



## RESEARCH ARTICLE

**REVISED** Polycistronic transcription of fused cassettes and identification of translation initiation signals in an unusual gene cassette array from *Pseudomonas aeruginosa* [version 3; referees: 2 approved]

Érica L. Fonseca, Ana Carolina Paulo Vicente

Laboratório de Genética Molecular de Microrganismos, Instituto Oswaldo Cruz, Rio de Janeiro, 4365, Brazil

**v3** First published: 28 Mar 2013, 2:99 (doi: [10.12688/f1000research.2-99.v1](https://doi.org/10.12688/f1000research.2-99.v1))  
 Second version: 07 Sep 2015, 2:99 (doi: [10.12688/f1000research.2-99.v2](https://doi.org/10.12688/f1000research.2-99.v2))  
 Latest published: 27 Nov 2015, 2:99 (doi: [10.12688/f1000research.2-99.v3](https://doi.org/10.12688/f1000research.2-99.v3))

**Abstract**

The gene cassettes found in class 1 integrons are generally promoterless units composed by an open reading frame (ORF), a short 5' untranslated region (UTR) and a 3' recombination site (*attC*). Fused gene cassettes are generated by partial or total loss of the *attC* from the first cassette in an array, creating, in some cases, a fusion with the ORF from the next cassette. These structures are rare and little is known about their mechanisms of mobilization and expression. The aim of this study was to evaluate the dynamic of mobilization and transcription of the *gcu14-bla<sub>GES-1</sub>-aacA4* gene cassette array, which harbours a fused gene cassette represented by *bla<sub>GES-1</sub>-aacA4*. The cassette array was analyzed by Northern blot and real-time reverse transcription-polymerase chain reaction (RT-PCR) in order to assess the transcription mechanism of *bla<sub>GES-1</sub>-aacA4* fused cassette. Also, inverse polymerase chain reactions (PCR) were performed to detect the free circular forms of *gcu14*, *bla<sub>GES-1</sub>* and *aacA4*. The Northern blot and real time RT-PCR revealed a polycistronic transcription, in which the fused cassette *bla<sub>GES-1</sub>-aacA4* is transcribed as a unique gene, while *gcu14* (with a canonical *attC* recombination site) has a monocistronic transcription. The *gcu14* cassette, closer to the weak configuration of cassette promoter (PcW), had a higher transcription level than *bla<sub>GES-1</sub>-aacA4*, indicating that the cassette position affects the transcript amounts. The presence of ORF-11 at *attI1*, immediately preceding *gcu14*, and of a Shine-Dalgarno sequence upstream *bla<sub>GES-1</sub>-aacA4* composes a scenario for the occurrence of array translation. Inverse PCR generated amplicons corresponding to *gcu14*, *gcu14-aacA4* and *gcu14-bla<sub>GES-1</sub>-aacA4* free circular forms, but not to *bla<sub>GES-1</sub>* and *aacA4* alone, indicating that the GES-1 truncated *attC* is not substrate of integrase activity and that these genes are mobilized together as a unique cassette. This study was original in showing the transcription of fused cassettes and in correlating cassette position with transcription.

**Open Peer Review**

Referee Status:

	Invited Referees	
	1	2
<b>REVISED</b> <b>version 3</b> published 27 Nov 2015	 report	
	↑	
<b>REVISED</b> <b>version 2</b> published 07 Sep 2015	 report	 report
	↑	↑
<b>version 1</b> published 28 Mar 2013	 report	 report

- 1 **Sally Partridge**, Sydney West Area Health Service Australia
- 2 **Béatrice Berçot**, Hôpital de Bicêtre France

**Discuss this article**

Comments (3)

**Corresponding author:** Érica L. Fonseca ([ericafon@ioc.fiocruz.br](mailto:ericafon@ioc.fiocruz.br))

**How to cite this article:** Fonseca ÉL and Vicente ACP. **Polycistronic transcription of fused cassettes and identification of translation initiation signals in an unusual gene cassette array from *Pseudomonas aeruginosa* [version 3; referees: 2 approved]** *F1000Research* 2015, 2:99 (doi: [10.12688/f1000research.2-99.v3](https://doi.org/10.12688/f1000research.2-99.v3))

**Copyright:** © 2015 Fonseca ÉL and Vicente ACP. This is an open access article distributed under the terms of the [Creative Commons Attribution Licence](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

**Grant information:** This work was supported by the CNPq and FAPERJ fellowship and Oswaldo Cruz Institute Grant.

**Competing interests:** No relevant competing interests were disclosed.

**First published:** 28 Mar 2013, 2:99 (doi: [10.12688/f1000research.2-99.v1](https://doi.org/10.12688/f1000research.2-99.v1))

**REVISED** Amendments from Version 2

Taking into account the Dr. Partridge recommendations, a new version of Figure 2 was provided in order to make clearer the differences between the canonical and truncated GES-1 *attC* sites. In this new version of the figure, all points raised by Dr. Partridge were addressed.

See referee reports

## Introduction

Class 1 integrons are capable of inserting, excising and rearranging gene cassettes by a site-specific recombination mechanism. These assembly platforms can also act as expression systems due to the presence of a promoter region (Pc), which drives the expression of genes captured by integron<sup>1</sup>. Moreover, naturally occurring integrons may have a second promoter (P2), which is activated by the insertion of three G residues between -35 and -10 hexamers<sup>1</sup>. Gene cassettes are generally promoterless units associated with a recombination site (*attC* or 59-be), which confers the ability of each structure to be mobilized independently<sup>2</sup>, and the Left Hand (LH –1L and 2L core sites) and Right Hand (RH – 1R and 2R core sites) domains from *attC* sites are crucial for this mobilization<sup>3</sup>. Studies focusing on gene cassette translation in the context of integrons are rare, however, it was recently showed that *attC* sites regulate the translation of downstream cassettes due to their peculiar sequences composed by imperfect inverted repeats that form stem-loop structures. These secondary structures prevent ribosome progression throughout mRNA, reflecting in a decreased expression of more distal genes regarding Pc<sup>4</sup>. Conversely, TIR (Translation Initiation Region)-deficient gene cassettes could have their expression promoted by the presence of ORF-11<sup>5</sup>. This ORF, when present, is found at the *attI* site preceding the gene cassette array. It codes for 11 amino acids and harbours its own Shine-Dalgarno (SD) sequence. Therefore, the ORF-11 recruits the ribosomes and, through an event of coupled translation, the subsequent TIR-deficient gene cassette could be expressed<sup>4,5</sup>. Gene cassettes can be found inserted in integrons or in other secondary sites, or free in the cytoplasm as a closed circle, in which the 5' end (5' UTR) and the *attC* recombination site are covalently linked<sup>6</sup>.

As demonstrated previously, several stress conditions could evoke the activation of the SOS response resulting in integron-integrase expression<sup>7</sup>. Therefore, under stress, the integrase activity increases, favoring the occurrence of integration/excision/rearrangements events.

Although rare, fused cassettes may be generated by partial or total loss of the first *attC*, retaining both complete coding regions and, therefore, creating permanent gene arrays comparable to bacterial operons<sup>8</sup>. The functionality of such structures has been indirectly inferred by the resistance profile of transformants carrying the fusion<sup>9</sup>; however, the transcription itself has never been verified.

This study showed the dynamics of fused cassette mobilization, the co-transcription of the *gcu14*-*bla*<sub>GES-1</sub>-*aacA4* cassette array and the effect of cassette position on transcription levels in *Pseudomonas aeruginosa* wild lineages carrying class 1 integrons. Moreover, the presence of translation signals in this gene cassette array was determined.

## Material and methods

An unknown Open Reading Frame (ORF), *gcu14* (gene cassette of unknown function), followed by the fused cassette *bla*<sub>GES-1</sub>-*aacA4*, created by partial loss of GES-1 *attC* were present in integrons from clinical *P. aeruginosa* isolates (PS1 and PS26)<sup>10</sup>. Total RNA was extracted and purified according to the manufacturer's instructions with the SV 96 Total RNA Isolation System (Promega). Northern blot using 7 µg of total RNA from PS1 and PS26 was performed in order to detect the transcript originated from *gcu14*-*bla*<sub>GES-1</sub>-*aacA4* cassette array. After electrophoresis in a denaturing-formaldehyde 1.5% agarose gel, the total RNA was transferred to the Hybond-N<sup>+</sup> nylon membrane (GE Healthcare) by upward capillary transfer. An amplicon of 519bp corresponding to part of the *bla*<sub>GES-1</sub> gene was used as a probe (Table 1) in hybridization assay. The GES probe was labeled with the AlkPhos Direct Labelling kit (GE Healthcare) and hybridized with the target RNA immobilized on the Hybond-N<sup>+</sup> membrane as recommended. The chemiluminescence was detected with the CDP-Star detection reagent (GE Healthcare) according to manufactures. Immediately after applying the detection reagents, the blot was drained, incubated five minutes at room temperature and exposed to the Hyperfilm ECL (GE Healthcare) for 60 minutes at room temperature.

In order to verify whether the relative position of gene cassettes on the variable region plays a role in transcription level, real-time RT-PCR reactions using the TaqMan System (Applied Biosystems) were performed with primers and probes detailed in Table 1. The *rpsL* gene of the *P. aeruginosa* chromosome was amplified by PCR (Table 1) and used as a reference gene for normalization. The relative quantification (RQ) results were presented as ratios of gene transcription between the target gene (cassettes) and the reference gene (*rpsL*), which were obtained according to the following equation:  $RQ=2^{-\Delta CT}$ , where CT is the value corresponding to the crossing point of the amplification curve with the threshold and  $\Delta CT=CT$  target gene minus CT reference gene. The effect of cassette position on gene transcription was considered significant when the ratios obtained between RQ values (RQ value of cassette 1/RQ value of cassette 2) were  $\geq 2.0$ , taking into account the standard deviation intervals.

In order to induce cassette excision from integrons, PS1 and PS26 strains<sup>10</sup> were submitted to thermal stress during the log growth phase to induce integrase activity. Cells were grown on Luria-Bertani (LB) broth medium (OXOID) at 37°C for two hours. Subsequently, the bacterial cultures were submitted to a heat shock at 4°C for 30 minutes and immediately incubated at 42°C for another 30 minutes. Briefly, the total DNA from PS1 and PS26 cultured under thermal stress were obtained with the Wizard Genomic DNA purification kit (Promega) following manufacturer recommendations and used as templates in inverse PCR reactions. The inverse PCR was performed with primers facing outwards towards the ends of *gcu14*, *bla*<sub>GES-1</sub> and *aacA4* so that only circular gene cassette configurations would be amplified. The reactions targeting the circular forms of *gcu14*, *bla*<sub>GES-1</sub>-*aacA4* and *bla*<sub>GES-1</sub>-*aacA4* fusion was performed with primers and combinations described in Table 1. The inverse PCR was performed using Platinum Taq DNA Polymerase reagents (Invitrogen), and the following components were added to a sterile 0.2-mL tube: 5 µL of 10X PCR buffer (1X final concentration); 1 µL of 10mM dNTP mixture (0.2 mM each); 1.5 µL of 50mM

**Table 1.** Primers used in conventional, inverse and real-time PCR reactions.

Primer	Primer sequence (5' – 3')	Size (bp)	Target
<b>Primers for conventional PCR</b>			
Ges F Ges R	GCGTTTTGCAATGTGCTC CCAGTTTTCTCTCCAACAACC	519	Internal fragment of <i>bla<sub>GES</sub></i> gene
<b>Primers for inverse PCR</b>			
Gcu14 FSQ Gcu14 RSQ	AGCAATCAACACACAGGGG CTCGCGTAAATGCACCGCTT	130	<i>gcu14</i> circular form
GES FSQ GES RSQ	CAAGTTATTACACAATCAT AGTGCGTGAATGAAGCGCAT	110	<i>bla<sub>GES-1</sub></i> circular form
AACA4 FSQ AACA4 RSQ	GCCAGGCATTCGAGCGAACAC ATTTAGCCACTCACATAGAGC	188	<i>aacA4</i> circular form
<b>Primers and probes for real time PCR (TaqMan)</b>			
RpsL F RpsL R Probe	GCCTGCGCTGCAAACT TTTCGGCGTGGTGTAT TCGTGGCGTATGCACC	67	<i>rpsL</i> transcripts
Gcu14 F Gcu14 R Probe	CATGCGCTTCTGGTTCGT ACGCCAGCTTGGATGCAA ATGCCACGAGACCTT	56	<i>gcu14</i> transcripts
Ges F2 Ges R2 Probe	GTGCAGCTTAGCGACAATGG CACAGAGTCGCCAATTTACGA AATTGCAGCAGGTCGGCC	99	<i>bla<sub>GES-1</sub></i> transcripts
AacA4 F AacA4 R Probe	CAAGCGTTTTAGCGCAAGAGT TCGGCTCTCCATTGAGCATTG CCGTCACCTCCATACATTG	59	<i>aacA4</i> transcripts
Ges F3 AacA4 R2 Probe	TCCTGAGCACGGACAAATAG TCATAGAGCATCGCAAGGTC TTCCGTCACACTGCGCCTCA	134	Polycistronic transcripts

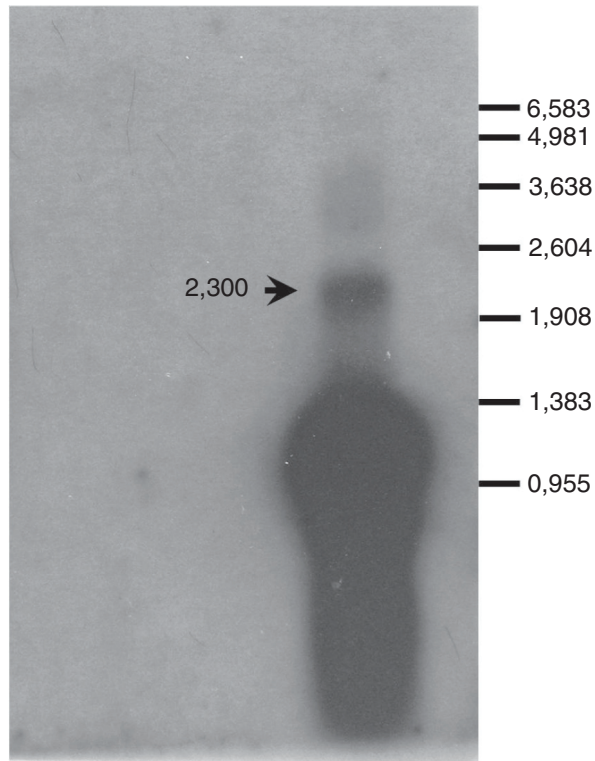
MgCl<sub>2</sub> (1.5 mM final concentration); 2 µL of 15 µM of each primer (30 µM each); 100 ng of template DNA; 0.3 µL of Platinum *Taq* DNA Polymerase (1U final concentration). The tubes were incubated in the Eppendorf MasterCycler (Eppendorf) at 94°C for 2 minutes and PCR amplification was performed in 40 cycles consisting of: 94°C for 30 seconds; 55°C for 30 seconds; and 72°C for 3 minutes. The amplicons generated with the inverse PCR were purified using Wizard SV Gel and PCR Clean-Up system kit (Promega) and directly sequenced on both strands. Sequencing reactions were performed with Big Dye Terminator RR Mix (Applied Biosystems) in an ABI 3730 XL DNA Analyzer (Applied Biosystems). Nucleotide sequences were compared to those available in the GenBank database accessible on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). All primers used in PCR, sequencing and real time RT-PCR are described in Table 1.

Analyses *in silico* were performed to search for a potential promoter for *gcu14* gene cassette. The 5'UTR from *gcu14* were submitted to the promoter predictor programs Neural Network for Promoter Prediction version 2.2 (Berkeley Drosophila Genome Project, <http://www.fruitfly.org/index.html>) and BPROM (SoftBerry, <http://linux1.softberry.com/berry.phtml>). Results with the highest scores were selected as candidates for a putative promoter.

## Results and discussion

The integrons analysed in this study harbored the weak Pc configuration (PcW)<sup>11</sup>, and they carry the *gcu14* as the first gene cassette (see reference 12 for nomenclature), which has not been reported so far. Considering that transcription initiates from the Pc promoter placed upstream the cassette array, both monocistronic and full length polycistronic transcripts could be identified. In fact, Northern blot and hybridization assays revealed a unique signal of approximately 2,300 bases, which corresponds to the co-transcription of the entire array (*gcu14-bla<sub>GES-1</sub>-aacA4*) (Figure 1). This result is in agreement with previous work in which the occurrence of transcripts containing more than one gene cassette was observed by Northern blot analysis<sup>1</sup>. Moreover, this finding gives support to the lack of *attC* function in terminating transcription of downstream gene cassettes as demonstrated previously<sup>4</sup>.

This fusion retained both entire coding regions and, due to a possible erroneous recombination event, the *bla<sub>GES-1</sub>-attC* site was replaced by part of the *attI1* site (DQ236170)<sup>10</sup>, reducing it to the 6bp from the 1L core site (Figure 2). Taking into account that the region responsible for stem-loop formation was missing in GES-1 *attC* and the participation of this site in terminating translation<sup>4</sup>, our findings indirectly suggested that *bla<sub>GES-1</sub>* and *aacA4* translation is occurring in a unique step.

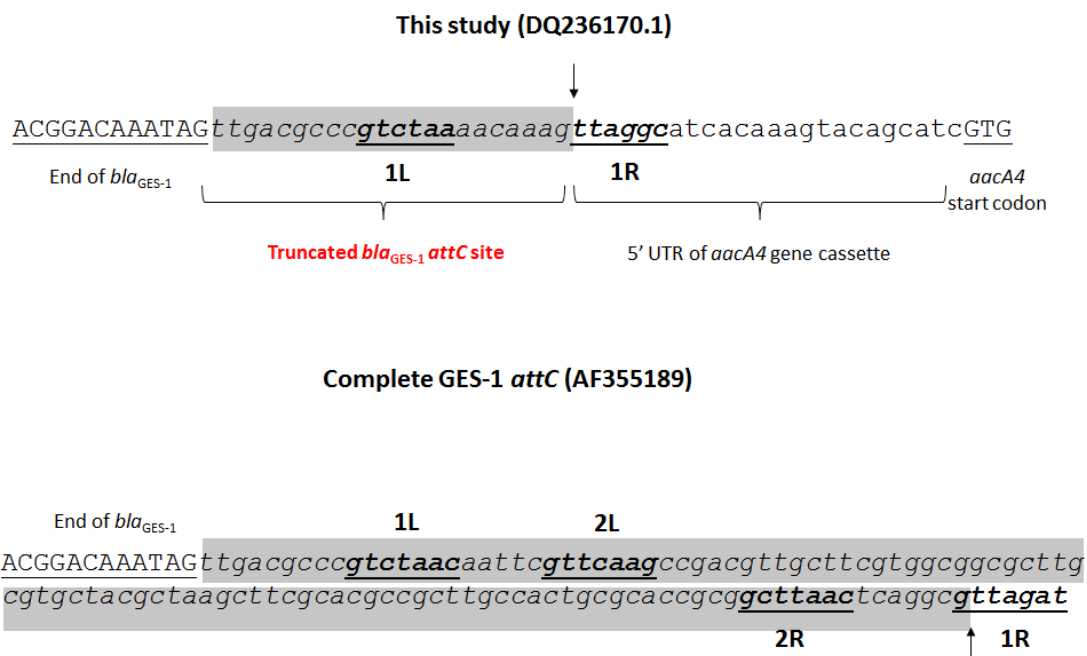


**Figure 1. Northern blot analysis of *gcu14-bla<sub>GES-1</sub>/aacA4* gene cassette array from *P. aeruginosa* class 1 integron.** The full length transcript (2,300 bases), corresponding to the entire gene cassette array, hybridized with the GES probe (arrow). The fragment sizes of the RNA marker (Promega) used in RNA electrophoresis are indicated.

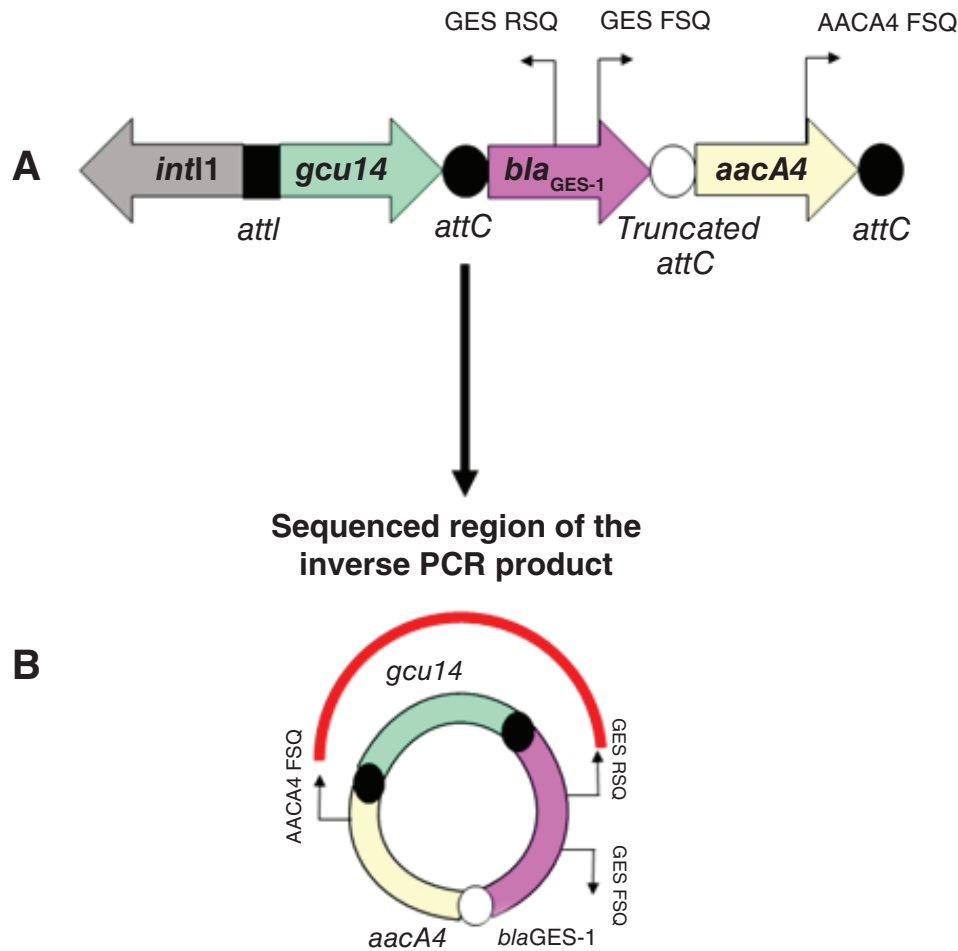
The strains were submitted to thermal stress conditions in order to verify the dynamics of mobilization of *gcu14-bla<sub>GES-1</sub>/aacA4* gene cassettes. Since the excision event depends on the recognition of the LH and RH domains of the *attC* site, and that the 2L core site and the entire RH domain are missing in GES-1 *attC* (Figure 2), it is expected that the *bla<sub>GES-1</sub>/aacA4* excision occurs only at the *aacA4 attC* site, and that this structure is excised together as a unique cassette.

Positive results were obtained for the *gcu14*, *gcu14-aacA4* and *gcu14-bla<sub>GES-1</sub>/aacA4* circular forms, but not for *bla<sub>GES-1</sub>* and *aacA4* alone. This finding indicates that the GES-1 *attC* is not functional and that the fused gene cassette is excised as a unique cassette. Moreover, the presence of *gcu14-aacA4* circular form suggests that this strain carries a second integron containing this gene cassette arrangement. Sequencing assessed the recombination point where excision occurred, confirming the occurrence of free circular forms (Figure 3). This is in agreement with the presence of the PcW configuration, in which the corresponding *intI1* gene codes for a high efficient integrase<sup>11</sup>. The lack of activity of a truncated *attC* had also been observed before when associated with *aadA1*<sup>13</sup>. However, Ramirez and colleagues<sup>14</sup> showed that the integrase was able to recognize and mediate excision of a truncated site associated to *aadA1*, indicating that the genetic context of such truncated sites could influence their role in IntI1 recognition and mobilization.

The relative quantification performed by real time RT-PCR revealed that PS1 and PS26 presented very similar RQ values for *gcu14-bla<sub>GES-1</sub>/aacA4* transcription (Figure 4). This result was expected since integrons from these two strains have the same backbone, including the Pc promoter, and are at the same genetic environment<sup>10</sup>.



**Figure 2. Sequences of the canonical (AF355189) and the truncated (this study) GES-1 attC sites.** The underlined uppercase letters represent the *bla<sub>GES-1</sub>* and *aacA4* partial coding regions. Italicized lowercase letters highlighted in grey define the *attC* sites, and their core sites (1L, 2L, 2R, 1R) are in boldface and underlined. The vertical arrows show the recombination point and the beginning of the next cassette.

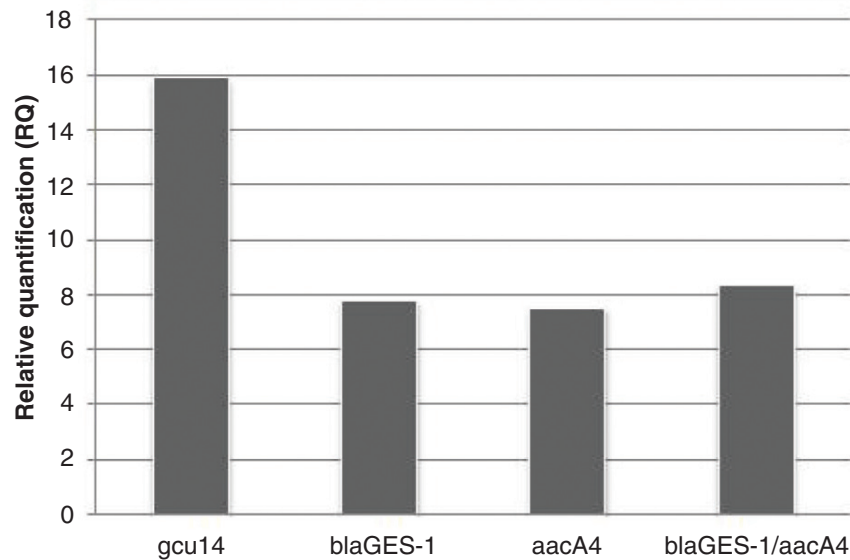


**Figure 3. Schematic representation of the free circular form cassette array resulted from the inverse PCR assay.** (A) inserted/linearized form of integron harbouring the fused cassettes. Arrows show the gene transcription orientation. Thin arrows represent the annealing sites of the inverse PCR and sequencing primers whose generated product corresponded to the cassette circular form illustrated in (B). (B) Illustration of the free circular form of gene cassettes represented in (A). Thin arrows show the primers used to obtain the inverse PCR product (GES FSQ and GES RSQ) and the primers used in sequencing (AACAA4 FSQ and GES RSQ) that revealed the excision in block of the entire gene cassette arrangement (*gcu14-bla<sub>GES-1</sub>/aacA4*) from integron (red curved line).

*gcu14*, the first cassette in integrons with the weak PcW configuration, presented approximately two-fold higher transcription when compared to *bla<sub>GES-1</sub>* and *aacA4* separately or when the fused cassette *bla<sub>GES-1</sub>/aacA4* was considered (Figure 4). The same RQ value obtained for *bla<sub>GES-1</sub>*, *aacA4* and the fusion reveals that these two ORFs are transcribed as a unique gene. The lower transcript amount of *bla<sub>GES-1</sub>/aacA4* compared to *gcu14* lies on the distance between these gene cassettes and Pc, which is one of the determinants influencing cassette transcription<sup>1,7</sup>, and it shows the effect of cassette position on expression levels.

A putative promoter for *gcu14* (-35 TTGATG [17 bp] -10 TGTTAC) was found 45 bp upstream from its start codon, which has the potential to influence transcription. Moreover, the ORF-11, which enhances the translation efficiency of downstream TIR-deficient

cassettes inserted in integrons<sup>5</sup>, was found at the *attI1* region preceding the TIR-deficient *gcu14* gene cassette. This ORF contained its own Shine-Dalgarno (SD) sequence placed 8 bp upstream of the ATG codon. The ribosome at the ORF-11 stop codon could, therefore, be carried along the mRNA by lateral diffusion, reinitiating translation at the *gcu14* start codon. A potential SD sequence was identified 10 bp upstream of the fused cassette *bla<sub>GES-1</sub>/aacA4*. In addition, the loss of the GES-1 *attC* region, which is involved in stem-loop formation, may enhance the chances of *aacA4* translation, since this *attC*, reduced to the 6bp of the 1L core site, no longer constitutes a physical barrier to ribosome progression<sup>4</sup>. Together, these findings create a scenario for the occurrence of *gcu14-bla<sub>GES-1</sub>/aacA4* expression in PS1 and PS26, which then provides a possible explanation for their resistance profile to  $\beta$ -lactams and aminoglycosides that has been observed elsewhere<sup>10</sup>.



**Figure 4. Transcription level of *gcu14-bla<sub>GES-1</sub>/aacA4* gene cassette array.** The relative quantification values obtained by real time RT-PCR are indicated for each gene cassette and for the fusion *bla<sub>GES-1</sub>/aacA4* when considered as a unique gene.

RQ fused cassette raw data for *Pseudomonas aeruginosa* isolates PS1 and PS26

2 Data Files

<http://dx.doi.org/10.6084/m9.figshare.651717>

#### Data availability

*Figshare:* Polycistronic transcription of fused cassettes and identification of translation initiation signals in an unusual gene cassette array from *Pseudomonas aeruginosa* doi: <http://dx.doi.org/10.6084/m9.figshare.651717><sup>19</sup>

#### Conclusions

Fused cassettes have been found in class 1 integrons<sup>9,13–18</sup>; however, the transcription of such structures has rarely been addressed. This work showed the transcription pattern of a fused cassette as a polycistronic mRNA and that these unusual structures are excised as a unique cassette. The mobilization in block of the entire *gcu14-bla<sub>GES-1</sub>/aacA4* array together with its active transcription, and the presence of translational signatures demonstrate the potential for dissemination and expression of multidrug resistance, in a one-step fashion, to other bacteria. Therefore, such events could represent a threat to public health and to the establishment of efficient antibiotic regimens.

#### Nucleotide sequence accession number

The sequence of the cassette array composed by the fusion has been deposited in the GenBank database under accession number [DQ236170](https://www.ncbi.nlm.nih.gov/nuclseq/DQ236170). The sequence obtained from the inverse PCR amplicon, showing the circular form gene organization, was submitted to GenBank under accession number [KT336477](https://www.ncbi.nlm.nih.gov/nuclseq/KT336477).

#### Author contributions

ELF and ACPV conceived the study and designed the experiments. ELF carried out the research and prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

#### Competing interests

No relevant competing interests were disclosed.

#### Grant information

This work was supported by the CNPq and FAPERJ fellowship and Oswaldo Cruz Institute Grant.

#### Acknowledgements

We thank those involved in PDTIS platform to enable us to do the sequencing and real-time relative quantification analysis work.

## References

1. Collis CM, Hall RM: **Expression of antibiotic resistance genes in the integrated cassettes of integrons.** *Antimicrob Agents Chemother.* 1995; **39**(1): 155–162.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
2. Collis CM, Hall RM: **Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase.** *J Bacteriol.* 1992; **174**(5): 1574–1585.  
[PubMed Abstract](#) | [Free Full Text](#)
3. Stokes HW, O’Gorman DB, Recchia GD, *et al.*: **Structure and function of 59-base element recombination sites associated with mobile gene cassettes.** *Mol Microbiol.* 1997; **26**(4): 731–745.  
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Jacquier H, Zaoui C, Sanson-le Pors MJ, *et al.*: **Translation regulation of integrons gene cassette expression by the attC sites.** *Mol Microbiol.* 2009; **72**(6): 1475–1486.  
[PubMed Abstract](#) | [Publisher Full Text](#)
5. Hanau-Berçot B, Podglajen I, Casin I, *et al.*: **An intrinsic control element for translational initiation in class 1 integrons.** *Mol Microbiol.* 2002; **44**(1): 119–130.  
[PubMed Abstract](#) | [Publisher Full Text](#)
6. Recchia GD, Hall RM: **Plasmid evolution by acquisition of mobile gene cassettes: plasmid pIE723 contains the aadB gene cassette precisely inserted at a secondary site in the incQ plasmid RSF1010.** *Mol Microbiol.* 1995; **15**(1): 179–187.  
[PubMed Abstract](#) | [Publisher Full Text](#)
7. Hocquet D, Llanes C, Thouverez M, *et al.*: **Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting.** *PLoS Pathog.* 2012; **8**(6): e1002778.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
8. Recchia GD, Hall RM: **Gene cassettes: a new class of mobile element.** *Microbiology.* 1995; **141**(Pt 12): 3015–3027.  
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Mendes RE, Toleman MA, Ribeiro J, *et al.*: **Integron carrying a novel metallo-beta-lactamase gene, bla<sub>IMP-16</sub>, and a fused form of aminoglycoside-resistant gene aac(6’)-30/aac(6’)-Ib’: report from the SENTRY Antimicrobial Surveillance Program.** *Antimicrob Agents Chemother.* 2004; **48**(12): 4693–4702.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
10. da Fonseca EL, Vieira VV, Cipriano R, *et al.*: **Emergence of bla<sub>GES-5</sub> in clinical colistin-only-sensitive (COS) Pseudomonas aeruginosa strain in Brazil.** *J Antimicrob Chemother.* 2007; **59**(3): 576–577.  
[PubMed Abstract](#) | [Publisher Full Text](#)
11. Jové T, Da Re S, Denis F, *et al.*: **Inverse correlation between promoter strength and excision activity in class 1 integrons.** *PLoS Genet.* 2010; **6**(1): e1000793.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
12. Partridge SR, Tsafnat G, Coiera E, *et al.*: **Gene cassettes and cassette arrays in mobile resistance integrons.** *FEMS Microbiol Rev.* 2009; **33**(4): 757–784.  
[PubMed Abstract](#) | [Publisher Full Text](#)
13. Partridge SR, Collis CM, Hall RM: **Class 1 integron containing a new gene cassette, aadA10, associated with Tn1404 from R151.** *Antimicrob Agents Chemother.* 2002; **46**(8): 2400–2408.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
14. Ramirez MS, Parenteau TR, Centron D, *et al.*: **Functional characterization of Tn1331 gene cassettes.** *J Antimicrob Chemother.* 2008; **62**(4): 669–673.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
15. Poirel L, Le Thomas I, Naas T, *et al.*: **Biochemical sequence analyses of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from Klebsiella pneumoniae.** *Antimicrob Agents Chemother.* 2000; **44**(3): 622–632.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
16. Céntron D, Roy PH: **Presence of a group II intron in a multiresistant Serratia marcescens strain that harbors three integrons and a novel gene fusion.** *Antimicrob Agents Chemother.* 2002; **46**(5): 1402–1409.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
17. Dubois V, Poirel L, Marie C, *et al.*: **Molecular characterization of a novel class 1 integron containing bla<sub>GES-1</sub> and a fused product of aac3-Ib/aac6’-Ib’ gene cassettes in Pseudomonas aeruginosa.** *Antimicrob Agents Chemother.* 2002; **46**(3): 638–645.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
18. Walsh TR, Toleman MA, Hryniewicz W, *et al.*: **Evolution of an integron carrying bla<sub>VIM-2</sub> in Eastern Europe: report from the SENTRY Antimicrobial Surveillance Program.** *J Antimicrob Chemother.* 2003; **52**(1): 116–119.  
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Fonseca ÉL, Vicente ACP: **RQ fused cassette raw data for Pseudomonas aeruginosa isolates PS1 and PS26.** *Figshare.* 2013.  
[Data Source](#)



# Open Peer Review

Current Referee Status:



---

## Version 3

Referee Report 30 November 2015

doi:10.5256/f1000research.8053.r11365



**Sally Partridge**

Sydney West Area Health Service, Sydney, NSW, Australia

The grey shading to indicate the attC site should not start until the start of 1L and the portion derived from attI1 is still not indicated.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.

---

## Version 2

Referee Report 18 November 2015

doi:10.5256/f1000research.3720.r10226



**Sally Partridge**

Sydney West Area Health Service, Sydney, NSW, Australia

This manuscript has been improved and the points I raised have been addressed, but I have some comments about the new Fig. 2:

1. The underlined uppercase letters indicating the blaGES-1 coding region should extend until the stop codon at position 914.
  2. ttagat of the IR site shown for the complete blaGES-1 attC is really part of the adjacent 3'-CS in AF355189 (most of the 1R core site belonging to the blaGES cassette (TTAGAC) is found at the start of the linear cassette). I would suggest ending this sequence after the first g of this site.
  3. The partial 1L of the truncated blaGES cassette (924-9), the nucleotides derived from the attI1 site (930-6) and 1R of the aacA4 cassette (937-42) should be indicated. I would suggest ending the sequence of the truncated cassette after 1R of aacA4, as the alignment of the complete attC with the start of the aacA4 cassette beyond this point is not really useful.
-

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response 19 Nov 2015

**Erica Fonseca**, Instituto Oswaldo Cruz, Brazil

Reviewer: The underlined uppercase letters indicating the blaGES-1 coding region should extend until the stop codon at position 914.

Response: Reviewer is right. This was corrected in the figure. Actually, the figure was modified in order to make clearer the differences between the canonical GES-1 attC and the truncated GES-1 attC found in this study.

Reviewer: ttagat of the IR site shown for the complete blaGES-1 attC is really part of the adjacent 3'-CS in AF355189 (most of the 1R core site belonging to the blaGES cassette (TTAGAC) is found at the start of the linear cassette). I would suggest ending this sequence after the first g of this site.

Response: We agree and this was corrected in the new figure.

Reviewer: The partial 1L of the truncated blaGES cassette (924-9), the nucleotides derived from the attI1 site (930-6) and 1R of the aacA4 cassette (937-42) should be indicated. I would suggest ending the sequence of the truncated cassette after 1R of aacA4, as the alignment of the complete attC with the start of the aacA4 cassette beyond this point is not really useful.

Response: We agree and this was modified in the new figure.

**Competing Interests:** There is no competing interests

Referee Report 28 September 2015

doi:[10.5256/f1000research.3720.r10563](https://doi.org/10.5256/f1000research.3720.r10563)



**Béatrice Berçot**

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Paris, France

I have read the updated paper which is clear and well written. Little is known in transcription of fused cassettes and these experimental data highlighted that the two genes are cotranscribed and mobilized together. The author answered all my request and I have not supplementary remarks

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.

## Version 1

Referee Report 21 May 2013

doi:10.5256/f1000research.903.r961

**Béatrice Berçot**

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Paris, France

This work is conducted to address the transcription of the fused gene cassette *bla<sub>GES-1</sub>/aacA4*. This arrangement is rare but not exceptional in a class 1 integron. In this work, the experimentation confirmed that the three gene cassettes harbored in this class 1 integron were expressed together from a unique polycistronic transcript.

It seems that the authors have observed a circular form containing the *gcu14* gene cassette associated with the *aacA4* gene cassette. Could they explain this arrangement? Is it possible that the *Pseudomonas* isolates harbored another class 1 integron containing the *gcu14* gene followed by the *aacA4* gene?

The authors have to provide in the paper the classical length of the *bla<sub>GES-1</sub> attC* site, which is 110bp and contained the 1L, 2L, 2R and 1R sequences-shown in bold.

TTGACGCCC **GTCTAAC** AATTC **GTTCAAGC**  
CGACGTTGCTTCGTGGCGGCGCTTGCGTGCTACGCTAAGCTTCGCACGCCGCTTGCCACTGCGCAC  
**GCTTAAC** TCAGGC **GTTAGNC**

In the *Pseudomonas* SP26 and PS1 isolates, the *bla<sub>GES-1</sub> attC* site is reduced to the 6 bp of 1 L box.

TTGACGCCC **GTCTAA** AACAAAGTTAGGCATCACAAAGTACAGCATC **GTG**

So, the distance from of the stop of *bla<sub>GES-1</sub>* to the start codon of the *aacA4* gene is 46bp. The translational start codon of *aacA4* gene is erroneous in the Genbank data base ([DC236170](#)). Indeed, it has been determined by N-terminal amino acid sequencing to be a GTG (in italic in the sequence above) and the beginning of the cassette was 24 bp before [[Hanau-Bercot B. et al., 2002](#)].

-Could the Genbank annotation be modified as explained above?

-The Appendix is not necessary and should be deleted.

-In the sentence page 4, line 13, "The single copy of *rpsL* gene of *Pseudomonas*...", should be replaced by "the *rpsL* gene of *Pseudomonas* (see [Bodilis et al., 2012](#))

-In the conclusion, page 6, change "never" to "rarely".

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response 05 Aug 2015

**Erica Fonseca**, Instituto Oswaldo Cruz, Brazil

It seems that the authors have observed a circular form containing the *gcu14* gene cassette associated with the *aacA4* gene cassette. Could they explain this arrangement? Is it possible that the *Pseudomonas* isolates harbored another class 1 integron containing the *gcu14* gene followed by the *aacA4* gene?

**Response:** Yes, the presence of the *gcu14*-*aacA4* circular form indicates that this strain harbours a second integron composed by the *gcu14*-*aacA4* arrangement. However, this result does not invalidate our main conclusion, which is that the truncated GES-1 *attC* site is not functional and this gene is only mobilized when recombination occurs in *aacA4 attC*. A brief explanation was included in the text (Results section).

The authors have to provide in the paper the classical length of the *bla*<sub>GES-1</sub> *attC* site, which is 110bp and contained the 1L, 2L, 2R and 1R sequences-shown in bold.

**Response:** A figure showing the truncated and the complete canonical *attC* from GES1 was included in the new version.

So, the distance from of the stop of *bla*<sub>GES-1</sub> to the start codon of the *aacA4* gene is 46bp. The translational start codon of *aacA4* gene is erroneous in the Genbank data base (DC236170). Indeed, it has been determined by N-terminal amino acid sequencing to be a GTG (in italic in the sequence above) and the beginning of the cassette was 24 bp before [Hanau-Bercot B. et al., 2002].

-Could the Genbank annotation be modified as explained above?

**Response:** We performed this modification and the updated sequence regarding the beginning of *aacA4* gene is already accessible on GenBank database under the same accession number (DQ236170).

-The Appendix is not necessary and should be deleted.

**Response:** The appendix is only for helping referees in their evaluation. It will not be published.

-In the sentence page 4, line 13, "The single copy of *rpsL* gene of *Pseudomonas*...", should be replaced by "the *rpsL* gene of *Pseudomonas* (see Bodilis et al., 2012).

**Response:** this was modified in the new version of the manuscript.

In the conclusion, page 6, change "never" to "rarely".

**Response:** this was modified in the new version of the manuscript.

**Competing Interests:** There is no competing interests

Referee Report 02 May 2013

doi:10.5256/f1000research.903.r920



**Sally Partridge**

Sydney West Area Health Service, Sydney, NSW, Australia

This paper looks at expression of genes in a gene cassette array that includes a fused cassette and at the excision of cassette from this array. Polycistronic transcripts from cassette arrays and the excision of other fused cassettes have previously been noted by others, as cited in this paper. The sequences in Appendix 1 need to be annotated properly, rather than just the results of searches given, and the point

illustrated by each sequence needs to be explained to make it possible to assess whether they support the conclusions drawn in the paper.

Some reorganisation (moving some information in the Results and Discussion to the Introduction) would help to make the paper easier to follow and the Conclusions section is very short and could be expanded. The English could also be improved and the manuscript checked for typos etc.

A few specific points also need correcting or clarifying:

- Abstract Line 5- fused cassettes do not always have fused orfs (e.g. *aacA1/orfG*)
- Abstract 6th Line from end and Results and Discussion p. 5 1st Line of 2nd paragraph – what is in the *gcu14-aacA4* circular form? Should this be *blaGES-1/aacA4*?
- Materials and Methods Line 1 – this needs rewording to explain that *gcu* is a gene cassette of unknown function.
- Results and Discussion p. 4 – the fusion here is the type where part of the *blaGES-14 attC* site is replaced by part of the *attI1* site (see ref. 14). The LH and RH domains are part of the *attC* site, rather than flanking it. The 5' UTR contains 6 bp of the *attC* site.
- Results and Discussion p. 5 – most of the 1L core site and the 1R core site of the *blaGES attC* site are present.
- Results and Discussion p. 6 – the deletion of part of the *attC* site doesn't bring the *aacA4* gene much closer to Pc and ref. 14 makes slightly different point (that expression of the downstream cassette may be enhanced).

Formatting “14” of *gcu14* and “A4” of *aacA4* should be in italics. Transposon, gene and species names etc are not correctly formatted in the references.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response 05 Aug 2015

**Erica Fonseca**, Instituto Oswaldo Cruz, Brazil

The sequences in Appendix 1 need to be annotated properly, rather than just the results of searches given, and the point illustrated by each sequence needs to be explained to make it possible to assess whether they support the conclusions drawn in the paper.

**Response:** The sequence resulted from inverse PCR product, showing the circular form of the *gcu14-blaGES-1-aacA4* array, was submitted to GenBank under accession number KT336477. This information was included in the text. Moreover, we provided a new figure showing a schematic representation of primer targeting sites used in inverse PCR in order to make easier for the reviewers comprehend our strategy and results.

Some reorganisation (moving some information in the Results and Discussion to the Introduction) would help to make the paper easier to follow and the Conclusions section is very short and could be expanded. The English could also be improved and the manuscript checked for typos etc.

**Response:** We agree that some points from results and discussion section would be more adequate in the introduction section, and they were moved in the new version of the manuscript (Page 3, lines 59-60 and 65-70; page 4, lines 71-76 in the new version).

The English was reviewed by a native spoken-English, and the conclusion was expanded.

A few specific points also need correcting or clarifying:

Abstract Line 5- fused cassettes do not always have fused orfs (e.g. *aacA1/orfG*)

**Response:** We agree and it was modified: We affirmed that only in some cases fused orfs can be created.

Abstract 6th Line from end and Results and Discussion p. 5 1st Line of 2nd paragraph – what is in the *gcu14-aacA4* circular form? Should this be *blaGES-1/aacA4*?

**Response:** The presence of the *gcu14-aacA4* circular form indicates that this strain harbours a second integron composed by the *gcu14-aacA4* arrangement. However, this result does not invalidate our main conclusion, which is that the truncated *GES-1 attC* site is not functional and this gene is only mobilized when recombination occurs in *aacA4 attC*. A brief explanation was included in the text (Results section).

Materials and Methods Line 1 – this needs rewording to explain that *gcu* is a gene cassette of unknown function.

**Response:** It was included in the text.

Results and Discussion p. 4 – the fusion here is the type where part of the *blaGES-1 attC* site is replaced by part of the *attI1* site (see ref. 14). The LH and RH domains are part of the *attC* site, rather than flanking it. The 5' UTR contains 6 bp of the *attC* site.

**Response:** We change the text emphasizing that the *attC* was replaced by part of *attI1*.

The referee is right; LH and RH domains are part of the *attC* site, rather than flanking it. This general idea concerning *attC* site and its domains was modified throughout the text.

Results and Discussion p. 5 – most of the 1L core site and the 1R core site of the *blaGES attC* site are present.

**Response:** In fact, only the 1L core site is present in the truncated *attC*. We included a figure (figure 2) in this new version showing this.

Results and Discussion p. 6 – the deletion of part of the *attC* site doesn't bring the *aacA4* gene much closer to *Pc* and ref. 14 makes slightly different point (that expression of the downstream cassette may be enhanced).

**Response:** The referee is right. This statement was removed.

Formatting “14” of *gcu14* and “A4” of *aacA4* should be in italics. Transposon, gene and species names etc are not correctly formatted in the references.

**Response:** All formatting errors were properly corrected.

**Competing Interests:** There is no competing interests

---

## Discuss this Article

Version 1

Author Response 05 Aug 2015

**Erica Fonseca**, Instituto Oswaldo Cruz, Brazil

Dear Authors,

Besides the comments already made by the reviewers, I would like to specify some points:

- The C to G mutations that converts the usual weak PcW variant into PcWTGN-10 has been shown to increase (instead of 'decrease', first part of the Results section).

**Response:** This sentence was removed.

- I have not checked which Intl1 had been used for the excision assay, usually these are made with the most efficient "PcW" Intl1 (Intl1R32\_H39); note that in our PLoS Genetics paper ([Jove et al. 2010](#)) we evidenced the Intl1 "PcWTGN-10" (IntlP32\_H39) were much less efficient for excision of gene cassettes, which could had been taken into account in your discussion.

**Response:** As properly noticed by you, our Pc configuration is the weak one, and this was changed in the text. However, we agree that including this discussion we will improve our manuscript (lines 182 and 184).

- The hypothesis of a synergistic effect between the Pc and P(gcu14) tandem promoters does not fit the observed phenotypes: since the resulting level of transcription observed for the downstream genes is lower I suggest that some transcriptional interferences occur rather than synergistic interactions. By the way in absence of any experimental evidence for the functionality of the gcu14 promoter, such hypothesis is overstated. It would have been interesting to check the level of transcription of the blaGES-1, aacA4 gene cassette when gcu14 is deleted.

**Response:** we totally agree with this observation, in fact our conclusion is overstated since no experimental assay was performed to test the promoter synergy. Therefore, we remove this part from the text. We also agree that would be interesting to verify the expression of the fused cassette in the absence of gcu14, however it was not the focus of this paper.

- First, the class 1 integron sequence of the PS1 strain in Genbank DQ236170 does not display the rare C to G mutation that converts a weak "PcW" promoter into a stronger "PcWTGN-10" as stated in the paper. Consequently you should write "the weak PcW configuration" instead of "weak PcWTGN-10". Also it means that its own Intl1 integrase is likely to be optimally efficient.

**Response:** Dr. Jové is absolutely right, the DQ236170 accession number presented the PcW configuration and not the PcWTGN-10 as stated in the manuscript, we apologize about this mistake. This was corrected in the text.

- Then, the sequence of the class 1 integron as deposited in the Genbank does not display the complete array of gene cassette (the end of the attCaacA4 is not available) which means that it is ambiguous whether there is a downstream third gene cassette or not. Consistently this array of gene cassette, although a novel one, has not been numbered in the INTEGRALL database (<http://integrall.bio.ua.pt/?acc=DQ236170>)

**Response:** We know that aacA4 was the last cassette in the array because this variable region was obtained using primers annealing in the attI1 and in the beginning of qacEΔ1 from 3'CS. It is true that the aacA4 attC is not completed in the GenBank accession number DQ236170. Therefore, spite of the

incomplete attC sequence we are sure that the aacA4 is the last cassette from this array.

- Lastly, as an element of reply to the reviewer Dr Bercot, I would like to notify that the so-called gcu14 GC has not been reported in other reports except in an environmental strain of Citrobacter (and the sequence is not 100% identical, Genbank FM998050).

**Response:** thanks for this observation. It was included in the text.

**Competing Interests:** There is no competing of interests

Reader Comment 23 May 2013

**Thomas Jové**, LGPB, Belgium

Dear authors,

I would like to add some further comments:

- First, the class 1 integron sequence of the PS1 strain in Genbank DQ236170 does not display the rare C to G mutation that converts a weak "PcW" promoter into a stronger "PcWTGN-10" as stated in the paper. Consequently you should write "the weak PcW configuration" instead of "weak PcWTGN-10". Also it means that its own IntI1 integrase is likely to be optimally efficient.

- Then, the sequence of the class 1 integron as deposited in the Genbank does not display the complete array of gene cassette (the end of the attCaacA4 is not available) which means that it is ambiguous whether there is a downstream third gene cassette or not. Consistently this array of gene cassette, although a novel one, has not been numbered in the INTEGRALL database (<http://integrall.bio.ua.pt/?acc=DQ236170>)

- Lastly, as an element of reply to the reviewer Dr Bercot, I would like to notify that the so-called gcu14 GC has not been reported in other reports except in an environmental strain of Citrobacter (and the sequence is not 100% identical, Genbank FM998050).

**Competing Interests:** None

Reader Comment 17 May 2013

**Thomas Jové**, LGPB, Belgium

Dear Authors,

Besides the comments already made by the reviewers, I would like to specify some points:

- The C to G mutations that converts the usual weak PcW variant into PcWTGN-10 has been shown to increase (instead of 'decrease', first part of the Results section).
- I have not checked which IntI1 had been used for the excision assay, usually these are made with the most efficient "PcW" IntI1 (IntI1R32\_H39); note that in our PLoS Genetics paper ([Jove et al. 2010](#)) we evidenced the IntI1 "PcWTGN-10" (IntIP32\_H39) were much less efficient for excision of gene cassettes, which could had been taken into account in your discussion.



- The hypothesis of a synergistic effect between the Pc and P(gcu14) tandem promoters does not fit the observed phenotypes: since the resulting level of transcription observed for the downstream genes is lower I suggest that some transcriptional interferences occur rather than synergistic interactions. By the way in absence of any experimental evidence for the functionality of the gcu14 promoter, such hypothesis is overstated. It would have been interesting to check the level of transcription of the blaGES-1, aacA4 gene cassette when gcu14 is deleted.
- Some years ago [Jacquier \*et al.\* \(2009\)](#) published data suggesting that attC sites rather influence the level of translation between each gene cassette while the level of transcription was unaffected.

**Competing Interests:** None

---