The bacterial type III secretion system-associated pilin HrpA has an unusually long mRNA half-life

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Received 23 April 2004; revised 22 June 2004; accepted 29 June 2004

Available online 6 July 2004

Edited by Pascale Cossart

Abstract Secondary structures affect mRNA stability and may play a role in protein secretion. We have studied the mRNA of *hrpA*, which codes for the major structural unit of the type III secretion system-associated pilus of *Pseudomonas syringae* pv. *tomato*, *Erwinia carotovora* and *Pseudomonas syringae* pv. *phaseolicola*. We show that *hrpA* mRNA has an unusually long half-live, approximately 33–47 min. We mapped regions in the transcript that affected *hrpA* mRNA accumulation. Apparently, sequences at both 5′ and 3′ ends affect accumulation. Altering the hypothetical, stable GC rich loop structure in the 3′ end of the transcript decreased transcript levels.

Keywords: *hrpA*; Type III secretion; mRNA stability; Half-life

1. Introduction

Type III secretion systems (TTSS) have been found in plant and animal pathogens, and they deliver so-called effector proteins into the host cytoplasm [1]. Effector proteins are translocated to the host cell by bacterial appendages termed needles and pili, through which the proteins travel [2,3]. The type III pilus of plant pathogenic *Pseudomonas syringae* pv. *tomato* (*Pst*), called the Hrp pilus, is composed of HrpA pilin subunits [4,5]. HrpA pilin itself uses the type III secretion route and the pilus is assembled from the tip [2]. Mutations in *hrpA* gene render the bacterium avirulent and unable to secrete effector proteins [4]. HrpZ, residing on the same operon with HrpA, belongs to harpins, which are proteins secreted by the type III systems and found to have pore forming activities on lipid membranes [6]. Pore forming activities may lead to nutrient release from the host and/or delivery of virulence factors to the host plants.

Signals that target the effector proteins to the TTSS channel have been under a great deal of debate. There seems to be no clear consensus sequence on the amino acid level. In 1997 Anderson and Schneewind [7] brought up the idea of an mRNA signal for the type III secretion of Yop proteins of *Yersinia*. Secretion signals of YopE and YopN were mapped to the first 15 codons. Frameshift mutations that altered their peptide sequences did not prevent secretion.

In 2001, Lloyd et al. [8] proposed, that the secretion signal for YopE lay in the N-terminus of the protein, not in the mRNA. Mutations made in the first few codons that changed the mRNA sequence while leaving the amino acid sequence non-altered allowed secretion, whereas mutations leaving the mRNA sequence essentially intact but changing the amino acid sequence reduced secretion drastically. Later they noted [9] that the N-termini of Yop proteins are amphipathic and showed that a synthetic sequence of such nature could serve as a targeting signal for type III secretion.

Some type III secreted proteins need a second secretion signal for directing them into the cytoplasm of the host cells [10,11]. This signal is, at least in some cases, recognised by a chaperone [12]. Chaperones maintain their substrates unfolded or partially folded so that they can fit into the TTSS channel [13]. Another role for these chaperones might be to deliver their substrates to the secretion apparatus [14], and to confer specificity of secretion between the TTSS and the flagellar systems [15].

HrpA belongs to the class of type III proteins, which are not translocated into the cytoplasm of their host plants cells, and no HrpA-specific chaperones have been identified. The secretion signal for HrpA is in the first 15 codons [16]. Changes made to the 5′ UTR or the secretion signal of *hrpA* sequence often result in lower amounts of mRNA [16,17], which complicates the study of secretion with HrpA.

Coupling of translation with secretion of TTSS-dependent proteins has been suggested [18]. An mRNA signal could be used to guide translation near the secretion apparatus. Translation and secretion of type III dependent proteins has been shown in some cases to occur simultaneously [19,20]. On the other hand, some TTSS-dependent proteins seem to be secreted primarily from a pre-maid pool [14]. Translation of HrpA can be uncoupled from secretion, but it is not known whether pre-made proteins can be secreted by TTSS [16].

Since mRNA may be important in the secretion of TTSS-dependent proteins, we studied the mRNA of HrpA from Pst DC3000. We determined the half-life of *hrpA* mRNA. We also studied the regions affecting the stability of the transcript. In addition, we compared half-lives of transcripts coding for Hrp-pilus major proteins from other plant pathogenic species,

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.072

Erwinia carotovora spp. *carotovora* (Ecc) and *P. syringae* pv. *phaseolicola* (*Psp*). All turned out to have an unusually long half-life.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study were *Escherichia coli* DH5α, *P. syringae* pv. *tomato* DC3000 and its *hrpA*$^-$ derivative [4] and for mRNA half-life studies the original rifampicin sensitive DC3000 isolate (D. Cuppels, London, Ont.) and *E. carotovora* spp. *carotovora* SSC1 [21]. Plasmids were introduced to *Pseudomonas* by triparental mating using pRK2013 as the helper plasmid [22] or by electroporation in the case of the rifampicin sensitive strain. Hrp-inducing medium [23] or Kings B [24] was used as culture media for *Pseudomonas*, LB and Hrp-inducing medium for *Erwinia* and LB for *E. coli*. *Pseudomonas* and *Erwinia* strains were grown at 28 °C and for Hrp-induction at 28 or 18 °C, and *E. coli* strains at 18, 28 or 37 °C. HrpA production in *E. coli* was induced with 1 mM IPTG.

2.2. Plasmid constructs

PCRs were done with PFU polymerase (Promega, US) and standard methods were used for making the DNA manipulations, transformations and conjugations [22,25]. Primers 5′-TCAAAAGCTTGCAGATCTGATTTT-3′ and 5′-CTCGAATTCACAACCTCCTCAAAG-3′ were used to PCR *Psp* Race 4 *hrpA* gene. These oligonucleotides introduce *Hin*dIII and *Eco*RI restriction sites, respectively. Primers 5′-GCGTCAAGGCGGCCGCCGACCGCAAC-3′ and 5′-CGGTCGGCGGCCTTGACGCTGTTG-3′ were used to create a *Not*I restriction site replacing nucleotides 186–193 of the gene for future cloning purposes. The PCR product was cloned into pDN18 plasmid and transcription was driven from a *Psp*-derived *hrpA* promoter. *hrpA* expressed in *E. coli* was as a transcriptional fusion driven from a *lac* promoter [16].

2.3. RNA techniques

RNA was isolated from exponentially growing cells using the hot phenol method [26]. The transcripts were separated in 1.5% agarose gels containing formaldehyde, blotted on Nylon membrane and detected with DIG labelled *hrpZ*, *pilA*, or *hrpA Pst*, *Psp* or *Ecc* antisense DNA probes (membranes, labelling and detection kits were purchased from Roche, Germany). Ribosomal RNA bands were visualised with ethidium bromide to confirm that equal amounts of RNA were loaded in each lane. Transcript half-lives were determined using the original rifampicin sensitive *Pst* DC3000 strain (D. Cuppels), *Ecc*, or *E. coli* after inhibiting de novo RNA synthesis with rifampicin (final concentration 150 mg/l). The strains were grown and maintained during the rifampicin treatment at 18, 28 or 37 °C as indicated in the text. Signals were quantified with the TINA 2.0 program (Raytest).

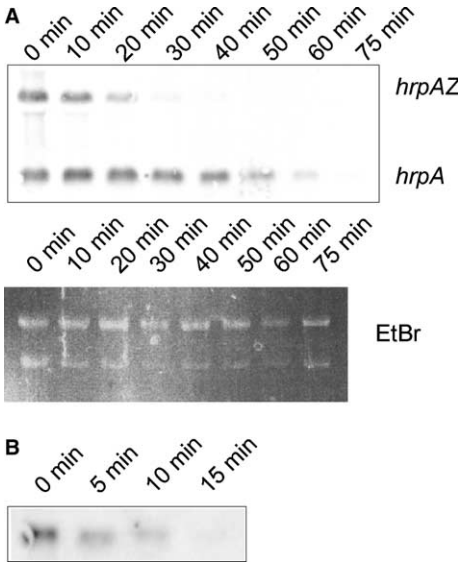

Fig. 1. Northern blot of DC3000 rifampicin sensitive strain after induction of Hrp system and cessation of RNA synthesis. Time is minutes after addition of rifampicin. (A, upper) *hrpA* probe at 28 °C, (A, lower) EtBr stain of the same gel confirming equal amounts of RNA per lane and (B) *pilA* probe at 18 °C.

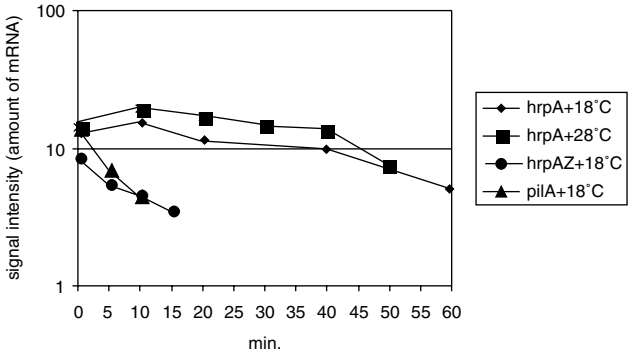

Fig. 2. Graph of the half-lives of *hrpA*, *hrpAZ* and *pilA* mRNAs. *x*-axis is time minutes after cessation of RNA synthesis, *y*-axis is band intensity quantified with TINA 2.0 program corresponding to the amount of remaining transcript. Some half-lives have been calculated in two temperatures, hence the degrees (Celsius).

3. Results and discussion

3.1. Half-lives of transcripts hrpA and hrpAZ

hrpA mRNA in *Pst* DC3000 is transcribed as two transcripts; *hrpA* alone (0.4 kb), and *hrpAZ* (1700 kb) [17]. We determined the half-lives of transcripts from genes coding for *hrpA*, *hrpAZ*, and as a control, *pilA*. *pilA* was used since it codes for a pilin for the type IV pilus [27], secreted by the type II secretion system. In the first experiment, samples were taken 0–35 min after cessation of de novo transcription. The mRNA half-life of *hrpAZ* was estimated to be 11 min (Figs. 1A and 2, and data not shown) and *pilA* half-life 6 min (Figs. 1B and 2), whereas the *hrpA* transcript signal did not appear to reduce at all. In the second experiment, samples were taken over a 75-min period. During the first 40 min, when the signal for *hrpAZ* was still visible, the *hrpA* signal remained fairly stable but after this time point the signal decreased somewhat faster (Fig. 2). The half-life of the *hrpA* transcript was estimated to be 40 min from the early time points and 20 min from the later time points (Figs. 1A and 2, and data not shown). The half-life of the *hrpA* transcript is thus discontinuous. The shorter half-life of the *hrpAZ* transcript compared with *hrpA* is presumably due to the different 3′ end. The average half-life of bacterial mRNAs is about 3–8 min [28,29].

A possible role for the long persistence of *hrpA* mRNA is that it may play a role in controlling the gene expression. Speed of degradation of transcripts may be used to control the relative amounts of proteins produced from the same operon. Indeed, HrpA is one of the major proteins produced and secreted via TTSS. Stable mRNA enables large quantities of protein to be produced compared with HrpZ. Also, HrpA protein is produced from two transcripts, *hrpA* and *hrpAZ*, which may add to the relative amount of proteins produced. In addition, *hrpA* is the first gene in its operon. In general, the 5′

ends of operons degrade more quickly than the 3′ ends due to the 5′ to 3′ directionality of degradation [30].

TTSS-dependent proteins may be translated post-transcriptionally from a pre-existing mRNA pool and the beginning of translation might be a control point for initiation of secretion. Uncoupling of transcription and translation may make mRNAs more prone to degradation by unmasking a nuclease cleavage site, which other vice would be shielded by a ribosome [31,32]. Half-lives of transcripts could thus be important in type III secretion.

3.2. Half-lives of hrpA from other bacterial species

We also determined half-lives of *hrpA* from a closely related strain *Psp* and from an unrelated species *Ecc* belonging to Enterobacteriaceae. The half-life of *Psp hrpA* was estimated to be 34 min and that of *Ecc hrpA* mRNA 47 min from the early time points, as the amount of the transcript decreased faster after 80 min similarly to *Pst hrpA* (data not shown). We used *Psp hrpA* cloned in *Pst* DC3000 since a rifampicin sensitive *Psp* strain was not available. Equivalently long half-lives of *hrpA* mRNA from different plant pathogenic species could reflect a common biological purpose for the extremely long half-lives.

3.3. The effect of temperature on the half-life of the transcript

Production of Hrp-dependent proteins is controlled by temperature as well as other abiotic factors. An elevated temperature might influence mRNA secondary structures and thus render it more vulnerable to attacks by nucleases. In order to study, the putative role of mRNA stability in temperature-dependent regulation, we measured the half-life of *hrpA* from *Pst* DC3000 at 28 °C. The half-life was long (approximately 33 min from time points 10 to 50 min) also at the higher temperature (Fig. 1A). The putative stabilising secondary structures of *hrpA* mRNA thus seem to be stable also at an elevated temperature and transcript stability of *hrpA* is not the key to temperature control of Hrp-dependent proteins.

3.4. The half-life of hrpA produced in E. coli

The half-life of *hrpA* mRNA from *Pst* expressed from the *lac*-promoter in *E. coli* was also extremely long over a temperature range 18–37 °C (data not shown). No *Pseudomonas*-specific factors thus control the long half-life and it seems to be a feature of the transcript itself.

3.5. The 3′ region of hrpA mRNA affects accumulation

Transcript stability is often dependent on mRNA secondary structures formed in the 5′ and 3′ non-translated regions. Secondary structures may protect the mRNA from degradation by nucleases [33]. mRNA cleavage usually starts at the 5′ end by an endonuclease followed by exonuclease cleavages. Net degradation occurs in the 5′–3′ direction. 3′ stem and loop structures can act as barriers to the exonucleases. Earlier studies have revealed, that the 5′ region of *hrpA* mRNA is important in the accumulation of the transcript [16,17]. In order to map other regions affecting the stability of *hrpA* mRNA, we studied the effect of 12 independent 15 bp insertion mutations (tgcggccgca + a 5-bp repeat of the insertion site sequence) [17] in the *hrpA* gene by determining the transcript levels of DC3000 *hrpA*$^-$ complemented with mutant *hrpA* in plasmid (Fig. 3A and B). Three of the insertions located in the 3′ UTR (mutant numbers 270, 250 and 350, see [17] and figure legend 3) had clearly lower mRNA levels than the wild type

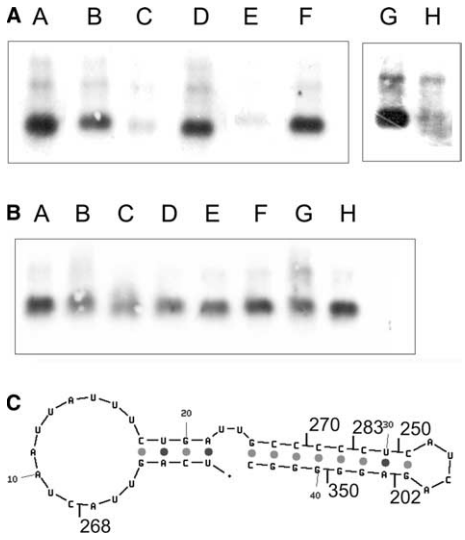

Fig. 3. (A, B) Northern blots of DC3000 *hrpA*$^-$ with insertion mutant *hrpA* plasmids hybridised with *neo* probe. Lanes A: wild type *hrpA*; lanes B–H mutant *hrpA*. Numbers represent mutant numbers published in [17], in parentheses are their locations (bp) from start codon. (A) Insertions in the 3′ region of *hrpA*. Locations of insertions from the start codon B: 283 (359), C: 250 (361), D: 202 (367), E: 350 (370), F: 321 (385), G: wild type and H: 270 (357). (B) Insertions throughout the gene *hrpA*. Locations of insertions B: 6 (44), C: 17 (110), D: 167 (170), E: 203 (228), F: 227 (290): G: 268 (338), H: 321 (385). (C) Mfold RNA secondary structure prediction using the *hrpA* transcript from transcription start point to the hypothetical termination loop. 3′ end of the transcript is shown. The larger numbers are mutant numbers.

hrpA (Fig. 3A). The *hrpA* 3′ sequence contains a GC rich inverted repeat that may form a stable stem-loop structure according to Mfold RNA secondary structure prediction [34] (Fig. 3C). The stem–loop was altered in those mutants that had reduced mRNA levels. This structure might also serve as a transcriptional termination loop. The insertion located at the last coding codon (268), also had slightly reduced amounts of mRNA (Fig. 3B). These mutants also produced lower levels of HrpA protein [17]. Insertions in other regions had no clear effect on the amount of *hrpA* mRNA produced (Fig. 3A and B).

Long half-lives are rare among bacterial mRNAs. There is a growing need in the biotechnological industry for new strategies to improve the production of heterologous proteins in biological systems. One strategy to achieve this could be to prolong the half-lives of the transcripts. The study of transcript half-lives in general, and especially that of stable transcripts, will be interesting from the point of view of the biotechnological industry. Half-lives of transcripts cannot be predicted on the basis of their sequence, such as the density of potential Rnase E cleavage sites or the folding of the 5′ and 3′ UTRs [29]. There is a tendency for longer-than-average mRNA half-lives among transcripts coding for proteins involved in cell-envelope maintenance and recycling of small molecules. The biological relevance of the long half-life of *hrpA* mRNA is not yet clear.

Acknowledgements: This work was funded by Academy of Finland (M.R. and S.T.), EU Framework IV Biotechnology programme (M.R.), Helsinki University's Research Funds (E.H.) and Viikki Graduate School in Biosciences (E.H. and A.R.). We thank Fred Fenel, Carbozyme Ltd, Espoo, Finland, for comments.

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