

## ORIGINAL ARTICLE

# Class 1 integrons are low-cost structures in *Escherichia coli*

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Resistance integrons are bacterial genetic platforms that can capture and express antibiotic resistance genes embedded within gene cassettes. The capture and shuffling of gene cassettes are mediated by the integrase *IntI*, the expression of which is regulated by the SOS response in *Escherichia coli*. Gene cassettes are expressed from a common *Pc* promoter. Despite the clinical and environmental relevance of integrons, the selective forces responsible for their evolution and maintenance are poorly understood. Here, we conducted pairwise competition experiments in order to assess the fitness cost of class 1 integrons in *E. coli*. We found that integrons are low-cost structures and that their cost is further reduced by their tight regulation. We show that the SOS response prevents the expression of costly integrases whose cost is activity dependent. Thus, when an integron is repressed, its cost depends mostly on the expression of its gene cassettes array and increases with *Pc* strength and the number of cassettes in the array. Furthermore, different cassettes have different costs. Lastly, we showed that subinhibitory antibiotic concentrations promoted the selection of integron-carrying bacteria, especially those with a strong *Pc* promoter. These results provide new insights into the evolutionary dynamics of integron-carrying bacterial populations.

*The ISME Journal* (2017) 11, 1535–1544; doi:10.1038/ismej.2017.38; published online 7 April 2017

## Introduction

The development and spread of antibiotic resistance among bacterial pathogens is a major public health issue (Levy and Marshall, 2004). Among the genetic elements involved in this spread, integrons are especially important, given their ability to capture and express resistance genes embedded within gene cassettes (Stokes and Hall, 1989).

Integrons are composed of three key elements: (i) an *intI* gene encoding an integrase that mediates the insertion, excision and shuffling of gene cassettes; (ii) an *attI* recombination site where cassettes are inserted; and (iii) a *Pc* promoter responsible for gene cassette expression (Cambray *et al.*, 2010). Several integron classes have been defined according to their integrase amino acid sequence (Mazel, 2006). Among them, class 1 integrons are highly prevalent in antibiotic-resistant Gram-negative bacteria (Escudero *et al.*, 2015).

In class 1 integrons, *intI1* gene expression is regulated by the SOS response (Guerin *et al.*, 2009), a bacterial stress response that can be triggered by various antibiotics (Baharoglu and Mazel, 2011).

This regulation involves a specific repressor, LexA protein, which prevents *intI1* gene transcription. Upon activation of the SOS response by a stress generating single-strand DNA, the LexA repressor is cleaved and integrase expression is thereby enabled.

Gene cassettes are expressed from the common promoter *Pc*; at least 13 different *Pc* variants have been described in class 1 integrons. Four variants predominate, namely *PcW*, *PcH1*, *PcW<sub>TGN-10</sub>* and *PcS*, from the weakest to the strongest (Jové *et al.*, 2010). Moreover, the closer a gene cassette is to the *Pc*, the stronger its expression (Collis and Hall, 1995). As *Pc* is located within the *intI1* gene, its polymorphism impacts the *IntI1* integrase amino acid sequence. Three *IntI1* variants are thus associated with the four *Pc* variants: *IntI1<sub>R32\_H39</sub>* (*PcW* or *PcH1*), *IntI1<sub>P32\_H39</sub>* (*PcW<sub>TGN-10</sub>*) and *IntI1<sub>R32\_N39</sub>* (*PcS*). A negative correlation between the strength of the *Pc* and the activity of its associated integrase has been shown: the weaker the *Pc*, the more active the integrase (Jové *et al.*, 2010).

Relatively few studies have focused on the evolution dynamics of class 1 integrons, with the exception of studies assessing the effect of antibiotic pressure on gene cassette reshuffling (Barraud and Ploy, 2015; Pérez-Valdespino *et al.*, 2016). Rearrangement of a gene cassette to a position closer to the *Pc* relies mainly on the excision of upstream cassettes rather than excision and reintegration of the cassette just ahead of the *Pc* (Barraud and Ploy, 2015). This suggests that gene cassette

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Received 23 November 2016; revised 30 January 2017; accepted 6 February 2017; published online 7 April 2017

rearrangements *via* excision are probably more cost-effective than excision and integration of a distal gene cassette closer to the Pc. Indeed, the 'cut and paste' mechanism leading to excision and reintegration of a gene cassette in the first position would require two recombination events, whereas excision of upstream cassettes requires only one recombination event. This raises questions as to the fitness cost associated with integrase activity and gene cassette expression.

Few studies have assessed the fitness cost of integrons. In particular, Starikova *et al.* (2012) assessed the impact of class 1 integrons in *Acinetobacter baylyi*. They showed that integron acquisition strongly reduced the fitness of *A. baylyi*, due to an active integrase and that inactivated integrases rapidly emerged to compensate for this biological cost. These data were used to create a mathematical model simulating the evolution of an integron-carrying bacterial population in response to fluctuating antibiotic pressure (Engelstadter *et al.*, 2016). The main conclusion was that gene cassette reshuffling, necessary to tolerate the fluctuating antibiotic pressure, could compensate for the integrase cost, enabling the maintenance of functional integrases within bacterial populations. However, both studies relied on data retrieved from *Acinetobacter* which lacks LexA protein (Hare *et al.*, 2012). Yet, evidence of SOS regulation was found for most integrons (Cambray *et al.*, 2011).

The aim of the present study was thus to assess the fitness cost of class 1 integrons in *E. coli*, a species in which integrase expression is regulated by the SOS response. We show that class 1 integrons are low-cost structures and that their cost is further reduced by the repressive system. LexA protein prevents the expression of costly integrases, whose cost is linked to their catabolic activity. We also show that the fitness of a given integron is dependent both on Pc polymorphism and the gene cassettes content. Finally, we show that subinhibitory antibiotic concentrations can select integron-carrying bacteria. These results provide new insights into the evolutionary dynamics of integron-carrying bacterial populations.

## Materials and methods

### *Bacteria and plasmids*

The bacteria and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani broth (LB) (MoBio Laboratories, Carlsbad, CA, USA) or Davis Minimal (DM) broth (Sigma Aldrich, St Louis, MO, USA) supplemented with 25 µg ml<sup>-1</sup> glucose (DM25 medium). Kanamycin (Km, 25 mg l<sup>-1</sup>) and tobramycin (Tob, various concentrations) were added when necessary.

All integrons were generated by assembly PCR, as described in Supplementary Figure S1, and cloned

into a pZA2 backbone *via* the *Xho*I and *Bam*HI restriction sites.

### *Construction of the MG1656 $\lambda$ att::gfp strain*

The MG1656  $\lambda$ att::gfp strain, expressing green fluorescent protein (GFP), was constructed by integrating a *km-frt-gfpmut3* cassette at the *E. coli*  $\lambda$ att site in a three-step PCR-based approach as previously described (Cherepanov and Wackernagel, 1995). Primers used are available in Supplementary Table S1.

### *Determination of minimal inhibitory concentrations*

MICs were determined by broth dilution with an inoculum of 10<sup>5</sup> CFU ml<sup>-1</sup> in DM25 medium. Each test was done at least three times.

### *Competition experiments*

The fitness of the MG1656 strain carrying a given plasmid, relative to the MG1656  $\lambda$ att::gfp strain containing another plasmid, was estimated in pairwise competition experiments (Lenski *et al.*, 1991). Briefly, independent pre-cultures of both competitors were grown in DM25 overnight and mixed at a 1:1 volume ratio the following day. Fresh DM25 cultures were then inoculated with this mix performing a 100-fold dilution. Cultures were then propagated for 4 days with a daily 100-fold dilution in fresh medium. Samples were taken each day to follow the relative frequency of the two competitors by flow cytometry.

### *Flow cytometry*

The relative frequency of GFP bacteria was determined with a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Measurements were performed directly on bacterial cultures. Bacterial cells were gated on the basis of their distribution on forward vs side scatter plots. GFP bacteria were then separated from non-GFP bacteria by using the fluorescent marker. Green fluorescence was collected in the FL1 channel (530 ± 15 nm). Thresholds were applied manually to determine the boundaries between the two populations, and remained constant throughout all the experiments. The relative frequency of the two competitors was calculated by counting 10 000 bacterial cells. All measurements were made in triplicate. Controls were performed to assess the level of false positives and false negatives in GFP quantification. Both were shown to be negligible and constant throughout independent daily measurements (Supplementary Data and Supplementary Figure S2).

**Table 1** Strains and plasmids

No.	Strains or plasmids	Genotype or description	Source
<i>Strains</i>			
	<i>Escherichia coli</i> MG1656	MG1655 (K-12F <sup>-</sup> λ <sup>-</sup> <i>ilvG</i> <sup>-</sup> <i>rfb-50 rph-1</i> ) Δ <i>lacMluI</i>	Espéli et al. (2001)
	<i>Escherichia coli</i> MG1656 <i>λatt::gfp</i>	MG1656 strain containing the <i>gfpmut3</i> gene at the <i>λatt</i> site	This study
	<i>Escherichia coli</i> MG1656 Δ <i>cpxl::km-frt</i>	<i>cpxl</i> gene replaced by the <i>km-frt</i> cassette in strain MG1656	Beloin et al. (2004)
<i>Plasmids</i>			
	pKOBEGA	λ red recombination system expression plasmid	Chaveron et al. (2000)
	pCP20	FLP flippase expression plasmid	Cherepanov and Wackernagel (1995)
	pZE1- <i>gfpmut3</i>	<i>gfpmut3</i> gene under the control of the constitutive p <sub>λ<sub>R</sub></sub> promoter	Gift from C Guet
	pZE- <i>km-frt-gfpmut3</i>	<i>km-frt</i> cassette amplified from MG1656Δ <i>cpxl::km-frt</i> using primers 7-8 (Supplementary Table S1) and cloned into pZE1R- <i>gfpmut3</i> ( <i>SacI</i> - <i>XhoI</i> )	This study
	p1W	integron <i>intI1-aac(6')-Ib-dfrA15-aadA1-catB9</i> with PcW promoter cloned into pSU38Δ <i>totlacZ</i>	Barraud and Ploy (2015)
	p1S	integron <i>intI1-aac(6')-Ib-dfrA15-aadA1-catB9</i> with PcS promoter cloned into pSU38Δ <i>totlacZ</i>	Barraud and Ploy (2015)
	p1W <sub>TGN-10</sub>	integron <i>intI1-aac(6')-Ib-dfrA15-aadA1-catB9</i> with PcW <sub>TGN-10</sub> promoter cloned into pSU38Δ <i>totlacZ</i>	Barraud and Ploy (2015)
	p16S <sup>a</sup>	integron <i>intI1-aac(6')-Ib-dfrA15-aadA1-catB9-aac(6')-Ib-dfrA15-aadA1-catB9</i> cloned into pSU38Δ <i>totlacZ</i>	Barraud and Ploy (2015)
	p8741	pBAD:: <i>intI1</i> <sub>Y312F</sub>	Loot et al. (2014)
<i>pZA2 and derivatives</i>			
1	pZA2	pZA2 plasmid, Km <sup>r</sup>	Lutz and Bujard (1997)
2	pW-4GC	integron <i>intI1-aadA1-catB9-aac(6')-Ib-dfrA15</i> with PcW promoter cloned into pZA2	This study
3	pH1-4GC	integron <i>intI1-aadA1-catB9-aac(6')-Ib-dfrA15</i> with PcH1 promoter cloned into pZA2	This study
4	pW <sub>TGN-10</sub> -4GC	integron <i>intI1-aadA1-catB9-aac(6')-Ib-dfrA15</i> with PcW <sub>TGN-10</sub> promoter cloned into pZA2	This study
5	pS-4GC	integron <i>intI1-aadA1-catB9-aac(6')-Ib-dfrA15</i> with PcS promoter cloned into pZA2	This study
6	pW PintI*-4GC	pW-4GC with inactivated PintI promoter (no <i>intI1</i> expression)	This study
7	pH1 PintI*-4GC	pH1-4GC with inactivated PintI promoter (no <i>intI1</i> expression)	This study
8	pW <sub>TGN-10</sub> PintI*-4GC	pW <sub>TGN-10</sub> -4GC with inactivated PintI promoter (no <i>intI1</i> expression)	This study
9	pS PintI*-4GC	pS-4GC with inactivated PintI promoter (no <i>intI1</i> expression)	This study
10	pW*PintI*-4GC	pW-4GC with inactivated Pc promoter and inactivated PintI promoter (no <i>intI1</i> expression nor cassette expression)	This study
11	pW*-4GC	pW-4GC with inactivated PcW promoter (silent mutation, IntI <sub>R32-H39</sub> integrase variant, no cassette expression)	This study
12	pW <sub>TGN-10</sub> *-4GC	pW <sub>TGN-10</sub> -4GC with inactivated PcW <sub>TGN-10</sub> promoter (silent mutation, IntI <sub>P32-H39</sub> integrase variant, no cassette expression)	This study
13	pS*-4GC	pS-4GC with inactivated PcS promoter (silent mutation, IntI <sub>R32-N39</sub> integrase variant, no cassette expression)	This study
14	pW*lexA*-4GC	pW-4GC with inactivated PcW promoter (no cassette expression) and IntI <sub>R32-H39</sub> constitutive expression (mutated LexA box)	This study
15	pW <sub>TGN-10</sub> *lexA*-4GC	pW <sub>TGN-10</sub> -4GC with inactivated PcW <sub>TGN-10</sub> promoter (no cassette expression) and IntI <sub>P32-H39</sub> constitutive expression (mutated LexA box)	This study
16	pS*lexA*-4GC	pS-4GC with inactivated PcS promoter (no cassette expression) and IntI <sub>R32-N39</sub> constitutive expression (mutated LexA box)	This study
17	pW <sub>Y312F</sub> *lexA*-4GC	pW*lexA*-4GC encoding an inactive integrase (catalytic amino acid Y312 mutated)	This study
18	pS-2GC	integron <i>intI1-aadA1-catB9</i> with PcS promoter cloned into pZA2	This study
19	pW*PintI*-2GC	pS-2GC with inactivated Pc promoter and inactivated PintI promoter (no <i>intI1</i> expression nor GCs expression)	This study
20	pS- <i>aadA1</i>	integron <i>intI1-aadA1</i> with PcS promoter cloned into pZA2	This study
21	pS- <i>catB9</i>	integron <i>intI1-catB9</i> with PcS promoter cloned into pZA2	This study
22	pS- <i>aac(6')-Ib</i>	integron <i>intI1-aac(6')-Ib</i> with PcS promoter cloned into pZA2	This study
23	pS- <i>dfrA15</i>	integron <i>intI1-dfrA15</i> with PcS promoter cloned into pZA2	This study
24	pW*PintI*- <i>aadA1</i>	pW- <i>aad</i> with inactivated Pc promoter and inactivated PintI promoter (no <i>intI1</i> expression nor GCs expression)	This study
25	pW- <i>aac(6')-Ib</i>	integron <i>intI1-aac(6')-Ib</i> with PcW promoter cloned into pZA2	This study

Abbreviation: GC, gene cassette. Bold was used to highlight the change of Pc variant between constructions.

### Calculating the selection coefficient

For each competition, the selection coefficient of the non-GFP strain was determined from the slope of the regression  $\ln [\text{freq}_{\text{nonGFP}}/\text{freq}_{\text{GFP}}]$  plotted against the time course in generations (Lenski et al., 1991).

Experiments were performed in at least 12 replicates. A single regression line was then fitted through the points from all replicates (Schrage et al., 1997; Rodríguez-Verdugo et al., 2013). Reported standard errors and *P*-values were calculated for the

regression coefficient. Error bars correspond to the 99% confidence interval. The fluorescent reporter used in the MG1656 *att::gfp* strain had no cost when compared with the MG1656 parental strain (Supplementary Data and Supplementary Table S2).

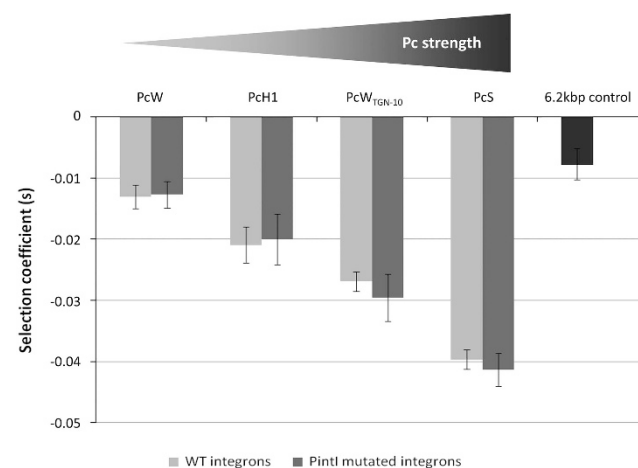
## Results

### Effect of Pc polymorphism on the cost of class 1 integrons in *E. coli*

We first assessed the impact of Pc polymorphism on the cost of class 1 integrons in *E. coli* by performing pairwise competition experiments between strains carrying and lacking the integron (Supplementary Figure S3A). Integrons differing only by their Pc sequence were used (plasmids 2–5; Table 1).

Whatever the Pc variant, integrons appeared to be costly for *E. coli* (Figure 1, light gray). The selection coefficients of the integron-carrying strains were all negative, ranging from  $-0.013 \pm 0.002$  to  $-0.04 \pm 0.003$  (all  $P$ -values  $< 1 \times 10^{-10}$ ), meaning that the integrons slowed bacterial growth by 1.3–4%. Interestingly, the cost of class 1 integrons appeared to correlate with the Pc strength: the stronger the Pc, the costlier the integron (Figure 1). This correlation was associated with higher resistance levels as we observed that stronger Pc variants (PcS and PcW<sub>TGN-10</sub>) exhibit higher resistance levels (Table 2).

We further examined whether or not the observed cost was due solely to gene cassette expression. We therefore performed competition experiments using the same above-mentioned plasmids but with



**Figure 1** Effect of Pc polymorphism on the cost of a class 1 integron in *E. coli*. MG1656 strains containing plasmid-borne integrons varying only by their Pc sequence were placed in competition with the MG1656 *att::gfp*+pZA2 strain (Supplementary Figure S3A). Experiments were performed with either wild-type (WT) integrons (plasmids 2–5 in Table 1) or PintI-mutated integrons (no *intI1* expression, plasmids 6–9 in Table 1). The 6.2 kbp control plasmid (no *intI1* expression or cassette expression, plasmid 10 in Table 1) was used to evaluate the effect of plasmid size between plasmid pZA2 (2.1 kbp) and the integron-carrying plasmids (6.2 kbp).

mutations in the PintI promoter which prevented *intI1* expression (plasmids 6–9; Table 1) (Guérin et al., 2011). The overall cost of the integron was similar whether PintI was mutated or not, suggesting that, in wild-type conditions in which *intI1* is repressed, the fitness cost of an integron is due to gene cassettes expression (Figure 1).

The strain with plasmid 10 was used as a control to assess the contribution of the 6.2 kbp plasmid to host fitness. In this plasmid, the PintI and Pc promoters were both mutated in order to relate its cost to its size alone (no expression of the *intI1* gene and gene cassettes; Guérin et al., 2011). The selection coefficient for this plasmid ( $s = -0.008 \pm 0.003$ ,  $P < 1 \times 10^{-8}$ ) showed that the 6.2 kbp plasmidic backbone itself slowed bacterial growth by 0.8% (Figure 1).

### The *IntI1* fitness cost is related to integrase catalytic activity

To assess the fitness cost of integrase expression, we used integron-carrying MG1656 strains in which the Pc promoter was mutated, thus preventing gene cassette expression without affecting the *IntI1* protein sequence (plasmids 11–16; Table 1) (Guérin et al., 2011). The integrons contained either the normally repressed *intI1* gene (plasmids 11–13) or the unrepressed *intI1* gene (mutated LexA binding site) (plasmids 14–16). Competition experiments were performed with each *IntI1* protein variant: *IntI1*<sub>R32\_H39</sub> (associated with the PcW or PcH1 promoter), *IntI1*<sub>P32\_H39</sub> (associated with PcW<sub>TGN-10</sub>) and *IntI1*<sub>R32\_N39</sub> (associated with PcS).

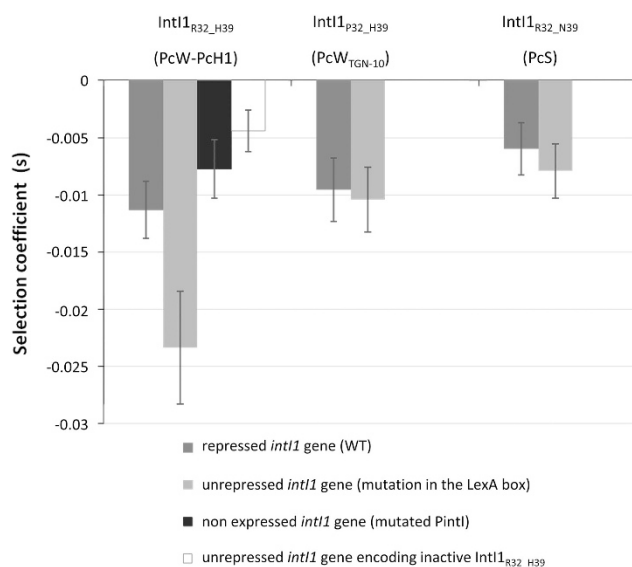
Whatever the Pc variant, when the *intI1* gene was repressed, its cost was not different from the control in which the PintI promoter was mutated (Figure 2). This confirmed that when *intI1* is repressed, the observed cost of an integron is only due to gene cassettes expression. When unrepressed (mutated LexA binding site), a twofold increase in fitness cost was observed with the integron encoding *IntI1*<sub>R32\_H39</sub> integrase (the integrase associated with the PcW sequence), rising from  $-0.011 \pm 0.002$  when

**Table 2** MICs of MG1656 strains containing the four gene cassettes with different Pc variants

	MIC ( $\mu\text{g ml}^{-1}$ )			
	SPT	CHL	TOB	TMP
MG1656+pZA2	2	4	0.25	–
MG1656+pW-4GC	128	16	4	–
MG1656+pH1-4GC	256	32	8	–
MG1656+pW <sub>TGN-10</sub> -4GC	512	64	16	–
MG1656+pS-4GC	512	128	16	–

Abbreviations: CHL, chloramphenicol; SPT, streptomycin; TMP, trimethoprim; TOB, tobramycin. Trimethoprim MICs were not determined due to solubility issues in DM25 medium.





**Figure 2** The cost of integrase expression. The MG1656 *latt::gfp* +pZA2 strain was placed in competition with MG1656 strains carrying plasmidic integrons with a mutated Pc promoter (no gene cassette expression, plasmids 11–17; Table 1). Mutations were silent, so as not to affect the IntI1 protein variant encoded by the *intI1* gene. The *intI1* gene was either repressed (plasmids 11–13) or unrepressed (plasmids 14–17). Derepression of the *intI1* gene was achieved by mutating the LexA box and thus preventing repressor binding. Plasmid 17 encoded an inactive integrase. Plasmid 10, with neither *intI1* nor gene cassette expression (Table 1), was used as a control.

repressed to  $-0.023 \pm 0.004$  when unrepressed ( $P < 1 \times 10^{-10}$ ; Figure 2). Interestingly, IntI1<sub>R32\_H39</sub> is also the most active integrase (Jové *et al.*, 2010). To determine whether the cost of this integrase was related to its catalytic activity, we used the MG1656 strain carrying an integron with a mutated *intI1* gene encoding a non-functional IntI1<sub>R32\_H39</sub> variant (IntI1<sub>Y312F</sub>) (plasmid 17; Table 1). The Y312F mutation was sufficient to abrogate the cost associated with the IntI1<sub>R32\_H39</sub> integrase (Figure 2).

#### Influence of the cassette array contents

As cassette arrays can vary by their size and gene content, we wondered whether these two parameters might impact the global cost of integrons.

We first analyzed the fitness cost in MG1656 strains carrying integrons with one, two or four gene cassettes under the control of the strong promoter variant PcS (plasmids 20, 18 and 5). As controls, plasmids 10, 19 and 24, in which the Pc and *PintI* promoters were both mutated, were used to evaluate the inherent cost related to plasmid size. We found that the cost of an integron increased with the number of gene cassettes (Figure 3a), rising eightfold from one to four gene cassettes. A minor part of this cost was related to plasmid size (Figure 3a, light grey).

To estimate the influence of the nature of the gene cassettes, we performed competition experiments with MG1656 strains carrying an integron in which

only one cassette was fully expressed from the strong Pc variant. Four gene cassettes were tested: *aadA1*, *catB9*, *aac(6′)-Ib* and *dfrA15* (plasmids 20–23; Table 1). As shown in Figure 3b, the cost of an integron was found to be dependent on the gene cassette it contained. The *aac(6′)-Ib* gene cassette was the costliest, with a selection coefficient of  $-0.017 \pm 0.002$  ( $P < 1 \times 10^{-10}$ ). Cassettes *catB9* and *aadA1* were slightly detrimental, with respective costs of  $-0.005 \pm 0.002$  ( $P < 1 \times 10^{-8}$ ) and  $-0.005 \pm 0.003$  ( $P < 1 \times 10^{-10}$ ). Given the detection limit of our approach (detection of fitness differences  $\geq |2 \times 10^{-3}|$ ), the *dfrA15* gene cassette appeared to bear no cost.

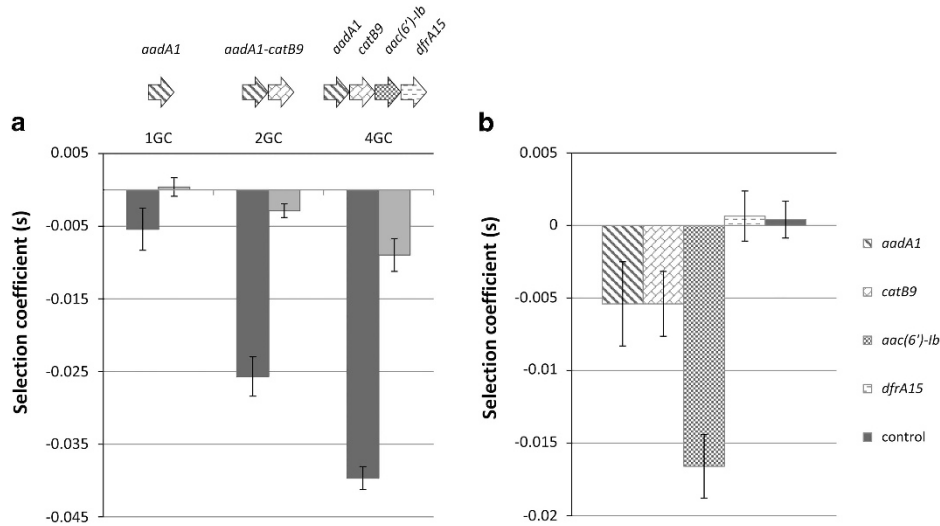
#### Impact of subinhibitory antibiotic concentrations

To study whether subinhibitory antibiotic concentrations favor bacteria carrying integrons with appropriate resistance gene cassettes, we analyzed the impact of a subinhibitory tobramycin (Tob) concentration on the cost of a four-cassettes integron containing the *aac(6′)-Ib* cassette in third position within the array (MIC  $4 \mu\text{g ml}^{-1}$ ). Competition experiments between resistant bacteria carrying the integron and susceptible strain lacking the integron were performed in the presence of various subinhibitory Tob concentrations. The chosen concentrations were 2.5 ( $0.1 \mu\text{g ml}^{-1}$ ) and 5 times ( $0.05 \mu\text{g ml}^{-1}$ ) lower than the MIC of the strain without the integron (MIC  $0.25 \mu\text{g ml}^{-1}$ ).

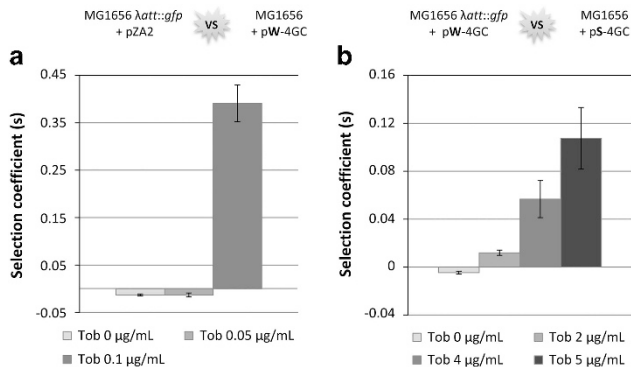
No effect was observed at  $0.05 \mu\text{g ml}^{-1}$  Tob but, at  $0.1 \mu\text{g ml}^{-1}$ , bacteria carrying the integron appeared superfit ( $s = 0.39 \pm 0.04$ ,  $P < 1 \times 10^{-10}$ ; Figure 4a). They grew 1.39 times faster than their integron-less counterparts, showing that subinhibitory antibiotic concentrations can favor the selection of bacteria containing an integron with an appropriate resistance gene and thereby promote the maintenance and dissemination of integrons.

To determine whether subinhibitory antibiotic concentrations could favor the maintenance of integrons with given promoter variants, we performed direct competition experiments between bacteria carrying integrons with either a weak Pc (PcW; plasmid 25, Table 1) or a strong Pc (PcS; plasmid 22, Table 1 and Supplementary Figure S3D), in the absence and presence of subinhibitory Tob concentrations (Figure 4b). Again, the chosen Tob concentrations were below the MIC of the most susceptible bacteria ( $8 \mu\text{g ml}^{-1}$ ).

In the absence of tobramycin, bacteria carrying the integron with the weak Pc were fitter than those carrying the integron with the strong Pc. However, when tobramycin was added, the selection coefficient became positive, showing that the bacteria carrying the integron with the strong Pc became fitter than those with the weak Pc. Tob concentrations from 2 to  $5 \mu\text{g ml}^{-1}$  were tested. Selection coefficients rose from  $0.012 \pm 0.002$  ( $P < 1 \times 10^{-10}$ ) at the lowest Tob concentration to  $0.107 \pm 0.003$



**Figure 3** Effect of the gene cassettes array content. (a) Effect of cassettes network size. MG1656 strains containing plasmidic integrons, varying only by the number of gene cassettes (GC) they carried (plasmids 5, 18 and 20; Table 1), were placed in competition with the MG1656 *latt::gfp*+pZA2 strain (Supplementary Figure S3B). For each condition (1, 2 or 4 gene cassettes), control plasmids with neither *intI1* nor cassette expression (plasmids 10, 19 and 24; Supplementary Table S1) were used to evaluate the inherent cost of carrying this plasmid size (light gray). (b) Influence of the nature of the gene cassettes. Competition experiments were performed between the MG1656 *latt::gfp*+pZA2 strain and MG1656 strains carrying plasmids with a one-cassette integron (plasmids 20–23; Table 1) (Supplementary Figure S3C). The cassette was *aadA1*, *catB9*, *aac(6)-Ib* or *dfrA15*. Plasmid 24 was used as a control to evaluate the carriage cost of this plasmid size.



**Figure 4** Effect of subinhibitory antibiotic concentrations on integron carriage. (a) Integron with PcW. Competition experiments were performed between GFP bacteria without an integron (MG1656 *latt::gfp*+pZA2) and non-GFP bacteria carrying an integron (MG1656+plasmid 2; Table 1). Different subinhibitory tobramycin (Tob) concentrations were added to the growth media during competition. These concentrations were chosen to be below the MIC of the most susceptible competitor ( $0.25 \mu\text{g ml}^{-1}$ ). (b) Integrons with different Pc. Competition experiments were performed between two bacteria carrying the same integron except for the Pc variant, one with a weak Pc promoter (GFP bacteria with plasmid 25; Table 1) and the other with a strong Pc promoter (non-GFP bacteria with plasmid 22; Table 1) (Supplementary Figure S3D). Subinhibitory Tob concentrations below the MIC of the more susceptible competitor ( $8 \mu\text{g ml}^{-1}$ ) were added to the growth media during competition.

( $P < 1 \times 10^{-10}$ ) at the highest Tob concentration, meaning that bacteria carrying the integron with the strong Pc grew 1.2–10.7% faster, depending on the antibiotic concentration (Figure 4b). These results show how integrons associated with strong Pc variants confer a major fitness

advantage in the presence of subinhibitory antibiotic concentrations.

## Discussion

This study shows that class 1 integrons are low-cost structures in *E. coli*. A previous meta-analysis involving 760 chromosomal resistance mutations and 49 resistance plasmids suggested that the average cost of chromosomal and plasmid-borne resistance was equivalent to a 21–9% decline in host fitness, respectively (Vogwill and MacLean, 2015). Thus with a 1.3–4% decrease in the host fitness, integron-mediated resistance would be 2–7 times less costly than plasmidic resistance, and 5–15 times less costly than chromosomal resistance. Furthermore, in this study we used a p15a replicon yielding approximately 15 plasmid copies per bacterial cell. In clinical and environmental settings, class 1 integrons are found mainly in low copy plasmids. Roughly, their cost might even be five times smaller for a natural integron-bearing plasmid with three copies such as the R388 plasmid (Avila and de la Cruz, 1988). In return for this very low fitness cost, integrons would allow *E. coli* to adapt more readily to antibiotic pressure.

This very low cost could explain why integrons are highly prevalent in *E. coli* and, probably, in many other *Enterobacteriaceae* (Ahmed et al., 2005; Guerra et al., 2006; Rao et al., 2006). Dissemination alone cannot explain the success of integrons. Indeed, while dissemination has contributed to expanding the host range of integrons, newly acquired genetic elements are not necessarily maintained in bacterial

populations. For instance, a previous study showed that various IncA/C plasmids harboring the *bla*<sub>CMY-2</sub> resistance gene were lost in the absence of antibiotic selective pressure (Subbiah *et al.*, 2011). Likewise, selective pressure alone cannot explain integron maintenance in bacterial populations. Indeed, integrons have been found in environments preserved from the effects of human activity (Wright *et al.*, 2008; Gaze *et al.*, 2011). The very low cost of integrons might therefore have favored their maintenance and spread in bacterial populations, notably in antibiotic-free environments. Moreover, this would help bacteria to retain resistance cassettes in the absence of antibiotic pressure, keeping them readily available in the event of antibiotic stress.

The low cost of class 1 integrons in *E. coli* could be explained by the tight regulation of integrase expression. Indeed, we found that LexA-mediated repression of *intI1* expression significantly lowered the cost of integrons (Figure 2). Regulatory systems that reduce the cost of resistance determinants have already been described. In *Enterococcus* for instance, the cost of the vancomycin resistance operon is lowered by a tight regulatory mechanism (Foucault *et al.*, 2010). Previously, the regulation of a tetracycline resistance operon derived from a Tn10 transposon was found to abrogate the cost of the structure in *E. coli* (Nguyen *et al.*, 1989). LexA regulation of integrase expression could therefore be an adaptive response designed to lower the cost of integrons and thereby to favor their maintenance and dissemination.

LexA-mediated repression of integrase expression might also be an adaptive response designed to prevent the loss of a costly protein. Truncated and inactivated *intI1* genes are commonly found in bacteria (Gillings *et al.*, 2005; Nemergut *et al.*, 2008). This loss of integrase functionality could be due to the burden they represent for the host. Yet, *in silico* evolutionary studies have shown the importance of integrase integrity and functionality for adaptation to environmental challenges (Engelstadter *et al.*, 2016). Interestingly, an *in silico* study correlated the loss of integrase functionality with the absence of LexA-mediated regulation in bacteria, suggesting that the integrase would be lost when *intI1* gene expression was not regulated by the SOS response (Cambray *et al.*, 2011). Further evidence of the protective role of LexA regulation came from evolutionary experiments with integron-containing *A. baylyi*, a species lacking the LexA repressor, in which frameshift mutations rapidly emerge to inactivate IntI1 protein and to restore host fitness (Starikova *et al.*, 2012). LexA repression could therefore have been needed to prevent the loss of a costly but adaptively important gene.

Interestingly, we found that the cost of a given integrase was related to its catalytic activity (Figure 2): integrase with high activity (IntI1<sub>R32\_H39</sub>) was costlier than those with low activity (IntI1<sub>P32\_H39</sub> and IntI1<sub>R32\_N39</sub>). Furthermore, a single mutation in

the catalytic amino acid codon of this highly active integrase was sufficient to abolish its cost. It has been proposed that the cost of an integrase may be related to the genomic instability it confers (Starikova *et al.*, 2012). Even though the IntI1 integrase mostly catalyzes recombination reactions between *attI* and *attC* sites carried by integrons and cassettes, it can also recognize non-canonical sites in the host genome and thereby create genomic instability (Recchia *et al.*, 1994). Recent work has notably highlighted the importance of *attI* × *attI* recombination *in vivo*. Despite having a lower recombination rate when compared with *attI* × *attC* reaction, *attI* × *attI* recombination involves sites that do not require a proper folding to become recombinogenic (in contrast with *attC* sites; Escudero *et al.*, 2016). Thus, *attI* × *attI* reactions could be more prevalent than previously thought. As a GNT is sufficient to become a secondary *attI* site (Recchia *et al.*, 1994), such recombination events are likely to be very important in integrase-mediated genome instability. In this context, a more active integrase would generate more recombination events and create more instability, thus being more deleterious for the host.

Our results also demonstrate that the cost of class 1 integrons correlates with the strength of the Pc promoter (Figure 1): the stronger the Pc (and thus the expression level of antibiotic resistance), the costlier the integron. Epidemiological studies revealed a high prevalence of integrons associated with weak Pc variants (PcW and PcH1) in *Enterobacteriaceae* (Vinué *et al.*, 2011; Wei *et al.*, 2013). This high prevalence could be explained by the observation that integrons with a weak Pc, encoding high-activity integrases (Jové *et al.*, 2010), are adaptively advantageous. Indeed, more efficient integrases would allow bacteria to adapt more efficiently to challenging environments by acquiring new gene cassettes. Besides, we show that integrons associated with weak Pc variants are also the least costly. Thus, weak Pc promoters would be doubly advantageous, favouring both integron maintenance and environmental adaptation.

Furthermore, the gene cassette contents also affect the cost of class 1 integrons: the higher the number of cassettes in the array, the costlier the integron (Figure 3a). This observation is consistent with previous reports on the cost of plasmidic resistance, showing that the average cost of a plasmid increases with the number of resistance genes it carries, independently of the size of the plasmid (Vogwill and MacLean, 2015). This could explain why class 1 integrons usually carry a limited number of gene cassettes (2 or 3 on average, and up to 10; Integrall database, <http://integrall.bio.ua.pt/>). However, this correlation between the cost of an integron and its size has to be balanced. Indeed, it has been shown that the level of gene cassette expression is directly dependent on its distance from the Pc promoter (Collis and Hall, 1995) and that the expression level is reduced by the action of *attC* secondary structure



which impedes translation activity (Jacquier *et al.*, 2009). Thus, beyond a certain number of cassettes, host fitness might increase only slightly as further cassettes are acquired.

The gene cassette content also seems to impact host fitness. In a model of a one-cassette integron, we found that different gene cassettes had different costs, *aadA1* and *dfrA15* being the least costly and *aac(6′)-Ib* the costliest (Figure 3b). Several explanations for these differences can be proposed. This could be linked to the recombinogenic activity of the *attC* sites. The low-cost *dfrA15* and *aadA1* gene cassettes, with their respective T-N<sub>6</sub>-C and T-N<sub>6</sub>-G extrahelical base motifs, are supposed to be highly recombinogenic (Larouche and Roy, 2011). In contrast, the costly *aac(6′)-Ib* gene cassette with the TC-N<sub>6</sub>-G *attC* site exhibiting low affinity for IntI1 might have the lowest recombination rate. Furthermore, previous studies have shown that the length of an *attC* site and the structure of its VTS (variable terminal sequence) can prevent proper folding of an *attC* site (Loot *et al.*, 2014). Thus, in our study, the following order of gene cassette recombination rates should be observed: *aadA1* (favorable extrahelical base motifs and short *attC* site) > *dfrA15* (favorable extrahelical base motifs but longer *attC* site) >>> *catB9* (extrahelical base motifs and long *attC* site) ≈ *aac(6′)-Ib* (very unfavorable extrahelical base motifs and moderate *attC* size). However, given the cost of each cassette (*aac(6′)-Ib* > *catB9* = *aadA1* > *dfrA15*), no clear correlation emerges between the recombinogenic activity of the *attC* site and the cost of the gene cassette. Another possibility is that the observed differences are related to the nature of the encoded resistance mechanisms. Indeed, a meta-analysis of chromosomal resistance mutations showed that the average cost of various resistance mechanisms could differ significantly (Vogwill and MacLean, 2015). It has been reported that the prevalence of *dfr* genes in clinical isolates remained unchanged for 2 years despite an 85% reduction in trimethoprim prescriptions, suggesting that these genes are highly stable even in the absence of selective pressure (Brolund *et al.*, 2010). We infer that the family of *dfr* genes might have particularly low cost. On the other hand, we found that the *catB9* and *aac(6′)-Ib* gene cassettes, which both encode acetyltransferases and have similar *attC* recombinogenic activities, had different costs (Figure 3b). Some acetyltransferases involved in aminoglycoside acetylation are able to acetylate peptidoglycans *in vitro* (Payie and Clarke, 1997). Thus, other putative targets of AAC(6′)-Ib protein might affect bacterial metabolism and thereby entail a higher cost.

We found that the *dfrA15* and *aadA1* gene cassettes entailed a negligible fitness cost. Interestingly, the *aadA* and *dfr* gene cassette families are highly prevalent in class 1 integrons (Vasilakopoulou *et al.*, 2009; Vinué *et al.*, 2011; Kheiri and Akhtari, 2016), reaching 63% in *E. coli* class 1 integrons (data from the Integrall database),

suggesting that their low cost could favor their maintenance in cassette networks. By contrast, *aac* gene cassettes, including the costly *aac(6′)-Ib* cassette, are only found in 11% of *E. coli* class 1 integrons (Integrall database).

The different costs of gene cassettes also raise the question of the impact of the order of the cassettes in a given array. A costly cassette located in first position, close to the Pc and therefore strongly expressed, would be highly deleterious for the host bacterium. By contrast, transfer of a low-cost cassette to first position might reduce the overall cost of the gene cassette array. An integron could therefore reduce its overall cost by reshuffling the gene cassettes into a more cost-effective order. This hypothesis is supported by the fact that, when present, low-cost *dfr* cassettes are often found in first position (70% of the time according to the Integrall database). However, other factors are also known to influence the position of gene cassettes. For example, some cassettes lack an efficient translation initiation region (TIR) (Hanau-Berçot *et al.*, 2002) and would need to be in first position in order to be properly expressed, relying on the translation of a short ORF11 peptide encoded by the *attI* site (Hanau-Berçot *et al.*, 2002). This would explain why, despite their cost, *aac(6′)-Ib* cassettes lacking an efficient TIR are often found in first position (60% of the time when present, Integrall).

Lastly, our experiments indicate that subinhibitory tobramycin concentrations favor the maintenance and enrichment of bacterial populations carrying an integron with the appropriate resistance gene cassette (Figure 4a). Furthermore, we found that integrons associated with strong Pc variants were fitter than those associated with weak Pc variants (Figure 4b). These findings are in agreement with a previous study showing that antibiotic concentrations up to several hundred times below the MIC can lead to enrichment in resistant bacteria (Gullberg *et al.*, 2011).

Altogether, these results reveal the cost-effectiveness of integrons in *E. coli*. In return for a low fitness cost, they allow their host bacteria to adapt readily to antibiotic pressure. This low cost would be due to the SOS repression system, which prevents the expression of a costly integrase. The low cost of integrons would also explain their success and prevalence in many bacterial species, and would make them easily selected in the presence of subinhibitory antibiotic concentrations.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

We thank C Ouk from the CIM technical platform (University of Limoges) for technical support with flow cytometry, M Baltazar for the MG1656 *λatt::gfp* strain



construct, C Guet and D Mazel for respectively providing us with the pZE1-*gfpmut3* and p8741 plasmids. This work was supported by grants from Ministère de l'Enseignement Supérieur et de la Recherche (MESR), Institut National de la Santé et de la Recherche Médicale (INSERM) and Fondation pour la recherche Médicale (FRM grant DEQ20150331742).

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