Pc promoter from class 2 integrons and the cassette transcription pattern it evokes

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Objectives: Integrons are considered expression systems due to the presence of Pc promoters that drive gene cassette transcription. The role and configurations of Pc are well known in class 1 integrons; however, this region has not yet been identified in class 2 integrons. This study aimed to characterize the Pc promoter from class 2 integrons and to determine the effect of gene cassette position on transcription driven by this promoter.

Methods: The class 2 cassette arrays from *Klebsiella pneumoniae* and *Vibrio cholerae* strains were determined by sequencing. Transcription analyses were performed by real-time RT–PCR and relative quantification was carried out by comparing the transcripts of each normalized gene inserted in the integron to each other. The resistance profile was determined by the disc diffusion method. The class 2 Pc promoter was characterized by 5′ rapid amplification of cDNA ends and promoter prediction programs.

Results: Sequence analysis revealed the presence of the dfrA1-sat2-aadA1-ybeA and sat2-aadA1-ybeA arrangements in *K. pneumoniae* and *V. cholerae* strains, respectively. Real-time RT–PCR showed that the transcription of the first cassettes was higher than that of distal ones in wild-type and recombinant strains. All strains were resistant, indicating cassette expression. The Pc promoter of class 2 integrons (−35 TTTAAT |16 bp| −10 TAAAAT) was determined based on in silico analyses and on the transcription start site sequence of the class 2 integron cassette array.

Conclusions: The Pc from class 2 integrons was characterized for the first time and the cassette position effect on transcription was demonstrated.

Keywords: intI2, real-time RT–PCR, 5′ RACE, *Klebsiella pneumoniae*, *Vibrio cholerae*

Introduction

Integrons are assembly platforms that code for an integrase able to insert, excise and rearrange gene cassettes by site-specific recombination. The class 2 integron occurs in the non-replicative transposon Tn7 and its relatives, and the majority of the class 2 integrase genes presents an internal stop codon (TAA) (intI2*), which results in the production of an inactive integrase unable to mediate recombination.1,2 In fact, the gene cassette arrays usually found in class 2 integrons are conserved, in contrast with class 1 integrons, which corroborates the lack of a dynamic recombination due to integrase inactivation.

Integrons are considered expression systems due to the presence of a promoter region (Pc) that controls transcription of the gene cassettes.3,4 It was shown experimentally that the transcription level of class 1 integron-inserted gene cassettes was influenced by their position in the array (cassette position effect on transcription), where the cassettes closer to the Pc were more transcribed than the distal ones.4–6 The well-characterized Pc promoters from class 1 integrons are embedded in the intI1 gene and several configurations have been identified with different expression strengths.5,7 The Pc promoter from class 2 integrons, the determinant of cassette transcription in such elements, has not yet been determined. Some pieces of evidence point to its location on the attI2 recombination site and not on the 5′ region of the integrase gene, as in class 1 integrons.1,5,8

So far, there are no data concerning the configuration and activity of the Pc promoter from class 2 integrons and the dynamics of the transcription of resistance gene cassettes inserted in these structures. Therefore, the objective of this study was to characterize the Pc promoter that controls cassette expression in class 2 integrons with regard to its functionality and transcription factor recognition. Also, we aimed to determine the effect of the gene cassette position on transcription in class 2 integrons found in clinical *Vibrio cholerae* and *Klebsiella pneumoniae* strains.
Materials and methods

Bacterial strains and DNA techniques

V. cholerae strains from the Brazilian Amazon region (L34, 1991) and from Ghana, Africa (VC97 and VC98, 1979), and K. pneumoniae strains from Rio de Janeiro, Brazil (KP48 and KP49, 2006) were positive for class 2 integrons and gene cassettes, and negative for class 1 and 3 elements. PCR reactions were performed using primers targeting the intI2 gene and conserved regions flanking the gene cassette array (INF2 and INB2), corresponding to the attI2 site and the tnsE gene from Tn7. Sequencing of intI2 and the gene cassette array was performed with the same primers used in the PCR. All primers are described in Table 1.

Plasmids and cloning assays

In order to determine class 2 Pc functionality and cassette transcription, a 3.1 kb fragment was cloned in both orientations into pGEM-T Easy vector, according to the manufacturer’s instructions (Promega). This fragment corresponded to the entire gene cassette array together with part of intI2*, obtained with primers INT2 F and INB2 (Table 1). The recombinant plasmid was used to transform the DH5α strain and transformants were selected on Luria–Bertani agar plates containing 100 mg/L of ampicillin. The presence of the insert was confirmed by PCR and sequencing with M13 primers (Table 1).

RNA isolation and first-strand cDNA synthesis

Total RNA from wild-type and recombinant strains was isolated using the SV Total RNA Isolation System (Promega), according to the manufacturer’s instructions. Before cDNA synthesis, RNA extracts were treated with Turbo DNA-free reagent (Ambion, Applied Biosystems), according to the supplier’s recommendations, to eliminate contaminant genomic DNA. After DNase treatment, RNA samples were quantified in a NanoDrop ND 1000 spectrophotometer and diluted to the same concentration (10 ng/μL) with sterile nuclease-free water. Total RNA extracts were submitted to cDNA synthesis with SuperScript III RNase H-reverse transcriptase (Invitrogen) and the resulting cDNA was used as a template in real-time PCR assays.

Real-time RT–PCR

The transcription of gene cassettes from class 2 integrons was evaluated by real-time RT–PCR using Power-SYBR Green PCR Master Mix (Applied Biosystems). Independent assays were performed in triplicate for each gene cassette from all samples to determine the reliability of the relative quantification (RQ). In order to correct for differences in the amount of starting RNA, the single-copy housekeeping rpoA and gapA genes from V. cholerae and K. pneumoniae, respectively, were used as reference genes for normalizing the RQ. Similarly, the gene coding for a β-lactamase (bla), present in the pGEM vector as a selective marker,
Susceptibility test

The expression of gene cassettes and Pc promoter functionality was also assessed by analysing the phenotype of wild-type strains and transformants, which was performed by the disc diffusion method (CLSI) using antibiotic substrates of the enzymes coded by gene cassettes found within class 2 integrons [trimethoprim (dfrA1), streptomycin and spectinomycin (aadA1)].

Determination of the transcription start site (TSS) of class 2 integron-associated gene cassettes by the 5’ rapid amplification of cDNA ends (5’ RACE) strategy

Since the class 2 integrons from K. pneumoniae and V. cholerae presented the same 5’ conserved segment, including intI2* and attI2, the 5’ RACE assay was performed with the integron from K. pneumoniae, which has the canonical gene cassette arrangement (dfrA1-sat2-aadA1). The TSS was determined by 5’ RACE strategy, according to manufacturer’s instructions. Briefly, 150 ng of the total DNA-free RNA was used as a template for first-strand cDNA synthesis, which was performed with a reverse primer (GSP1) for the dfrA1 gene (Table 1), the first cassette in the array from K. pneumoniae. The target site of this specific primer in mRNA was submitted to four promoter predictor programs: Neural Network for Promoter Prediction 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;11 and Prokaryotic Promoter Prediction (http://bioinformatics.biol. rug.nl/webswsoftware/ppp/ppp_start.php). Results with the highest scores were selected as candidates for putative class 2 Pc promoters.

Nucleotide sequence

The entire class 2 integron sequences from L34, VC97/VC98 and KP48/KP49 were deposited in the GenBank database with accession numbers DQ196320, GU570569 and GU570570, respectively.

Results and discussion

Sequence characterization of class 2 integrons

Sequence analysis of INF2-1NB2 amplicons revealed the presence of the canonical arrangement dfrA1-sat2-aadA1-ybeA (previously named orfX) in two unrelated K. pneumoniae strains (Figure 1), while V. cholerae strains harboured the sat2-aadA1-ybeA gene cassette array. All these cassette arrangements were embedded in Tn7, since such amplicons were obtained in part from a specific region placed in this transposon (tnsE). The ochre premature stop codon (TAA) was found at nucleotide position 600 in all intI2*.

The class 2 integron from L34 presented the boundaries of the insertion sequence IS1, composed of 24 bp imperfect inverted repeats, without the transposase gene, suggesting its previous insertion. These inverted repeats were placed immediately before attI2, 10 nucleotides upstream of intI2*. Recent works have identified a complete IS1 inserted in different class 2 integrons.8,12

In spite of the distribution of class 2 integrons in Enterobacteriaceae, this class of integrons is rare in K. pneumoniae and V. cholerae. Here, class 2 integrons in V. cholerae O1 strains (L34, VC97 and VC98) from Brazil and Ghana, and its emergence in K. pneumoniae species are reported.

Transcription analyses of gene cassettes from class 2 integrons

The gene cassette arrays were successfully cloned in the opposite orientation relative to Ppc and the cloned arrays were submitted to RQ analyses to determine the real transcription of the gene cassettes.

Quantitative analysis revealed the cassette position effect on the transcription level (Table 2). Overall, the cassettes closer to the promoter presented greater transcript levels. The exception was verified in VC98 (Table 2), where the first (sat2) and the second (aadA1) cassettes presented the same mRNA levels, probably due to the presence of extra gene copies of the second cassette. This hypothesis was confirmed by the result obtained for the VC98 respective recombinant, which carried a unique copy of aadA1, and showed a remarkable position effect related to the first cassette (Table 2).

The wild-type and recombinant strains harbouring the class 2 integrons were resistant to trimethoprim, streptomycin and spectinomycin (Table 2), indicating cassette expression.
Determination of the TSS by the 5′ RACE method

The TSS of the class 2 integron gene cassette array and, consequently, the putative class 2 Pc promoter were determined based on the sequences of the 5′ RACE fragments and conserved motifs. A 450 bp fragment, corresponding to one transcript, was obtained. Sequence analyses of three clones revealed a TSS in the 450 bp insert, whose +1 position was located 192 bp upstream from the initiation codon of dfrA1 (Figure 1). Therefore, the −35 and −10 hexamers identified immediately upstream of this TSS would be considered the promoter region. Further in silico analyses were performed to determine the promoter sequence related to the TSS identified here.

Class 2 cassette promoter prediction and characterization

The class 2 integron Pc promoter has yet to be precisely identified. Here, we determined a sequence very likely to be an active promoter (−35 TTTAAT |16 bp| −10 TAAAT), having presented the highest threshold values using four different computer-assisted promoter prediction programs. This putative Pc promoter was embedded in the attI2 site from K. pneumoniae and V. cholerae class 2 integrons (Figure 1), and no reliable promoter was identified in the integrase gene sequence. This result corroborated other studies that suggested that Pc would be placed in attI2 instead of the integrase gene,1,5,8 as encountered for Pc from class 1 integrons.

The distance between a promoter and the TSS is usually 7–10 nucleotides.10 The potential Pc promoter identified here (−35 TTTAAT |16 bp| −10 TAAAT) is five nucleotides upstream from the TSS determined by 5′ RACE (Figure 1), which supports this region as the Pc responsible for resistance gene cassette transcription.

This putative Pc from class 2 integrons resembles those σ70-dependent promoters, which have the consensus sequence −35 TTGACA |16–18 bp| −10 TATAAT. On average, σ70-dependent promoters preserve 8 of the 12 canonical

Table 2. Gene cassette transcription and resistance profile of wild-type and recombinant strains

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Gene cassette transcription</th>
<th>Antibiotic resistance phenotype</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>dfrA1</td>
<td>sat2</td>
</tr>
<tr>
<td>L34</td>
<td>—</td>
<td>4.62</td>
</tr>
<tr>
<td>VC97</td>
<td>—</td>
<td>4.32</td>
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<tr>
<td>VC98</td>
<td>—</td>
<td>4.55</td>
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<tr>
<td>DHS5x (pL34/sat2-aadA1)</td>
<td>—</td>
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<tr>
<td>DHS5x (pVC97/sat2-aadA1)</td>
<td>—</td>
<td>5.06</td>
</tr>
<tr>
<td>DHS5x (pVC98/sat2-aadA1)</td>
<td>—</td>
<td>4.78</td>
</tr>
<tr>
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</tr>
<tr>
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<td>5.00</td>
<td>2.14</td>
</tr>
<tr>
<td>DHS5x (pKP49/dfrA1-sat2-aadA1)</td>
<td>5.19</td>
<td>2.09</td>
</tr>
</tbody>
</table>

TMP, trimethoprim; STR, streptomycin; SPT, spectinomycin; S, susceptible; R, resistant.
The Pc determined in this study conserved eight nucleotides related to the consen-
sus, where three nucleotides in the −35 hexamer and one nucleotide in the −10 hexamer were different. In fact, the −35 hexamer is more heterogeneous in sequence, while the −10 element plays a more essential role in the interaction between the promoter and the RNA polymerase. 13

We performed an in silico analysis and observed that the majority of class 2 integron sequences available in GenBank containing the attI2 site presented the putative Pc promoter identified here. Only five entries had nucleotide differences in the hexamers. Four sequences presented a single point mutation in the −10 region (TAAAGT). One sequence had a probable inactive promoter due to a deletion of the last 2 bp from the −35 region, which created a point mutation (TTTAT) and reduced the spacer region from 16 to 14 bp, which is crucial for promoter activity. 5 In fact, all subject sequences were >99% identical in the attI2 site and most nucleotide differences, when present, were within the putative Pc promoter region. Such differences could probably influence the strength of the promoter, as previously noticed elsewhere for Pc promoters from class 1 integrons. 3–5

The in silico analyses also identified other putative promoters, but they were not confirmed experimentally. These results show the limitation of in silico promoter predictor programs, since a large number of false positive predictions were returned, as reported elsewhere. 10 Predictions in silico are valuable when applied together with experimental assays, such as 5’ RACE, to define a promoter region.

Ribosomal binding sites were identified in the beginning of dfrA1 (Figure 1) and adaA1 cassettes. Therefore, these regulator signals could be contributing to the translation of the downstream gene cassettes, which is reinforced by the resistance to trimethoprim (K. pneumoniae), streptomycin and spectinomycin displayed by the wild-type and recombinant strains (Table 2).

The putative class 2 Pc promoter (−35 TTTAAT [16 bp] −10 TAAAGT) was proven to be functional through determination of the TSS from the class 2 integron gene cassette array, the produc-
tion of cassette transcripts and antibiotic resistance. The determination of gene cassette transcription patterns from class 2 integrons provides insight concerning the dynamics of such structures and their contribution to the emergence of resist-
ance in bacteria of clinical importance, such as V. cholerae and K. pneumoniae.

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Transparency declarations
None to declare.

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