Approval

Not required

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.11.012.

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