Promoter activity of ORF-less gene cassettes isolated from the oral metagenome

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Abstract

Integrons are genetic elements consisting of a functional platform for recombination and expression of gene cassettes (GCs). GCs usually carry promoter-less open reading frames (ORFs), encoding proteins with various functions including antibiotic resistance. The transcription of GCs relies mainly on a cassette promoter (P_C), located upstream of an array of GCs. Some integron GCs, called ORF-less GCs, contain no identifiable ORF with a small number shown to be involved in antisense mRNA mediated gene regulation.

In this study, promoter sequences were identified, using *in silico* analysis, within GCs PCR amplified from the oral metagenome. The promoter activity of ORF-less GCs was verified by cloning them upstream of a *gusA* reporter, proving they can function as a promoter, presumably allowing bacteria to adapt to multiple stresses within the complex physico-chemical environment of the human oral cavity. A bi-directional promoter detection system was also developed allowing direct identification of clones with promoter-containing GCs on agar plates. Novel promoter-containing GCs were identified from the human oral metagenomic DNA using this construct, called pBiDiPD.

This is the first demonstration and detection of promoter activity of ORF-less GCs and the development of an agar plate-based detection system will enable similar studies in other environments.

Introduction

Integrons are bacterial genetic elements able to integrate and express genes present on gene cassettes (GCs) [1-3]. They consist of two main components; a functional platform and a variable array of GCs. The functional platform, located on the 5' end of an integron, consists of an integrase gene (*intI)*, and its promoter (P_{intI}), an *attI* recombination site and a constitutive cassette promoter (P_C) for the expression of GCs [4]. IntI is a site-specific tyrosine integrase that catalyses the insertion and excision of GCs via recombination mainly at *attI* and the *attC*, the latter located at the joint of excised, circularised GCs. The integrase gene; *intI,* is normally transcribed in the opposite direction to GCs within an integron (Fig 1A). However, some integrons have integrase genes transcribed in the same directions as their GCs. These are called unusual integrons or reverse integrons (Fig 1B), and have been identified in *Treponema denticola*, *Chlorobium phaeobacteroides* and *Blastopirellula marina* [5, 6].

The second part of an integron is an array of GCs. Each usually contains a single promoterless open reading frame (ORF) and an *attC* recombination site [7]. The proteins encoded by GCs are diverse and include those associated with antibiotic resistance, virulence, and metabolism [2, 8]. When a GC is excised from integron, it forms a non-replicative mobile genetic element, which can be a substrate for integrase mediated recombination between *attI* (on the integrons) and *attC* (on the circular GC). This directionality of recombination is favoured over *attC*:*attC* recombination, resulting in the usual insertion of a newly integrated GC immediately next to the P_C promoter in the first position of GC array, ensuring maximal expression [9-11].

The expression of integron integrases is controlled via the SOS response; there is a LexA-binding site located in the P_{intI} [12]. In the absence of stress, the transcriptional repressor LexA binds to P_{intL} and prevents the transcription of *intI*. The SOS response is activated upon the accumulation of single-strand DNA (ssDNA), generated during DNA damage, DNA repair, transformation, conjugation and certain antibiotic exposure e.g. trimethoprim and fluoroquinolones [13-15]. RecA recognises ssDNA and polymerises into RecA nucleofilaments, which induce autocleavage of LexA, releasing P_{intI} from repression and allowing *intI* transcription [12, 16]. By controlling the expression of IntI, bacteria can reshuffle their GCs at the precise moments of need (stress), thereby avoiding accumulation of random recombination events that could be deleterious to the host cell [17, 18].

As most of the GCs do not contain a promoter, their expression usually relies on the P_C promoter. The level of expression of GCs varies depending on the distance from P_C, as the strength of expression decreases when GCs are located further from P_C [19]. This ensures that a recently acquired GC will be immediately expressed ensuring rapid adaptation due to stress-induced repositioned gene within the integron GC array. There are also some GCs that contain their own promoters, ensuring constitutive expression of their genes regardless of the P_C promoter and their position within the integron array; examples include *cmlA1* (chloramphenicol resistance), *qnrVC1* (quinolone resistance), *ere(A)* (erythromycin resistance) and many of the GCs encoding toxin-antitoxin (TA) systems [20-23].

Integron GCs have been identified from environments such as soils, marine sediments, seawater and more recently from human oral metagenomes [24-28]. In our previous study on the detection of integron GCs in the human oral metagenome, we found 13 ORF-less GCs out of 63 identified GCs (20%) [28]. ORF-less GCs have been shown to encode regulatory RNAs, for example the trans-acting small RNA (sRNA)-Xcc1, encoded by the ORF-less GC of a *Xanthomonas campestris* pv. *campestris* integron, which is involved in regulation of virulence [29]. Whilst promoter activity of ORF-less GCs has been discussed, this has not been experimentally demonstrated [8].

In this study, we performed *in silico* analysis to identify promoter sequences in the GCs identified in our previous study on the oral metagenome. Promoter activity was experimentally determined by cloning the selected GCs upstream of the *gusA* reporter gene and measuring β-glucuronidase enzyme activity. Furthermore, we devised a GC-based promoter detection strategy utilising PCR and subsequent cloning between divergently orientated reporter genes. With this system, the successful cloning of amplicons from promoter-containing GCs can be visualised directly on agar plates, allowing the direct isolation of GC PCR amplicons with promoter activity from metagenomic DNA.

Results

in silico analysis of the promoter sequences on the ORF-less GCs.

Among 63 GCs previously identified from human oral metagenomic DNA, 13 were predicted to be ORF-less GCs [28]. Using BPROM promoter prediction software, all ORF-less GCs were predicted to contain promoters on both strands, suggesting that these GCs can transcribe genes in flanking GCs (Table 1). In this study, we have defined the sense strand as the same strand containing the P_C promoter (Fig 1).

Determination of promoter activity of the ORF-less GCs using the β-glucuronidase assay.

Five GCs were chosen for experimental expression analysis. GC TMB4 (amplified with primers targeting *intI* and *attC*) was selected as it is ORF-less and located in the first position of the integron array [28]. ORF-less GCs MMU23 and MMB37 were selected as they had the highest overall score predicted by BPROM. Finally, GCs SSU17 and MMB3 were selected as controls, to represent GCs with an ORF.

As BPROM predicted putative promoter sequences on both strands, promoter activity of the selected GCs was determined by directionally cloning upstream of a promoterless β-glucuronidase (*gusA*) gene on pCC1BAC-*lacZα-gusA* (Fig 2) in both directions. For the first position GC; TMB4, three different constructs were made: TMB4 P_C promoter, TMB4 GC, and TMB4 P_C-GC constructs. As the TMB4 P_C promoter was not identical to the P_C of *T. denticola* integron [30], the P_C of another integron GC; TMB1 [28], which was identical to it [30], was included. As the selected GCs were likely derived from *Treponema* spp., two experimentally verified *T. denticola* promoters, P_{TdTro} and P_{Fla}, were also included as controls showing that *T. denticola* promoters can be recognised in our *E. coli* host [31, 32]. P_{Fla} and P_{Tdtro} were selected as they rely on different sigma factors. P_{Tdtro} is recognised by sigma factor 70 (σ^{70}) that is responsible for the transcription of most genes during growth in both *E. coli* and *Treponema* spp. [31, 33], while P_{Fla} is recognised by sigma factor 28 (σ^{28}), involved in the expression of flagella-related genes in motile bacteria [32, 34]. This will determine the limitations of our assay in recognising promoters associated with different types of sigma factors. The results are shown in figure 3. MMB37 and MMB3 had promoter activity on one strand, while MMU23 and SSU17 had no promoter activity, compared to the negative control. The TMB4-P_C, TMB4 GC, TMB4 P_C-GC and TMB1- P_C constructs, all showed promoter activities on both strands. The P_{Tdtro} from *T. denticola* showed strong promoter activity on both sense and antisense strands, verifying that promoters from *T. denticola* are recognised by *E. coli*. As the P_C promoter sequences on TMB1 and TMB4 samples were different at several nucleotides, it was shown that TMB4-P_C had higher promoter activities than the TMB1-P_C in both directions (Fig 3).

Detection of promoter-containing GCs from oral metagenome.

The pCC1BAC-*lacZα-gusA* plasmid, developed for the above enzymatic assay, had the potential to be used in an agar plate-based detection strategy to detect amplified integron GCs with promoter activity on either strand of DNA. This construct is called the Bi-Directional Promoter Detection plasmid (pBiDiPD). To verify the utility of pBiDiPD, and also to detect novel GCs containing promoter sequences in the human oral metagenome, integron GCs were amplified with SUPA4-*Nsi*I/SUPA3-*Nhe*I and MARS5-*Nsi*I/MARS2-*Nhe*I primers [28], and cloned into pBiDiPD. By spreading transformants on LB plates containing X-gal/IPTG and 4-methylumbelliferyl β-D-glucuronide (MUG), clones containing inserts with promoter activity in either direction could be identified. The clones with GCs containing a promoter on the sense strand showed blue fluorescence when visualised under UV light, reflecting the activity of β-glucuronidase enzymes catalysing MUG to yield the blue-fluorescent 4-methylumbelliferyl. Clones with promoter activity on the antisense strand resulted in blue colonies as a result of β-galactosidase enzymes catalysing X-Gal into a blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo (Fig 4).

After screening clones from both amplicon libraries (amplified with SUPA3-SUPA4 and MARS2-MARS5 primers), 23 different GCs with promoter activities were identified (Table 2). Fourteen of these were similar to the GCs identified in the previous study with >86% nucleotide identity [28]. Among the recovered promoter-containing GCs, 9 out of 23 were novel including sample SSU-Pro-20, SSU-Pro-27, SSU-Pro-32, SSU-Pro-46, SSU-Pro-65, MMU-Pro-5, MMU-Pro-24, and MMU-Pro-53. Artefactual PCRs were discounted by detecting the consensus R' (1R) core sites [GTTRR(Y)R(Y)Y(R)] and the complementary R'' (1L) core sites [R(Y)Y(R)Y(R)YAAC] of *attC* located downstream from the *attC* forward primers and upstream from the *attC* reverse primers, respectively (Supplementary Table 1) [35].

The GCs can be categorised into two groups, one predicted to encode toxin-antitoxin systems in 12 out of 23 GCs, including plasmid stabilization protein (toxin)-prevent-host-death protein (antitoxin), BrnT (toxin)-BrnA (antitoxin), VapC (toxin)-AbrB/MazE/SpoVT family protein (antitoxin), RelE/ParE family (toxin)-XRE transcriptional regulator (antitoxin). The second group contained ORF-less GCs, which could be found in 7 samples, all reported in the previous study, except sample MMU-Pro-53. Most of the samples (14 out of 23 GCs) showed the promoter activity only on the sense strand. Samples with promoter activity only on the antisense strand were MMU-Pro-6, MMU-Pro-63, and MMU-Pro-65, while 6 out of 23 GCs exhibited promoter activity on both strands.

Discussion

Integrons are important disseminators of antimicrobial resistance genes and therefore, it is important to understand the diversity of GCs and how their expression is controlled. Even though most of the GCs contained a single ORF, ORF-less GCs have also been found [24, 27, 28, 36, 37].

In this study, we determined promoter activity from GCs isolated by PCR from metagenomic DNA by measuring promoter activity from multiple GC containing constructs. As the ORF-less GCs were recovered from the oral metagenome, there is little information regarding the original host. Therefore, we chose to test the promoter activities by using an *E. coli* surrogate. Nucleotide sequence analysis suggested that these GCs were likely to be derived from *Treponema* spp., therefore, the ability of *E. coli* to utilise *T. denticola* promoter sequences was determined by including the experimentally verified

T. denticola promoter, P_{TdTro} [31] which showed high activity on both sense and antisense strands, providing confidence that *E. coli* could be used. However, as no promoter activity was detected from P_{Fla}, it suggested that our enzymatic assay cannot detect promoters associated with σ^{28} from *Treponema* spp., which could be due to an inability for the *E. coli* host to recognise the *Treponema* σ^{28} promoter sequence.

Promoter activities of the ORF-less GCs were confirmed and quantified by using a β-glucuronidase enzymatic assay. This is the first time that the promoter activity of ORF-less GCs has been demonstrated *in vitro*, as shown by the activity on the sense strand of the MMB37 and both strands of the TMB4. A study on the *Vibrio* integron, containing a 116-cassette array, showed that most of the GCs are transcribed [38]. Therefore, ORF-less GCs could be responsible for transcription of the other GCs not transcribed by P_C.

For the TMB4 GC (ORF-less GC in the first position), it was initially hypothesised that the promoter could help increase the expression of the downstream GCs. This was shown when P_C promoter was coupled with a second promoter (P_2) (found in 10% of class 1 integron and located 119 bp downstream from P_C), could result in a significantly higher expression of GCs [39, 40]. The constructs of TMB4 P_C and TMB4 P_C+GC were therefore included in the assay to determine whether having a promoter GC at the first position could help promote the expression of downstream GCs. The results show that coupling promoter GC in the first position slightly increased the expression of reporter genes (Fig. 3). However, this was not significant (*p*-value >0.99 by using ordinary one-way ANOVA followed by Bonferroni's post-hoc).

The lack of additive promoter activity can be explained by more competition for enzymes involved in transcription such as RNA polymerases (RNAP) or sigma factors to be available for transcription from each promoter, resulting in lower transcriptional level [41]. Another, not mutually exclusive possibility is transcriptional interference (TI) between the four promoters on the TMB4 Pc+GC construct. We have experimentally shown promoter activity of TMB4 P_C and TMB4 GC constructs on both strands, indicating convergent TI is a possibility.

In usual integrons, P_C is in *intI*, which is convergent to the integron integrase promoter P_{IntI} downstream (Fig 1), resulting in TI. The TI between P_C and P_{IntI} has been shown to control the expression of integrase and the subsequent recombination of GCs. The weaker strength of P_C could result in higher expression of integrase, which increases recombination of GCs [42, 43]. This relationship of P_C and *intI* might also apply to the reverse integrons found in *T. denticola,* even though their position and direction of P_{intI}, P_C and *intI* gene are in a different orientation compared to the usual integrons (Fig 1).

Due to the lack of additive promoter activity when Pc and an ORF-less GC promoter were tested in tandem we hypothesised that there is an alternative selective advantage for having an ORF-less, promoter-containing GC in the first position on an integron GC array.

The expression level of cassette genes located further down in the array normally decreases due to the formation of a stem-loop structure on mRNA at *attC* sites, which impede the progression of the ribosome [44]. It was previously shown that the level of streptomycin resistance was reduced four times, when the *aadA2*-containing GC was located in the second position [45]. However, our data shows that the insertion of an ORF-less, promoter-containing GC in the first position did not decrease the *gusA* expression significantly (considered as a proxy for the expression of gene(s) in the second GC), i.e. comparing the data for TMB4 P_C and TMB4 P_C+GC. Therefore, we hypothesised that promoter-containing GCs could act as a genetic clutch, where the expression of the original first GC is partially disengaged from the P_C promoter and replaced by the one on the ORF-less promoter containing GC (Fig 5A). This can prevent a significant change in expression of the first GC while a new, first GC is sampled from the pool of GCs in order to adapt to an additional stress concurrent with the selective pressure requiring expression of the first GC. This system would work as a genetic clutch with the insertion of any GC containing a promoter in the same direction as P_C, so it could be the insertion of either ORF-less GCs such as TMB4 GC, or other promoter-containing GCs such as the multiple TA-containing GCs we have identified; providing another selective advantage to retaining them and explaining their varied position within the GC array.

A genetic clutch within an integron can be of benefit to bacteria when they are exposed to multiple environmental stresses such as two different antibiotics simultaneously. The first resistance gene (green ORF in Fig 5Biii) can be expressed by the P_C promoter, while the second resistance gene (blue GC), located in the third position, is expressed by P_C and the promoter GC. Therefore, allowing bacteria to survive in both the presence of both drugs.

As the other ORF-less GC MMU23 showed no promoter activity it may have other functions or carry a promoter that can be recognised in its native host but not in *E. coli*, or require other sigma factors. For the ORF-containing GC MMB3 sample, the promoter activity was found on the sense strand. This GC was predicted to carry toxin-antitoxin (TA) ORFs, including the PIN toxin and ribbon-helix-helix antitoxin domains, which were shown to contain their own promoter. Sample SSU17 and MMU23, which showed no promoter activity, can be considered as a control; illustrating that not all of GCs amplified from the oral metagenome exhibited promoter activity within our assay.

The pCC1BAC-*lacZα-gusA* plasmid, developed for the enzymatic assay, also had potential to be used for the detection of promoter activity in either direction from GCs. The clones with promoters on the sense strand can be detected under UV light and showed blue fluorescence because β-glucuronidase can cleave the substrate, MUG, on the plate, which produces a fluorescence compound called methylumbelliferone. For the clones carrying promoters on the antisense strand, they can be detected by blue-white screening as β-galactosidase can cleave X-gal, producing an intensively blue product called 5,5’-dibromo-4,4’-dichloro-indigo, which can be viewed by eye under normal light.

To verify the application of pCC1BAC-*lacZα-gusA* plasmids as promoter detection system, integron GCs were amplified from the human saliva metagenome by using SUPA3-SUPA4 and MARS2-MARS5 primers, which amplify integron GCs from the oral metagenome [28]. After cloning the amplified GCs between both reporter genes, two groups of GCs were identified with promoter activities: ORF-less GCs and TA-containing GCs. By detecting 7 clones containing ORF-less GCs with promoter activity it further supported that one of the functions of ORF-less GCs in integrons is to provide promoter activities.

TA-containing GCs are abundant in chromosomal integrons (CIs), which were suggested to have a role in preventing random deletion of GCs and stabilising the large arrays CIs [22, 46, 47]. TA systems normally encode a stable toxin and a labile antitoxin [48], therefore TA cassettes have to carry their own promoters to ensure their expression. These were found in CIs of *Treponema* spp., such as the HicA-HicB TA-containing GC in the fourth position within the GC array (Accession number NC_002967) in the CI from *T. denticola* [30]. As most of the GCs amplified with our primers were homologous with *Treponema* spp., these TA-containing GCs should be present in our oral metagenome and were detected by our pBiDiPD based on their promoter activities.

Two of the GCs, SSU-Pro-9 and MMU-Pro-18, were similar to the MMB3 and MMB37 GCs, respectively, which were shown by the β-glucuronidase enzyme assay to have promoter activity on the sense strand. The phenotypes of SSU-Pro-9 and MMU-Pro-18 colonies also showed only a blue fluorescence phenotype, reflecting the promoter activity on the sense strand, which corresponded with the enzymatic assay results of MMB3 and MMB37.

To summarise, the promoter activities of the ORF-less integron GCs were experimentally demonstrated by using a robust β-glucuronidase enzyme assay, confirming that one of the functions of ORF-less GCs is to provide promoters for the expression of ORF containing GCs, in addition to expression from P_C. The dual reporter plasmid; pBiDiPD, was developed for the direct visualisation of clones containing gene cassettes with promoter activity on agar plates. This can be applied as a detection system for promoter activity for any other DNA fragments.

Materials and methods

in silico **analysis of the human oral cavity gene cassettes and the construction of pCC1BAC-*lacZα*-GC-*gusA* constructs.**

All of the ORF-less GCs and some of the GCs containing ORFs, identified in the previous study [28], were predicted for putative promoter sequences by using the web-based software BPROM in the Softberry package [49].

Construction of pUC19-GC-*gusA* and pCC1BAC-*lacZα*-GC-*gusA* constructs.

To determine the promoter activity of the selected GCs, the constructs were initially cloned into the EcoRI and KpnI restriction sites on pUC19-P*tet*(M)-*gusA* plasmid [50]. The selected GCs were amplified from the pGEM-T easy vector containing the GC amplicon from a previous study [28], as shown in Supplementary Fig 1, by using primer listed in Supplementary Table 2.

Due to a significant difference in the plasmid copy number in some constructs of the pUC19-GC-*gusA*, new constructs were prepared based on a low copy number CopyControl™ pCC1BAC™ vector (Epicenter, UK) as it will be maintained in *E. coli* cell as one plasmid per cell and enable us to control the plasmid copy number to be similar between each construct. The construct was designed to contain two reporter genes, β-galactosidase *lacZα* and β-glucuronidase *gusA* genes (Fig 2 and Supplementary Fig 2). As *lacZα* on pCC1BAC contained T7 promoter sequence, it was first deleted by using Q5® Site-Directed Mutagenesis Kit (New England Biolabs, UK). The backbone of pCC1BAC was amplified with pCC1BAC-del*LacZ*-F1 and pCC1BAC-del*LacZ*-R1, and the amplified products were treated with a Kinase-Ligase-DpnI (KLD) enzyme mix, following the instructions from the manufacturer. The KLD-treated product was then transformed into *E. coli* α-Select Silver Efficiency competent cells (Bioline, UK) following the instructions from the manufacturer. The pCC1BAC-del*LacZ* plasmid was then extracted from *E. coli* by using QIAprep Spin Miniprep Kit (Qiagen, UK), following the manufacturer's instructions.

The *lacZα* reporter gene was amplified from the pUC19 vector (New England Biolabs, UK) with *LacZ*-F1 and *LacZ*-R1 primers. For *gusA* reporter gene, it was amplified from pUC19-P*tet*(M)-*gusA* with *gusA-F1* and *gusA-R1* primers. A bidirectional terminator, modified from *lux* operon, was added to *LacZ*-F1 and *gusA*-R1 primers, resulting in two bi-directional terminators flanking the *lacZα-gusA* reporter genes [51]. This was done to prevent transcriptional read-through from the promoter in the plasmid backbone and to also prevent promoters from the inserts interfering with the expression of genes on the plasmid backbone. The *lacZα* and *gusA* amplicons were digested with NsiI restriction enzymes (New England Biolabs, UK) and ligated together by using T4 DNA ligase (New England Biolabs, UK). The *lacZα-gusA* ligated product was directionally cloned into the pCC1BAC-del*LacZ* plasmid by digesting them with AatII and AvrII restriction enzymes and ligated together, resulting in pCC1BAC-*lacZα-gusA* plasmid.

The selected GCs were amplified from each pUC19-GC-*gusA* constructs by using primer listed in Supplementary Table 1. The amplicons were double digested with *Nsi*I and *Nhe*I and directionally cloned into a pre-digested pCC1BAC-*lacZα-gusA* plasmid, then transformed into *E. coli* α-Select Silver Efficiency competent cells.

Determination of β-glucuronidase enzymatic activity.

The β-glucuronidase enzymatic assay was performed to measure the promoter activity based on the expression of *gusA*, following the protocol described previously with some modifications [52]. The overnight cultures of *E. coli* containing the reporter constructs were prepared in LB broth supplemented with 12.5 µg/mL chloramphenicol. The OD_{600} of each overnight culture was measured. An aliquot of 1 mL of the overnight culture was centrifuged at 3000 x *g* for 10 min and discarded the supernatant. The cell pellets were incubated at -70°C for 1 hr and resuspended in 800 µl of pH 7 Z buffer (50 mM 2-mercaptoethanol, 40 mM $NaH_2PO_4{\cdot}H_2O$, 60 mM $Na_2HPO_4{\cdot}7H_2O$, 10 mM KCl, and 1mM $MgSO_4{\cdot}7H_2O$) and 8 µl of toluene. The mixture was transferred to a 2 ml cryotube containing glass beads (150–212 µm in diameter) (Sigma, UK) and vortexed twice for 5 min each with an incubation on ice for 1 min in between. The glass beads were then removed by centrifugation at 3000 x *g* for 3 min. One-hundred microliters of cell lysate were mixed with 700 µl of Z-buffer, then incubated at 37°C for 5min. One-hundred sixty microliters of 6 mM ρ-nitrophenyl-β-D-glucuronide (PNPG) was then added to the reaction and incubated at 37°C for 5 min. The reactions were stopped by adding 400 µl of 1 M Na_2CO_3 and centrifuged at 3000 x *g* for 10 min to remove cell debris and glass beads. The absorbance of the supernatant was measured with a spectrophotometer at the wavelength of 405 nm. Three biological replicates of the β-glucuronidase enzymatic assay were performed. The β-glucuronidase Miller units were calculated from $\frac{A_{405}\times 1000}{OD_{600}\times time\ (\mathrm{min})\times 1.25\times \mathrm{volume(mL)}}$ [53].

Statistical analysis.

The average and standard deviation of β-glucuronidase concentration were calculated from three biological replicates, which were used for the columns and error bars in figure 3, respectively. The statistical comparisons between the negative control (pCC1BAC-*lacZ-gusA*) to the other constructs were performed by using ordinary one-way ANOVA with either Dunnett's post-hoc test (to compare each construct with a negative control) or Bonferroni's post-hoc test (to compare constructs between themselves). The groups with statistically significantly difference from the control had the *p*-value of less than 0.05.

Recovery of promoter-containing GCs from the human oral metagenome

The integron GCs were amplified from the human oral metagenome by using as described previously with SUPA4-NsiI-SUPA4-NheI and MARS5-NsiI-MARS2-NheI primers [28]. The human oral metagenomic DNA was previously extracted from the saliva samples collected from 11 volunteers in the Department of Microbial Diseases, UCL Eastman Dental Institute [28]. The Ethical approval for the collection and uses of saliva samples was obtained from University College London (UCL) Ethics Committee (project number 5017/001).

The amplified products were purified and digested with NsiI and NheI and ligated with the pre-digested pCC1BAC-*lacZα-gusA* plasmid. The ligated products were transformed into *E. coli* α-Select Silver Efficiency competent cells by heat shock. Cells were spread on LB agar supplemented with 12.5 µg/mL chloramphenicol, 80 µg/mL X-gal, 50µM IPTG, and 70 µg/mL 4-methylumbelliferyl-β-D-glucuronide (MUG). After incubation at 37°C for 18 hr, the colonies with β-galactosidase activity from *lacZ* was detected by blue-white screening on the agar plate, and the β-glucuronidase activity from *gusA* was visualisation under UV light. Colonies exhibiting either activity were selected and subcultured on fresh agar plates. The inserts were amplified by colony PCR using *lacZ*-F2 and *gusA-F2* primers and sequenced by sequencing service from Genewiz (Genewiz, UK).

Sequence analysis and nomenclature of promoter-containing GC amplicons.

DNA sequences were visualised and analysed by using BioEdit version 7.2.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The contigs from sequencing reactions were combined by using CAP contig function in the software. The sequences were then matched to the nucleotide and protein database by using BlastN and BlastX from the National Centre for Biotechnology Information (NCBI), respectively. The criteria for the sequence analysis of integron GC were the same as described in the previous study [28]. Two additional criteria for the verification of GCs detected with pCC1BAC-*lacZα-gusA* were included. Any clones containing incomplete GCs, caused by digestion at internal NsiI and NheI restriction sites on the GCs, were excluded from the dataset. Also chimeric inserts, which were the ligation products between digested amplicons, were also excluded.

The promoter-containing GCs were named as described in the previous study [28]. The first and second letters represented the forward primer and reverse primer used in the amplification. The third letter represents the source of the human oral metagenomic DNA which is U for the United Kingdom. This was followed by term “Pro”, indicating the presence of promoter activity, and the number of the clone. For instance, SSU-Pro-1 stands for the first clone amplified from the UK oral metagenome by using

SUPA3 and SUPA4 primers. The sequences of these GCs were deposited in the DNA database with the accession number from MH536747 to MH536769.

Conflict of interest

Nothing to Declare

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Tables

Table 1; The putative promoters for ORF-less GCs and GCs with an ORF (SSU17 and MMB3) predicted using BPROM.

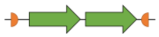
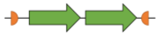
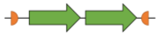
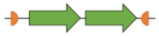
Clones	Strand	-10 box	-35 box	Score		
				-10 box	-35 box	Linear discriminant function (LDF)* (Overall score)
TMB4	+	AGGTATAAT	ATAAGA	89	-10	9.78
	-	CATTATTTT	TTGACA	41	66	7.60
SSU9	+	AATTATAAT	TAAAAA	74	0	7.04
	-	TAGTATAAT	TTTATT	80	34	7.11
MMU2	+	AATTATAAT	TTAAAA	74	37	8.36
	-	TAGTATAAT	TTTATT	80	34	8.90
MMU11	+	ATGTAAAAT	TTGCTG	75	47	11.34
	+	AACTATACT	AGGAAA	59	-7	5.99
	-	AAATAAAAT	TTTTCA	56	34	6.96
	-	CTATAAATT	TTTCAA	44	36	3.24
MMU19	+	AGGTATAAT	TAGAAA	89	23	9.07
	+	TTGAAAAAT	TTGCGG	44	32	3.43
	-	TATTATAAT	TTTCCT	79	37	9.10
MMU23	+	AATTATAAT	TAAAAG	74	-6	9.84
	+	TTTTATTAT	TTGATG	72	52	6.05
	-	TATTATAAT	TTTCCT	79	37	8.66
	-	TAGTATAAT	TTTATT	80	34	8.05
MMB2	+	AATTATAAT	TATAAG	74	-2	8.71
	+	TATTATAAT	TTGATG	79	52	7.88
	-	TATTATAAT	TTTCCT	79	37	9.10

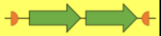
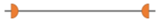
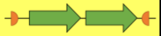
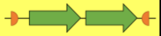
Clones	Strand	-10 box	-35 box	Score		
				-10 box	-35 box	Linear discriminant function (LDF)* (Overall score)
	-	TATTATAAT	TTTATT	79	34	8.84
MMB5	+	AATTATAAT	TTAAAA	74	37	8.36
	-	TAGTATAAT	TTTATT	80	34	7.95
MMB20	+	AATTATAAT	TAAAAG	74	-6	9.09
	-	TATTATAAT	TTTCCT	79	37	9.10
MMB32	+	TATTATAAT	TTGATG	79	52	6.28
	+	AGATATAAA	GTGTAA	39	14	4.84
	-	TATTATAAT	TTGATT	79	53	6.61
	-	TTTTATTTT	TTAAAA	52	37	5.11
MMB36	+	AATTATAAT	TTAAAA	74	37	6.94
	+	TATTATAAT	TTGATG	79	52	6.45
	-	TATTATAAT	TTTATT	79	34	7.44
	-	TTTTAAAAT	TTGACT	79	61	6.13
MMB37	+	AATTATAAT	TAAAAG	74	-6	9.11
	+	TTATATAAT	TTGATG	75	52	8.55
	-	TAGTATTAT	TTTATT	66	34	10.48
	-	TATTATAAT	TTTCCT	79	37	9.10
SSU17	+	CTTTATAAT	ATGAAT	82	25	7.80
	+	TGATAAAAT	GTGAAA	75	27	4.62
	-	TGATATAAT	TTTATT	82	34	9.34
	-	TGATTAGAT	TTTATG	21	33	5.10
MMB3	+	CTGTATATT	TTGATA	63	58	6.74
	+	ATTTATGAT	ATGAAA	65	30	5.18
	-	ATGTATTGT	TTGATG	44	52	6.64

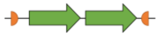
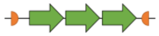
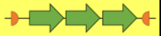
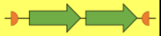
Clones	Strand	-10 box	-35 box	Score		
				-10 box	-35 box	Linear discriminant function (LDF)* (Overall score)
	-	GCATATAAT	TTCTCT	65	28	4.75

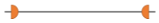
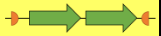
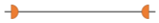
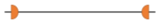
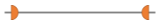
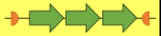
* The LDF takes into account motifs found in promoters: -10 and -35 boxes, a distance between -10 and -35 boxes, and frequencies of certain nucleotides represented in transcription start sites. It can be approximated as log(<likelihood of a site being promoter>/<likelihood of a site not being promoter>) [49].
** The selected samples for the enzymatic assay are highlighted in yellow.

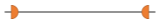
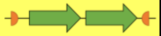
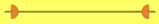
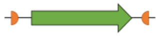
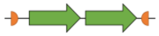
Table 2. Characterisation of the human oral integron GCs containing promoter sequences detected by pBiDiPD.

Gene cassettes	Primer pair	Cassette Size (bp)	Orientation*	BlastN		BlastX				Promoter activity		Accesion number
				Closest homologue	Percentage identity (%)/ Coverage (%)	Closest homologue	ORF size (bp)	Percentage identity (%)/Coverage (%)	Accession number of the homologous proteins (BlastX)	Sense Strand (*gusA*)	Antisense strand (*lacZ*)	
SSU-Pro-7	SUPA3-SUPA4	1001		SSU22	98/95	Prevent-host-death protein (Phd_YefM antitoxin superfamily) [*Treponema vincentii*]	264	97/100	WP_006188308.1	Y	N	MH536747
						XRE family transcriptional regulator [*Treponema vincentii*]	441	98/100	WP_006188306.1			
SSU-Pro-9	SUPA3-SUPA4	834		MMB3	98/99	Hypothetical protein (antitoxin, ribbon-helix-helix domain protein) [*Treponema putidum*]	246	67/100	WP_044978234.1	Y	N	MH536748
						Twitching motility protein PilT (PIN toxin domain) [*Treponema putidum*]	414	71/100	AIN93467.1			
SSU-Pro-13	SUPA3-SUPA4	855		MMB39	98/99	Toxin RelE [*Treponema medium*]	357	95/100	WP_016522532.1	Y	N	MH536749
						Transcriptional regulator (Antitoxin, XRE family) [*Treponema medium*]	330	95/100	WP_016522533.1			
SSU-Pro-16	SUPA3-SUPA4	925		SSU28	98/100	AbrB/MazE/SpoVT family DNA-binding domain-containing protein (Antitoxin) [*Treponema putidum*]	231	96/100	WP_044979179.1	Y	N	MH536750

Gene cassettes	Primer pair	Cassette Size (bp)	Orientation*	BlastN		BlastX				Promoter activity		Accesion number
				Closest homologue	Percentage identity (%)/ Coverage (%)	Closest homologue	ORF size (bp)	Percentage identity (%)/Coverage (%)	Accession number of the homologous proteins (BlastX)	Sense Strand (*gusA*)	Antisense strand (*lacZ*)	
						Endoribonuclease MazF (Toxin) [*Treponema denticola*]	336	99/100	WP_010694033.1			
SSU-Pro-20	SUPA3-SUPA4	1263		MMU28	77/42	Prevent-host-death protein (Phd_YefM antitoxin superfamily) [*Treponema sp.* JC4]	249	75/88.3	WP_009103386.1	Y	N	MH536751
						Plasmid stabilization protein (ParE toxin superfamily) [*Treponema sp. JC4]*	147	57/43.8	WP_009104800.1			
SSU-Pro-24	SUPA3-SUPA4	425		SSU9	99/100	-	-	-	-	Y	Y	MH536752
SSU-Pro-27	SUPA3-SUPA4	753		*Treponema putidum* strain OMZ 758	93/100	BrnT family toxin [*Treponema sp.*]	273	99/100	WP_002666393.1	Y	N	MH536753
						CopG family transcriptional regulator (BrnA antitoxin) [*Treponema denticola*]	288	99/100	WP_044909778.1			
SSU-Pro-32	SUPA3-SUPA4	972		No significant similarity found.	-	RelE/ParE family toxin [*Treponema denticola*]	354	98/100	WP_002683264.1	Y	N	MH536754
						XRE family transcriptional regulator [*Treponema denticola*]	273	100/100	WP_002683262.1			

				BlastN		BlastX				Promoter activity		
Gene cassettes	Primer pair	Cassette Size (bp)	Orientation*	Closest homologue	Percentage identity (%)/ Coverage (%)	Closest homologue	ORF size (bp)	Percentage identity (%)/Coverage (%)	Accession number of the homologous proteins (BlastX)	Sense Strand (*gusA*)	Antisense strand (*lacZ*)	Accesion number
SSU-Pro-34	SUPA3-SUPA4	832		SSU5	99/100	Hypothetical protein (antitoxin, ribbon-helix-helix domain protein) [*Treponema putidum*]	246	67/100	WP_044978234.1	Y	N	MH536755
						PIN domain-containing protein [*Treponema putidum*]	414	71/100	WP_044978236.1			
SSU-Pro-39	SUPA3-SUPA4	1137		MMU25	99/99	Hypothetical protein [uncultured bacterium]	462	99/100	ANC55535.1	Y	N	MH536756
						Hypothetical protein [*Treponema maltophilum*]	213	88/100	WP_016526060.1			
						PemK/MazF family toxin [*Fibrobacter sp. UWCM*]	342	80/100	WP_022932935.1			
SSU-Pro-46	SUPA3-SUPA4	971		No significant similarity found	-	Hypothetical protein [*Treponema socranskii*]	267	80/100	WP_021329686.1	Y	N	MH536757
						Hypothetical protein [*Treponema socranskii*]	228	84/100	WP_021329641.1			
						DUF4160 domain-containing protein [*Treponema sp.* C6A8]	276	67/100	WP_027729334.1			
SSU-Pro-65	SUPA3-SUPA4	811		*Treponema sp.* OMZ 838	91/21	AbrB/MazE/SpoVT family DNA-binding domain-containing protein (Antitoxin) [*Treponema denticola*]	228	93/100	WP_010693782.1	Y	N	MH536758

Gene cassettes	Primer pair	Cassette Size (bp)	Orientation*	BlastN		BlastX				Promoter activity		Accesion number
				Closest homologue	Percentage identity (%)/ Coverage (%)	Closest homologue	ORF size (bp)	Percentage identity (%)/Coverage (%)	Accession number of the homologous proteins (BlastX)	Sense Strand (*gusA*)	Antisense strand (*lacZ*)	
						VapC family toxin [*Treponema denticola*]	402	93/100	WP_010693784.1			
MMU-Pro-4	MARS5-MARS2	520		MMU2	99/100	-	-	-	-	Y	Y	MH536759
MMU-Pro-5	MARS5-MARS2	983		*Treponema putidum* strain OMZ 758	94/78	Prevent-host-death protein (Phd_YefM antitoxin superfamily) [*Treponema denticola*]	240	98/98.8	WP_002669519.1	Y	Y	MH536760
						RelE/StbE family addiction module toxin [*Treponema denticola*]	318	94/100	WP_002688980.1			
MMU-Pro-6	MARS5-MARS2	737		MMB36	86/100	-	-	-	-	N	Y	MH536761
MMU-Pro-18	MARS5-MARS2	634		MMB37	95/100	-	-	-	-	Y	N	MH536762
MMU-Pro-22	MARS5-MARS2	431		MMU19	91/100	-	-	-	-	Y	Y	MH536763
MMU-Pro-24	MARS5-MARS2	904		No significant similarity found	-	Universal stress protein [*Marinobacter sp.*]	348	30/54.1	WP_008177208.1	Y	Y	MH536764
						Hypothetical protein [*Methylobacter tundripaludum*]	213	79/100	WP_031438379.1			
						Prevent-host-death protein [*Treponema pedis*]	84	76/27.8	WP_024469914.1			

Gene cassettes	Primer pair	Cassette Size (bp)	Orientation*	BlastN		BlastX				Promoter activity		Accesion number
				Closest homologue	Percentage identity (%)/ Coverage (%)	Closest homologue	ORF size (bp)	Percentage identity (%)/Coverage (%)	Accession number of the homologous proteins (BlastX)	Sense Strand (*gusA*)	Antisens e strand (*lacZ*)	
MMU-Pro-31	MARS5-MARS2	574		MMB5	88/70	-	-	-	-	Y	N	MH536765
MMU-Pro-48	MARS5-MARS2	817		*Treponema sp.* OMZ 838	91/25	AbrB/MazE/SpoVT family DNA-binding domain-containing protein [*Treponema denticola*]	228	93/100	WP_010693782.1	Y	N	MH536766
						VapC family toxin [*Treponema denticola*]	402	93/100	WP_010693784.1			
MMU-Pro-53	MARS5-MARS2	430		No significant similarity found	-	-	-	-	-	Y	Y	MH536767
MMU-Pro-63	MARS5-MARS2	927		SSU8	99/99	Hypothetical protein [*Treponema denticola*]	531	98/93.7	WP_002692239.1	N	Y	MH536768
MMU-Pro-65	MARS5-MARS2	896		MMU27	99/100	Hypothetical protein [uncultured bacterium]	399	99/84.2	ANC55539.1	N	Y	MH536769
						Hypothetical protein [uncultured bacterium]	357	99/100	ANC55540.1			

[*] The orange half circles and green arrow boxes are representing *attC* sites and ORFs, respectively.

[**] The GCs found in this study are highlighted in yellow. Those not highlighted were also detected in Tansirichaiya et al. (2016) [28].

Figure Legends

Figure 1: A generalised structure of (A) usual integrons and (B) unusual, or reverse integrons. The green arrows indicate the primer binding sites on the unusual integron structure of *T. denticola*. The grey and blue open arrowed boxes represent integrase gene (*intI*) and the open reading frames (ORFs), respectively, pointing in the direction of transcription. The promoters, P_{intI} and P_C, were represented by black arrows. The recombination sites, *attI* and *attC*, were represented by yellow and orange circles, respectively.

Figure 2: The structure of pCC1BAC-*lacZα-gusA*_plasmid. The green, blue and orange open arrowed boxes represent *lacZα, gusA* and chloramphenicol resistance gene, respectively, pointing in the direction of transcription. The black lines indicate the position of restriction sites on the plasmid. The red circles indicate bidirectional transcriptional terminators.

Figure 3: The promoter activity from pCC1BAC-*lacZα*-GC-*gusA* constructs estimated by β-glucuronidase enzyme assays. Error bars indicate the standard errors of the means from three replicates. The asterisks (*) indicate the constructs were statistically significantly different from the negative control group (pCC1BAC-*lacZα-gusA*) with the *p*-value <0.05 by using ordinary one-way ANOVA followed by Dunnett's multiple comparison tests.

Figure 4: The detection of the integron GCs by using pBiDiPD. A.) Blue-white screening to detect for the clones with promoter activity on the antisense strand, B.) Exposing the colonies under the UV light to detect clones with promoter activity on the sense strand. The positive (+) and negative (-) colonies were the *E. coli* containing pCC1BAC-*lacZα*-TMB4-Pc-*gusA* (with experimentally proven promoter activities on either strand of DNA and pCC1BAC-*lacZα-gusA* (no promoter activity), respectively

Figure 5: The proposed genetic clutch. (A) When a promoter-containing GC inserts into the first position, it can act as a genetic clutch by disengaging the original first GC (blue arrow) from PC promoter and replaced with the one on promoter GC. When a new GC (green arrow) inserts, it can be

expressed by PC promoter, while the blue GC is expressed by promoter-containing GC and PC promoter. (B) The expression level of gene cassettes with and without a genetic clutch. The estimated levels of expression of the blue ORF in i.) the first, ii.) the second and iii.) the third position were shown in the bar chart. The solid bars represent the situation when promoter-containing GC was inserted upstream of the blue GC, while the gridded bars represent the situation when no promoter-containing GC was inserted. The asterisks indicate the experimentally verified expression level, suggested by the results in Figure 3 (TMB4 PC and TMB4 PC+GC). The expression of the blue ORF was hypothesised to be decreased when more GCs are inserted without the presence of a promoter-containing GC as a genetic clutch (gridded bars), based on the data from previous study [45].

565 **Figure 1**

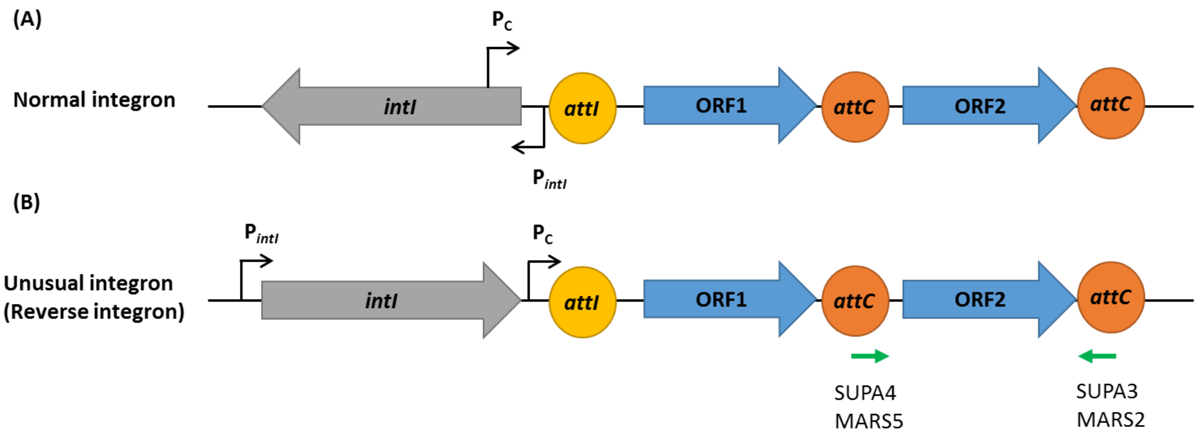


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Figure 2.

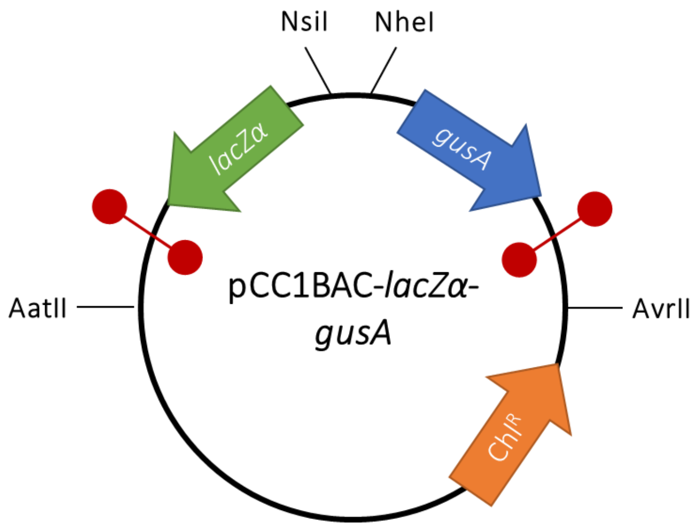


Figure 3.

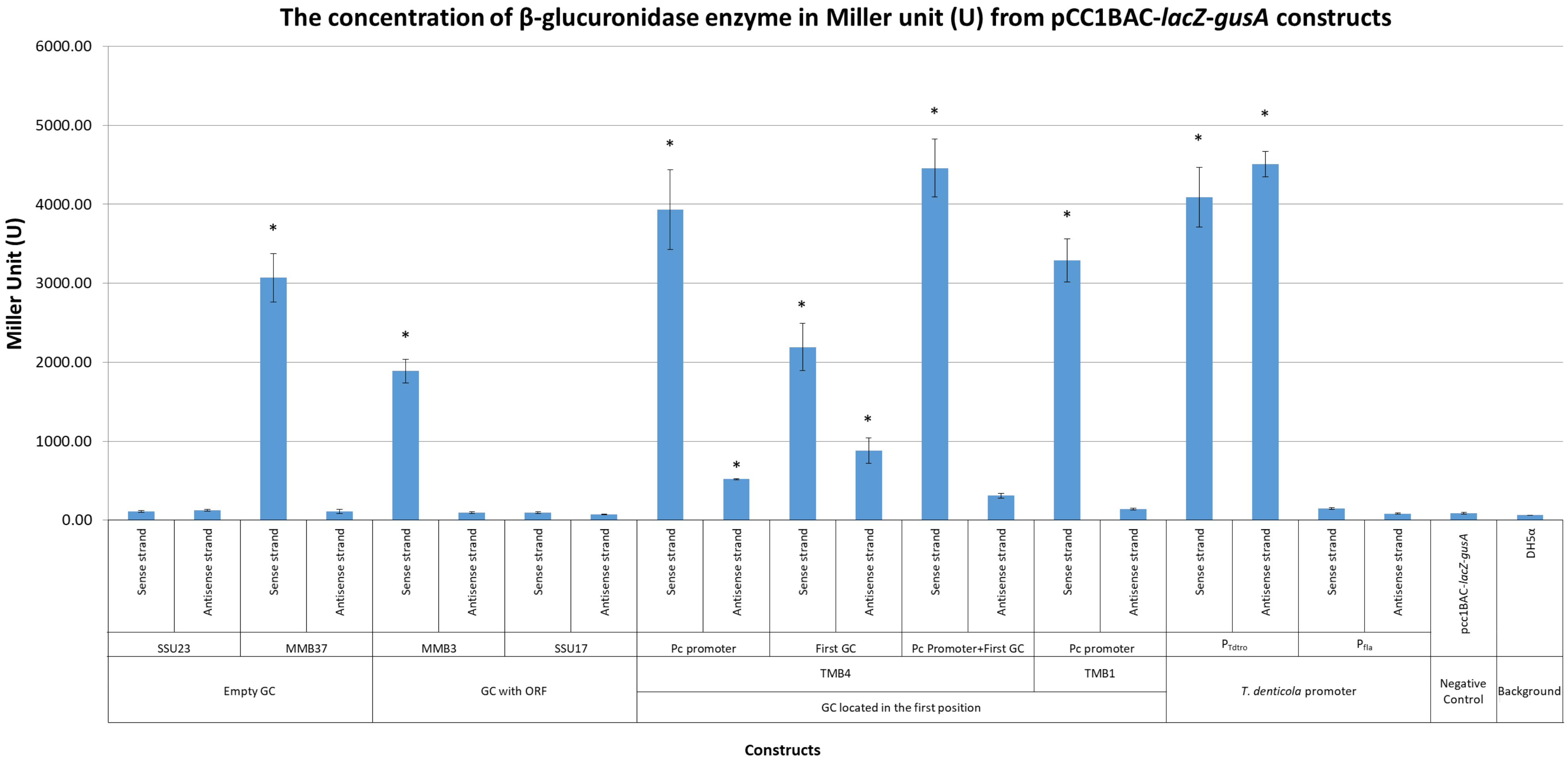

The concentration of β-glucuronidase enzyme in Miller unit (U) from pCC1BAC-*lacZ-gusA* constructs
Miller Unit (U)
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
*
Sense strand
Antisense strand
SSU23
MMB37
MMB3
SSU17
Pc promoter
First GC
Pc Promoter+First GC
Pc promoter
P_{Tdtro}
P_{fla}
pcc1BAC-*lacZ-gusA*
DH5α
Empty GC
GC with ORF
TMB4
TMB1
GC located in the first position
T. denticola promoter
Negative Control
Background
Constructs

Figure 4.

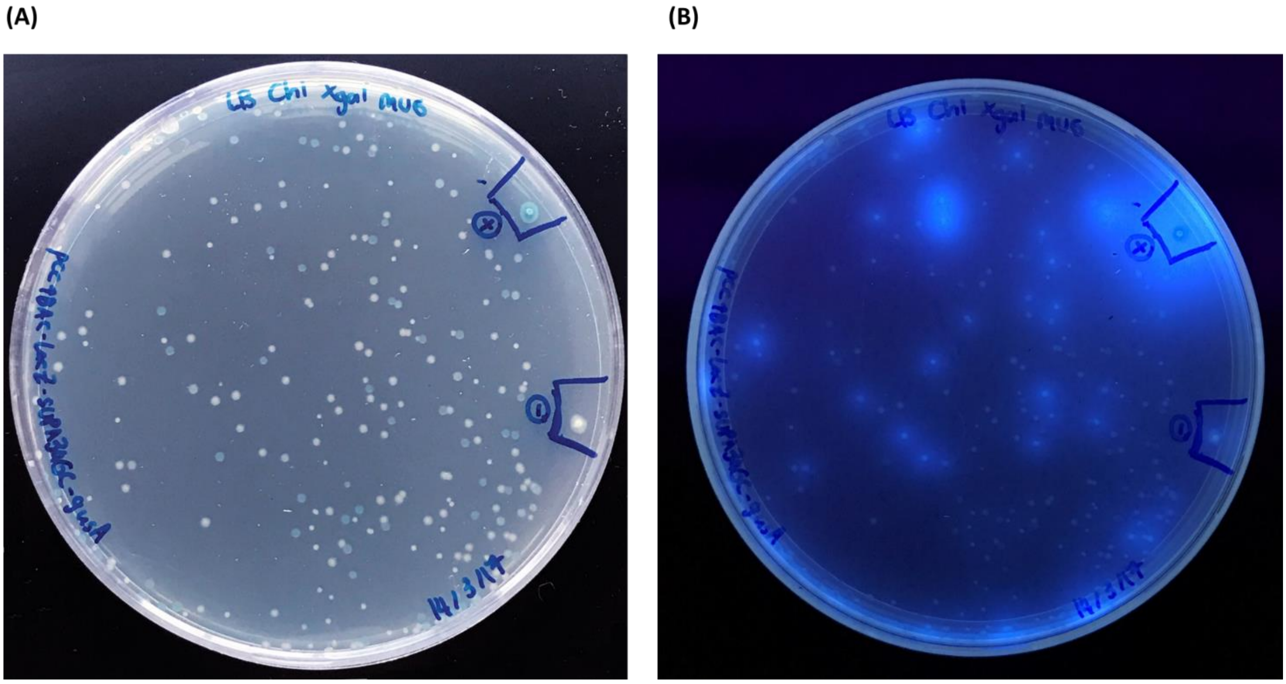
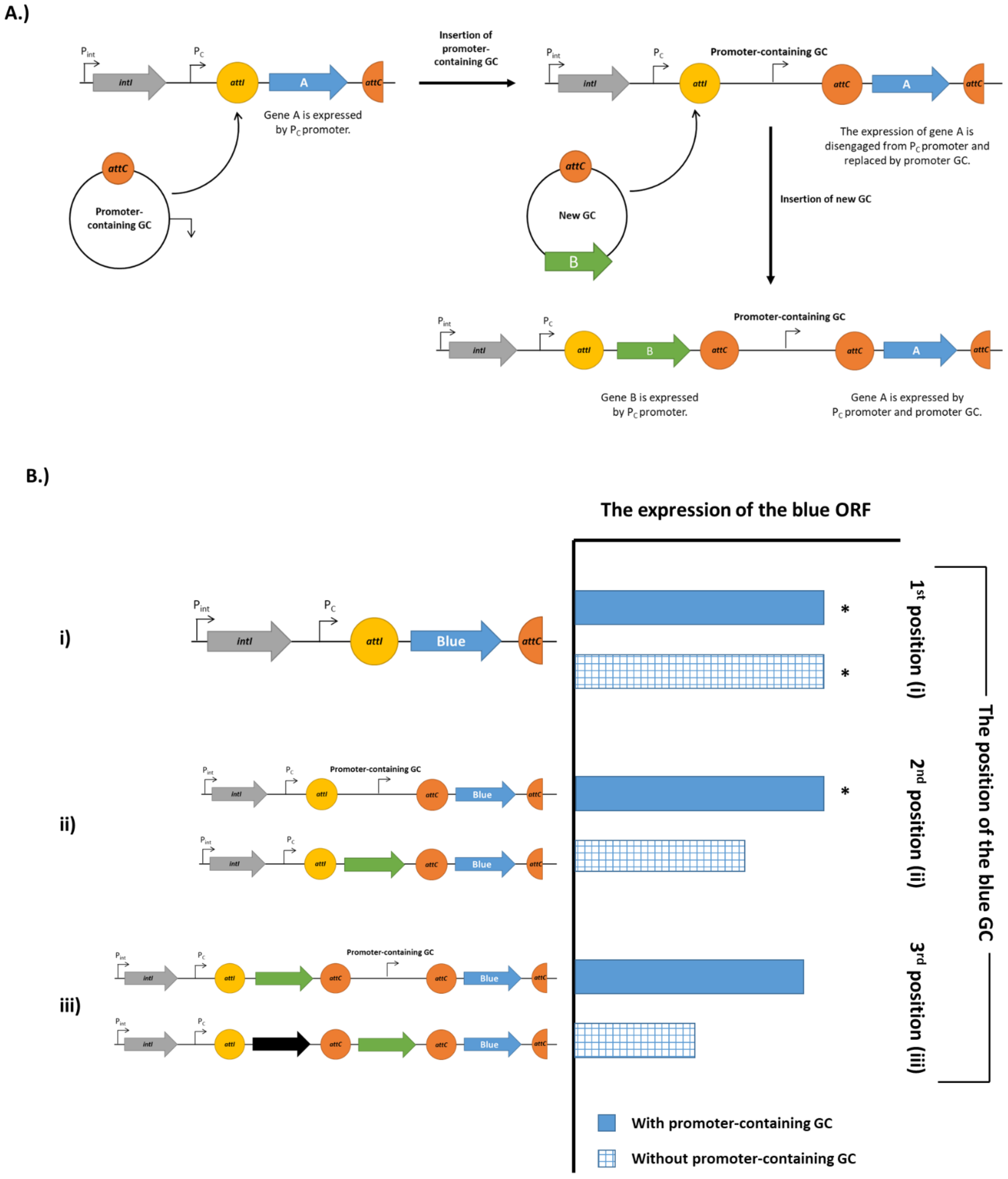


Figure 5.


A.)
P_int
P_C
intI
attI
A
attC
Gene A is expressed by P_C promoter.
attC
Promoter-containing GC
Insertion of promoter-containing GC
Promoter-containing GC
The expression of gene A is disengaged from P_C promoter and replaced by promoter GC.
attC
New GC
B
Insertion of new GC
Promoter-containing GC
Gene B is expressed by P_C promoter.
Gene A is expressed by P_C promoter and promoter GC.
B.)
The expression of the blue ORF
i)
Blue
ii)
iii)
*
*
*
1st position (i)
2nd position (ii)
3rd position (iii)
The position of the blue GC
With promoter-containing GC
Without promoter-containing GC