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Functional Characterization of a Cassette-Specific Promoter in the Class 1 Integron-Associated *qnrVC1* Gene

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Integrons are natural expression vectors due to the presence of an intrinsic promoter (P_c). Although rare, gene cassettes can harbor their own promoter. This study determined the functionality of an internal promoter in the *qnrVC1* cassette whose presence was suggested by a level of transcription similar to that of the preceding cassette (*aadA2*) and confirmed by *in silico* analysis. Its functionality was determined by 5′ rapid amplification of cDNA ends (RACE) and cloning into promoter-probe vectors. P_{qnrVC} was found in the *qnrVC* cassette family, stressing its role in contributing to resistance manifestation.

The integron is a genetic element which produces an integrase capable of inserting, excising, and rearranging gene cassettes by site-specific recombination. This element is also considered a natural expression system due to the presence of a promoter (P_c) that controls the transcription of gene cassettes inserted in the array (12). Although gene cassettes are generally promoterless functional units, internal cassette-specific promoters have been identified by *in silico* analyses and by the resistance phenotype changes of transformed *Escherichia coli* (1, 2, 9, 11, 13). In general, the expression level of cassette-associated genes is influenced by their relative position in the array (cassette position effect), where genes closer to P_c are more expressed than distal ones (3), and by the presence of cassette-specific promoters.

The *qnrVC1* cassette was previously found in a class 1 integron, preceded by the *aad*A2 cassette, from a clinical Brazilian *Vibrio cholerae* strain (4). Different from other gene cassettes, which have a specific *attC* recombination site, *qnrVC1* was associated with an *attC* typically found in *Vibrio* chromosomal integrons (CIs). More precisely, its *attC* corresponded to a *Vibrio parahaemolyticus* repeat (VPR), which is one of the signatures of the *V. parahaemolyticus* CI. Moreover, although *qnrVC1* has been found in a class 1 integron, it is more similar to the chromosomal *qnr* genes from *Vibrionaceae* than to the plasmid-borne determinants, such as *qnrA1*.

This work fully characterized and determined the functionality of an internal cassette promoter distributed among the *qnrVC* alleles that was present in several genetic elements, ensuring their expression and the emergence of the resistance phenotype.

The transcription of the *aadA2-qnrVC1* array was assessed by real-time reverse transcription (RT)-PCR using Power SYBR green PCR master mix (Applied Biosystems). The transcription of gene cassettes was measured in both the wild *V. cholerae* strain (VC627) and in an *E. coli* DH5α strain transformed with the pGEM-T Easy vector (Promega) harboring the *aadA2-qnrVC1* insert. The single-copy housekeeping *rpo*A gene from *V. cholerae* and the β-lactamase (*bla*) gene, present in the pGEM vector as a selective marker, were used for normalizing the transcription of wild-type and cloned genes, respectively (Table 1). The use of the *bla* gene eliminates any interference of plasmid copy number on relative quantification of cloned genes. The criteria to interpret the results are described elsewhere (5). Similar relative quantification values were obtained for the *aadA2* (2.68 ± 0.04 for the wild-type strain and 2.77 ± 0.09 for recombinant strains) and *qnrVC1* (2.51 ± 0.1 for the wild-type strain and 2.60 ± 0.07 for recombinant strains) genes, indicating similar transcript amounts. These results may be explained by (i) the occurrence of a read-through of the RNA polymerase along this array producing a full-length transcript (3) or (ii) the presence of an internal specific cassette promoter controlling *qnrVC1* transcription.

Analyses *in silico* were performed to verify the presence of internal cassette-specific promoters of *aadA2* and *qnrVC1* using four promoter prediction programs: Neural Network for Promoter Prediction (NNPP) version 2.2 (Berkeley Drosophila Genome Project, http://www.fruitfly.org/index.html), BPROM (SoftBerry, http://linux1.softberry.com/berry.phtml), prokaryotic promoter analysis using SAK (6), and Prokaryotic Promoter Prediction (PPP) (http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php). No reliable promoter was found for *aadA2*; however, a putative internal promoter for *qnrVC1* was determined with high scores by all programs. This promoter (−35 TTGAGA |16 bp| −10 TAG TCT) (Fig. 1), named P_{qnrVC} here, presented high similarity with *E. coli* σ^{70}-dependent promoters (−35 TTGACA |16 to 18 bp| −10 TATAAT) once it preserved 8 (underlined above) of the 12 canonical nucleotides of the −35 and −10 hexamers (10). Most gene cassettes feature a short 5′ untranslated region (UTR); for example, this region in the *aadA2* gene cassette has only 9 bp. The *qnrVC1* cassette presented an unusual 5′ UTR of 216 bp, supporting the presence of expression signals such as a promoter and a translation initiation region (TIR). A Shine-Dalgarno sequence was identified seven nucleotides upstream from the *qnrVC1* start codon (Fig. 1).

Interestingly, P_{qnrVC} had been found in *qnrVC2*, *qnrVC3*, and *qnrVC4* (4, 7, 14). The 5′ UTRs of these alleles are very similar in sequence and length to that of *qnrVC1*. Moreover, not only *qnrVC* alleles, but all BLAST hits obtained in January 2012, showed conservation of P_{qnrVC}. These results demonstrate that P_{qnrVC} is a general property of these *qnr* determinants.

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TABLE 1 Primers used in this study

Primers	Sequence (5′–3′)	Target
Real-time RT-PCR		
TR AADA2 F	GCGATGAGCGAAATGTAGTG	*aadA2* transcription
TR AADA2 R	GGCAGGTAGGCGTTTTATTG	
TR QNRVC F	CTTCTCACATCAGGACTTGCA	*qnrVC1* transcription
TR QNRVC R	AATCGCACCCTTCCAATG	
TR RPOA F	GAACAAATCAGCACGACACA	*V. cholerae rpo*A transcription
TR RPOA R	CACAACCTGGCATTGAAGA	
TR AMP F	TTTATCCGCCTCCATCCA	*bla* transcription from the pGEM vector
TR AMP R	AGCCATACCAAACGACGAG	
Conventional PCR and sequencing		
INT1P F	AAACCTTGCGCTCGTTC	Class 1 integron variable region
INB	AAGCAGACTTGACCT	
INF	GGCATCCAAGCAGCAAG	
M13 F	CGCCAGGGTTTTCCCAGTCACGAC	Cloned insert into pGEM vector
M13 R	TCACACAGGAAACAGCTATGAC	
T7 GFP Reverse	TAATACGACTCACTATAGGG GGGTAAGCTTTCCGTATGTAGC	Cloned insert into pGLOW
5′ RACE		
GSP1—QNRVC	CACAGCCTTGTACTCTAAAC	First-strand cDNA synthesis
GSP2—QNRVC	ACACCACGGCTTAAATCTGA	PCR for accessing the TSS
AUAP	GGCCACGCGTCGACTAGTAC	
Promoter regions (promoterless probe-vector)		
pQL896-P_c F	GAGCTCGAATTCAAACCTTGCGCTCGTTC	P_c promoter region
pQL896-P_c R	CTGCAGAAGCTTGTTGCTGCTCCATAACATCA	
pGLOW-P_c F	AAACCTTGCGCTCGTTC	
pGLOW-P_c R	GCATACTGCAATCATCCTGTTCGGTCAAGGTTCTGGA	
pQL896-QNR F	GAGCTCGAATTCCCGGCGTTATGTGCTTTCT	Putative *qnrVC* promoter region
pQL896-QNR R	CTGCAGAAGCTTTGCAAGTCCTGATGTGAGAAG	
pGLOW-QNR F	TTGGCTAAAACGGGGTGT	
pGLOW-QNR R	GCATACTGCAATCATCCTCATGCTGTGGCTCCAAAA	

The functionality of P_{qnrVC} was experimentally assessed by cloning into promoterless vector-probe plasmids (pLQ and pGlow) to test its ability in driving expression of a resistance gene (*cat*) and a reporter gene (*gfp*). The strong P_c version from the class 1 integron was also cloned as a positive control. These promoters were amplified with modified primers (Table 1) and were cloned into the polylinker region of the pLQ896 and pLQ897 vectors (2), kindly provided by Paul H. Roy. Recombinants transformed with both pLQ896-P_c and pLQ896-P_{qnrVC} were able to grow in 25 mg/liter of chloramphenicol and presented MIC values of ≥256 mg/liter, while *E. coli* DH5α, used as a control strain, had a MIC of 8 mg/liter.

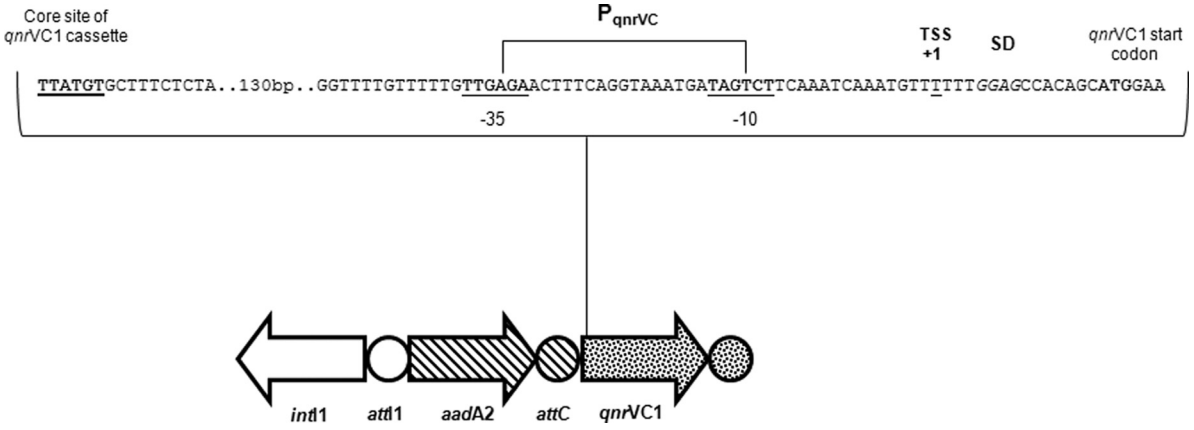

FIG 1 Schematic representation of the transcription/translation motifs found in the class 1 integron harboring the *qnrVC1* gene cassette. The recombination core site representing the beginning of the *qnrVC1* cassette is highlighted in bold and thickly underlined. The P_{qnrVC} and the first base (+1) of its corresponding TSS (underlined base) are shown. The Shine-Dalgarno (SD) sequence and the start codon of *qnrVC1* are indicated in italics and in boldface, respectively.

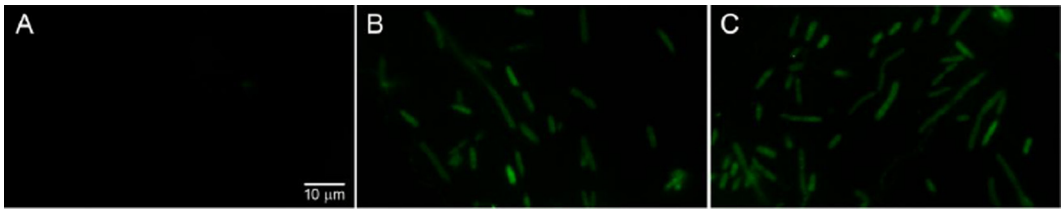

FIG 2 *In vitro* P_{qnrVC} promoter activity in the *qnrVC1* gene cassette. Panel A shows an image of *E. coli*::pGlow-TOPO TA, corresponding to the recircularized vector used as a negative control for background fluorescence. Panels B and C show the green fluorescent protein (GFP)-fluorescent *E. coli*::pGlow-P_c and *E. coli*::pGlow-P_{qnrVC}, respectively, resulting from transformation of *E. coli* TOP10 cells with the pGlow-TOPO TA containing the promoter regions fused into the ATG codon of *gfp*.

The P_{qnrVC} and P_c were amplified with primers modified as recommended (Table 1) and cloned into the pGlow-TOPO TA expression kit (Invitrogen). After fixation, ampicillin-resistant fluorescent clones harboring the constructions pGlow-P_{qnrVC} and pGlow-P_c were visualized in an Axio Imager M1 fluorescence microscope (Carl Zeiss) with a 60× objective lens by using a fluorescein isothiocyanate (FITC) filter (excitation/emission peaks of 489 nm/509 nm). Only background fluorescence was observed in the negative-control recombinant (*E. coli* transformed with recircularized pGlow vector) (Fig. 2). This *in vitro* analysis is one more demonstration of P_{qnrVC} functionality.

In order to determine whether *qnrVC1* transcription begins under the control of its putative internal promoter, its transcription start site (TSS) was assessed by the 5′ rapid amplification of cDNA ends (RACE) strategy as previously described (5). Sequence analysis revealed a TSS with the +1 position located 14 bp upstream from the initiation codon of *qnrVC1*. P_{qnrVC} was placed 14 bp upstream from this TSS (Fig. 1), an interval which is considered an acceptable distance between a promoter and its start site (8).

The lack of a promoter region is a remarkable characteristic of gene cassettes, and the identification of structures harboring such motifs is, so far, rare. The possibility of internal cassette promoters is, in fact, an extra element in the evolution of the antimicrobial resistance phenotype, minimizing the position effect, since the cassette-specific promoter guarantees the transcription of even genes that are distal relative to P_c.

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