Differential mRNA stability of the \textit{vapAICD} operon of the facultative intracellular pathogen \textit{Rhodococcus equi}

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Abstract

The gene encoding virulence associated protein A (VapA) is clustered with three \textit{vapA} homologues (\textit{vapICD}) within the pathogenicity island of the virulence plasmid of \textit{Rhodococcus equi}. Northern blot analysis showed a \textit{vapA} transcript of 700 nucleotides (nt) suggesting that \textit{vapA} is a monocistronic transcript. However, using the more sensitive RT-PCR, it was shown that \textit{vapA} is cotranscribed with the downstream \textit{vapICD} genes forming a 2.3-kb operon. This initial transcript is subsequently processed to give rise to a 700 nt \textit{vapA} transcript with a half-life of 7.5 min. In contrast, the \textit{vapI}, \textit{vapC} and \textit{vapD} transcripts have an average half-life of 1.8 min, identical to that of the five cistronic \textit{virR} operon located upstream of the \textit{vapA} operon. It is speculated that the need for differential gene expression arises from the different localisation of the Vap proteins. VapA is tethered to the surface of the cell wall, whereas VapC and VapD are secreted, diffusible proteins. The intercistronic region between \textit{vapC} and \textit{vapD} harbours two short ORFs (OrfA, OrfB). These ORFs are translationally coupled to \textit{vapC} and \textit{vapD} in which the start codon overlaps the stop codon of the preceding gene.

Introduction

The actinomycete \textit{Rhodococcus equi} is responsible for pyogranulomatous pneumonia in foals, commonly known as rattles (Muscatello \textit{et al}., 2007). Although foals are the primary host of \textit{R. equi}, it sporadically infects other animals and immunocompromised humans (Meijer & Prescott, 2004). \textit{Rhodococcus equi} primarily gains access to its host via inhalation of contaminated dust particles followed by infection of alveolar macrophages (Meijer & Prescott, 2004). Survival and proliferation of \textit{R. equi} within macrophages is dependent on its ability to prevent maturation and acidification of the phagolysosome, eventually resulting in the necrotic death of the macrophage (Zink \textit{et al}., 1987; Hondalus & Mosser, 1994; Lührmann \textit{et al}., 2004; Fernandez-Mora \textit{et al}., 2005; Toyooka \textit{et al}., 2005).

\textit{Rhodococcus equi} harbours a virulence plasmid containing a 27.5-kb pathogenicity island (Takai \textit{et al}., 2000). A number of the pathogenicity island genes have to date only been identified in \textit{R. equi}, most notably a family of seven virulence associated protein (\textit{vap}) genes (Takai \textit{et al}., 2000; Polidori & Haas, 2006). In addition, the pathogenicity island contains two pseudo-\textit{vap} genes (Takai \textit{et al}., 2000; Russell \textit{et al}., 2004). Foals infected with \textit{R. equi} invariably have high antibody titres against VapA: a small cell surface associated lipoprotein that is essential, but not sufficient, for virulence and proliferation in macrophages (Takai \textit{et al}., 1991; Giguère \textit{et al}., 1999; Jain \textit{et al}., 2003).
The regulation of VapA expression is dependent on a number of environmental parameters, including temperature, pH and oxidative stress (Takai et al., 1992, 1996; Benoit et al., 2002; Ren & Prescott, 2003). The vapA gene is transcribed from a single promoter, 226-bp upstream of the vapA initiation codon (Russell et al., 2004). The activity of the vapA promoter is dependent on the LysR type transcriptional regulator VirR, which is encoded within a five cistronic operon located upstream of vapA (Russell et al., 2004; Byrne et al., 2007). In addition to VirR, this operon encodes a response regulator that is required for virulence and likely controls transcription of vapA (Ren & Prescott, 2004; Russell et al., 2004).

Three vapA homologues (vapI, vapC, vapD) are located downstream of vapA. (Takai et al., 2000; Byrne et al., 2001). The function of these Vap proteins remains elusive. However, all vapA positive R. equi strains tested to date are also positive for vapC and vapD, suggesting that these play a role in virulence (Byrne et al., 2001). Recent data appear to show that vapA is transcribed as a monocistronic mRNA, despite the fact that it is clustered with three homologues that are transcribed in the same direction (Benoit et al., 2002; Russell et al., 2004). Considering the pivotal role VapA plays in infection, the aim of the current study was to analyse the transcriptional organisation of the vapA cluster in greater detail. The results show that vapA is cotranscribed together with the downstream vapICD genes. However, the vapA transcript is significantly more stable than the downstream vapICD transcript, resulting in accumulation of vapA mRNA.

**Materials and methods**

**Bacterial strains and growth conditions**

*Rhodococcus equi* ATCC33701 and its virulence plasmid cured derivative were grown in Luria–Bertani (LB) broth (Sambrook & Russell, 2001). Growth of *R. equi* at 37 °C and pH 6.5 (inducing conditions) was used to induce virulence plasmid gene expression, whereas these genes were transcribed at low levels following growth at 30 °C and pH 8.0 (noninducing conditions). For solid media, agar was added to 1.5% (w/v).

**DNA manipulations**

Chromosomal DNA was isolated as described (Nagy et al., 1995). Plasmid DNA was isolated via the alkaline lysis method of Birnböim and Doly (Birnböim & Doly, 1979) or using the Wizard Plus SV miniprep as described by the manufacturer (Promega). DNA fragments were isolated from agarose gels using the Genelute DNA purification kit as described by the manufacturer (Sigma-Aldrich). PCR was carried out using Taq DNA polymerase (Promega) as described by the manufacturer. Other DNA manipulations were done in accordance with standard protocols (Sambrook & Russell, 2001).

**RNA isolation and reverse transcriptase real-time PCR**

RNA was isolated from *R. equi* as described previously (Russell et al., 2004). Reverse transcriptase reactions using random primers (Promega) were performed with 1 U ImProm II Reverse Transcriptase following the manufacturer’s recommendations with 100 ng of total RNA as template in a final volume of 20 μL. The product was subsequently used in a normal PCR reaction, using 2 μL of the reaction mixture as a template for PCR amplification with Taq DNA polymerase (Promega) as described by the manufacturer. For quantitative PCR, the product was amplified using the Quantitect SYBR green real-time kit following manufacturer’s instructions (Qiagen). Reaction mixtures were subjected to 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s in a LightCycler (Roche) with temperature transition rates of 20 °C s⁻¹. Melting curve analysis was performed at 50–95 °C (temperature transition, 0.2 °C s⁻¹) with stepwise fluorescence detection following amplification. Cycle threshold Ct values were obtained and used to calculate the number of RNA copies µg⁻¹ of total RNA using a standard curve of known amounts of DNA target with r² coefficients larger than 0.997 in the range of 5 × 10⁻¹⁵–5 × 10⁸ molecules per reaction. *gyrB* mRNA was used as a housekeeping gene to compare the amount of RNA in each reaction. The data reported in this paper represent the results of three independent experiments in which each sample was analyzed in duplicate. The sequences of oligonucleotides used for real-time PCR are listed in Table 1.

**mRNA half-life determination**

RNA was isolated from *R. equi* in the mid-logarithmic phase of growth (OD₆₀₀nm = 0.5) at selected intervals following inhibition of transcription by the addition of 200 μg mL⁻¹ rifampicin (Sigma). The number of mRNA copies was determined using reverse transcriptase real-time PCR, followed by linear regression to determine the half-life. The 16S rRNA gene was used for internal normalization in this study.

**Northern hybridization**

Following electrophoresis in a denaturing formaldehyde gel (Sambrook & Russell, 2001), RNA was transferred to a positively charged membrane according to the manufacturer’s instructions (Roche). The vapA probe used in Northern analysis was synthesized using the oligonucleotides Vap1 and Vap2 (Table 1) and Taq DNA polymerase (Promega) in the presence of 0.2 mM of dATP, dCTP and dGTP; 0.13 mM
**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence (5’–3’)</th>
<th>Purpose</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA F200</td>
<td>ACCAAGCGAGAGTGACGTTA</td>
<td>Quantification of 16S rRNA gene</td>
<td>Miranda-Casolengoe et al. (2005)</td>
</tr>
<tr>
<td>16S rRNA R200</td>
<td>ACTTCAAGTCTGGCCGATTAC</td>
<td>Quantification of 16S rRNA gene</td>
<td>Miranda-Casolengoe et al. (2005)</td>
</tr>
<tr>
<td>012F</td>
<td>CAGTACGGCAAGTCCCGAGGA</td>
<td>Quantification of vapA</td>
<td>This study</td>
</tr>
<tr>
<td>012R</td>
<td>CACCGGCGTTGTACTGGAAC</td>
<td>Quantification of vapA</td>
<td>This study</td>
</tr>
<tr>
<td>013F</td>
<td>GTAGCTCTCCCTGCGGGAAT</td>
<td>Quantification of vapA</td>
<td>This study</td>
</tr>
<tr>
<td>013R</td>
<td>GCTCGTTGACAGCAGTGGAAT</td>
<td>Quantification of vapI</td>
<td>This study</td>
</tr>
<tr>
<td>014F</td>
<td>GTGCGAGTGTCCTATGCTT</td>
<td>Quantification of vapD</td>
<td>This study</td>
</tr>
<tr>
<td>014R</td>
<td>CAGTACGACGTTCACGGAGA</td>
<td>Quantification of vapC</td>
<td>This study</td>
</tr>
<tr>
<td>015NF</td>
<td>CTTTTGGCTGTACGGTACCTT</td>
<td>Quantification of vapD</td>
<td>This study</td>
</tr>
<tr>
<td>015NR</td>
<td>TCGACGTGATCAATGAAATAT</td>
<td>Quantification of vapD</td>
<td>This study</td>
</tr>
<tr>
<td>Vap1</td>
<td>GCACGTTGACAGCAGTGGAAT</td>
<td>vapA probe</td>
<td>Russell et al. (2004)</td>
</tr>
<tr>
<td>Vap2</td>
<td>GGCGCTGGAGGCGTTGTGTCAGTACCGT</td>
<td>vapA probe</td>
<td>Russell et al. (2004)</td>
</tr>
</tbody>
</table>

**Results**

**VapA is coordinately transcribed with vapICD**

The virulence plasmid (accession number AP001204) harbours four homologous vap genes (vapICD) located between 12546 and 15329 nucleotides (nt) (Takai et al., 2000). Growth at low pH and high growth temperature increases the activity of the vapA promoter in a VirR dependent manner (Russell et al., 2004). To determine the effect of temperature and pH on the transcription of the genes downstream of vapA, R. equi was grown under inducing (pH 5.5, 37°C) and noninducing (pH 8.0, 30°C) conditions; mRNA levels for each gene in the vapA cluster was subsequently determined by reverse transcriptase real-time PCR. A fivefold increase of transcript levels was observed for vapA, vapI and vapC, whereas vapD transcription was upregulated threefold when comparing noninducing to inducing growth conditions. (Fig. 1).

**vapA is cotranscribed with vapICD**

The vapA gene is clustered with three homologous genes located that display the same regulatory pattern (Fig. 2a). It is therefore likely that this vapICD gene cluster is transcribed as an operon. Initially a Northern blot was carried out using the vapA gene as a homologous probe. The results show that vapA transcription gave rise to a single 700-nt transcript (Fig. 2b). This is sufficiently large to accommodate vapA but not the downstream homologues, indicating that vapA is transcribed as a monocistronic transcript as was concluded previously (Benoit et al. 2002; Russell et al., 2004). However, this does not rule out that vapICD is transcribed as single message which is subsequently processed. Therefore, RT-PCR was employed, which has a lower detection threshold than Northern blotting. Following reverse transcription of mRNA, the resulting cDNA was subsequently amplified by PCR using primers located within the 5’-end of vapA and the 3’-end of vapD. This yielded a 2269-bp product; an amplicon was not observed when the reverse transcription step was omitted. The data thus showed that vapA is cotranscribed with the downstream vapICD genes (Fig. 2c).

**dTTT and 0.07 mM digoxigenin 11-dUTP. The reaction mixture was incubated at 94°C for 2 min and was subsequently subjected to 30 cycles of 94°C for 30 s, 50°C for 45 s, 74°C for 1 min, followed by an incubation at 74°C for 7 min. Prehybridization, hybridization and chemiluminescent detection of the labeled probe using Digoxigenin Easy Hyb and CDP-Star (Roche) were done according to the manufacturer’s recommendations.**

**RNA structure prediction**

RNA structures were predicted using the Vienna RNA secondary structure server (http://www.tbi.univie.ac.at/~ivo/RNA/) as described (Hofacker, 2003).

**Fig. 1.** Regulation of vapA operon gene transcription by temperature and pH. mRNA was isolated from Rhodococcus equi grown at noninducing (30°C, pH 8.0) or inducing (37°C, pH 6.5) growth conditions, followed by absolute quantification of the mRNA molecules using reverse transcription real-time PCR. Transcription levels are indicated for each gene of the vapA operon following growth at noninducing (grey bars) and inducing (black bars) conditions.
Analysis of the vapAICD operon

The intergenic regions between the vapAICD genes is relatively large (vapA-vapI, 240 bp; vapI-vapC, 371 bp; and vapC-vapD, 340 bp Fig. 2a) and were therefore analyzed to identify a possible function. vapI, originally annotated as orf13, encodes a naturally occurring C-terminal truncated Vap protein, in which the carboxy terminal part is missing as a result of a frame shift mutation and introduction of a stop codon in the original intact Vap coding sequence (Polidori & Haas, 2006). The relatively large spacing between vapI and vapC (371-bp) encodes the C-terminus of the full-length parent vapI gene, which is 92% identical to VapE. The intergenic region between the precursor gene of vapI and vapC is only 16 bp, showing that these two genes were closely linked before the advent of the frameshift mutation.

The large intergenic region in between vapC and vapD harbours two short ORFs encoding peptides of 12 (OrfA, start at 14491 nt) and 47 (OrfB, start at 14526 nt) amino acids. Interestingly, vapC, OrfA and OrfB are translationally coupled, in which the start codons of orfA and orfB overlap the stop codon of the preceding gene (Fig. 2b).

The vapA–vapI intergenic region does not contain any obvious ORFs that might be functional. However, it does contain a 14-bp inverted repeat capable of forming a stable hair-pin structure that is part of a larger stable RNA structure (Fig. 3, free energy of $-51.62 \text{ kcal mol}^{-1}$) located between 13117–13214 nt. A stretch of thymidines, characteristic for a rho-independent terminator structure, is absent, suggesting that the hairpin is not involved in transcription termination.

The vapA transcript is more stable than that of vapICD

The vapA gene is flanked by a 5′ untranslated region (UTR) of 226 bp (Russell et al., 2004) and a downstream intercistronic region that can be folded into a stable mRNA structure. Both structures may be involved in stabilizing the vapA transcript (Grunberg-Manago, 1999). To analyze the...
stability of the transcripts arising from the vapAICD cluster, the mRNA half-lives of each gene were determined following inhibition of transcription by addition of rifampicin (Table 2). The half-lives of the vap transcripts fell into three categories: vapI and vapC transcripts were the most unstable, followed by vapD and vapA. The half-lives of the latter were fivefold longer than those of the vapI and vapC transcripts. Growth temperature or pH did not affect the stability of the vapA transcript.

### Discussion

The vapA gene of *R. equi* is a major immunogenic protein, and essential for virulence of *R. equi* in foals (Meijer & Prescott, 2004). The current model of vapA transcriptional regulation is that transcription occurs from a single promoter 226 bp (reported 69 bp in *R. equi* 85F) upstream of the vapA initiation codon (Benoit et al., 2002; Russell et al., 2004), in response to temperature, pH, oxidative stress and the concentrations of iron, magnesium and calcium ions (Meijer & Prescott, 2004). In both the *R. equi* strains ATCC33701 and 85F, vapA mRNA was previously detected as a c. 700-bp transcript, suggesting that it is monocistronic (Benoit et al., 2002; Russell et al., 2004). Using a more sensitive technique, it was shown that vapA is cotranscribed with the downstream located vapA homologues vapICD, and is thus part of a 2.3-kb polycistronic operon. A 2.3-kb transcript harbouring vapA could not be observed using Northern blotting, suggesting that the vast majority of the vapAICD transcripts are processed, leading to the formation of the 700-bp vapA transcript.

The suggestion that the initial 2.3-kb transcript is processed is further supported by the differences in half-lives of the individual vap transcripts within the vapAICD operon. The vapICD transcripts display an average half-life (1.8 ± 0.5 min) that is identical to that of the five-cistronic virR operon of *R. equi* (1.8 ± 0.5 min), which also harbours the vapA homologue vapH (Byrne et al., 2007). In contrast, the half-life of the vapA transcript is fourfold longer than the average half-life of the other pathogenicity island transcripts characterized to date (present study and Byrne et al., 2007). Although not proven, it seems likely that the 226-bp 5'-UTR of vapA and its downstream vapAI intergenic RNA structure are responsible for increasing the stability of the vapA transcript relative to that of vapICD, as this has been shown for a large number of other bacterial systems (Gurberg-Manago, 1999). Interestingly, the 5'-UTR of the unstable virR transcript is only 53 bp (Byrne et al., 2007). An important reason for differential mRNA stability within a polycistronic transcript is to attain differential gene expression. For example, differential mRNA stability of the six cistronic puf operon of *Rhodobacter capsulatus* allows for different stochiometries of reaction centre complexes and lightharvesting pigments (Heck et al., 2000). In most cases of differential stability of a polycistronic transcript, functionally related but different, nonhomologous proteins are expressed at different levels. In contrast, the vapAICD operon encodes four homologous proteins. A probable explanation for the need for different expression levels of apparently four similar proteins may lie in their cellular localization. VapA is a lipid-modified, surface anchored protein (Takai et al., 1992; Tan et al., 1995), whereas VapC and VapD (but not VapA) are encountered within the culture supernatant (Byrne et al., 2001). Whatever the function of these virulence proteins, the cell appears to require more surface anchored VapA than diffusible VapC and VapD.

The vapCD intergenic region contains two small ORFs, that do not share sequence similarity with other proteins, which is true for most proteins encoded within the pathogenicity island, including the Vap proteins. Genes encoding very small proteins have a variety of functions including signaling in, for example, quorum sensing (Lazzarella et al., 1997), antimicrobial compounds such as lantibiotics (Sahl & Bierbaum, 1998) or transport of solutes. One of the smallest functional genes identified to date is kdpF encoding a peptide of 29 amino acids involved in K+ transport (Gaßel et al., 1999). Because of their small size and often poor similarities to other proteins it is hard to predict whether small ORFs are functional. Considering the translational coupling of OrfA and OrfB to vapC, it appears likely that these small ORFs identified here are functional. However, as is the case with the Vap proteins, their function remains elusive.

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### References


