Research Note

The rpoN Gene of Xanthomonas campestris pv. vesicatoria Is Not Required for Pathogenicity

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We have cloned the rpoN region of the pepper and tomato pathogen Xanthomonas campestris pv. vesicatoria to analyze its role in pathogenicity and hpr gene activation. Open reading frame 3 (ORF3) from this region is predicted to encode a protein that is up to 45% identical and 65% similar to previously described RpoN proteins and was shown to be essential for the utilization of nitrate as sole nitrogen source. The X. campestris pv. vesicatoria rpoN gene is, however, not required for pathogenicity and hpr gene expression.

Additional keywords: ATP-binding protein, sigma factor σ54.

The interaction with plants of all major gram-negative plant-pathogenic bacteria except Agrobacterium is controlled by hpr (hypersensitive reaction and pathogenicity) genes. hpr genes are required for bacterial growth and development of disease symptoms in susceptible host plants, and to induce a hypersensitive reaction (HR) in resistant host or nonhost plants (for reviews see Willis et al. 1991; Bonas 1994). Expression of most hpr genes is suppressed during growth in complex media but is induced in planta and in defined, synthetic media that contain a low concentration of organic nutrients, in particular nitrogen (Bonas 1994).

Regulation of hpr gene expression has been studied most extensively in Pseudomonas syringae, where it involves three regulatory genes: hprR, hprS, and hprL. The HprR and HprS proteins are highly similar and belong to the large family of two-component response regulators (Grimmel et al. 1995). They positively regulate expression of hprL, which in turn activates all other hpr genes (Xiao et al. 1994). A preliminary report indicates that induction of hpr transcription depends on the rpoN gene (Fellay et al. 1991). rpoN (often designated ntrA) encodes the alternative sigma factor σ54, which has been identified in many prokaryotes and which is distinct from the major sigma factor, σ70. RpoN is required for transcription of a wide range of genes involved in diverse physiological functions such as nitrogen metabolism, dicarboxylic acid transport, xylene degradation, and synthesis of flagellar and pilus components (see Merrick 1993 for a recent review).

Research in our laboratory is focused on regulation and function of hpr genes of Xanthomonas campestris pv. vesicatoria, the causal agent of bacterial spot disease of pepper and tomato. In X. campestris pv. vesicatoria, six structural hpr loci, hprA to hprF, are clustered in a 23-kb region (Bonas et al. 1991). Expression was found to be induced in planta and under certain in vitro conditions (Schulte and Bonas 1992; Wengelnik et al. 1996a). In this communication, we investigated whether rpoN plays a role in X. campestris pv. vesicatoria hpr gene function.

Southern hybridization with the 1.6-kb PvuI-SmaI fragment of the Alcaligenes eutrophus rpoN gene (clone pCH148; Römermann et al. 1989) as a probe revealed that X. campestris pv. vesicatoria strain 85-10 (Bonas et al. 1989) carries a single hybridizing sequence on a 5.3-kb EcoRV-BamHI fragment (data not shown). Hybridization was performed at 65°C, followed by washing at low stringency—1× SSPE (0.15 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH 7.4), 0.1% sodium dodecyl sulfate. EcoRV-BamHI digested, size-fractionated genomic DNA of strain 85-10 was cloned into pBluescript SK (Stratagene, La Jolla, CA) with the use of standard techniques (Maniatis et al. 1982). Plasmid pB405, which contains the rpoN homologous 5.3-kb EcoRV-BamHI fragment, was identified by colony hybridization with the A. eutrophus rpoN gene used as a probe. Figure 1 shows the structural organization of the X. campestris pv. vesicatoria rpoN region, 2.7 kb of which was sequenced.

Three open reading frames (ORFs) were identified as potential coding sequences. Inspection of the sequence did not reveal similarity with consensus promoter sequences, nor were rho-independent transcription terminators identified. Comparison with the RpoN sequence of A. eutrophus showed that ORF3 corresponds to the rpoN gene (see below). The deduced polypeptides corresponding to ORF1 and ORF2 show significant similarities to genes that in several bacteria are in equivalent positions upstream of the rpoN gene (Merrick 1993). The ORF1 product, for which we did not obtain the N-terminal sequence, shares 25% identity and 50% similarity with the C-terminal 118 amino acids of a putative 185-amino

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Nucleotide sequence data have been submitted to GenBank (accession number U67179).
acid protein from Escherichia coli; the corresponding gene is in a similar position with respect to rpoN (Powell et al. 1995). ORF2, which is 717 nucleotides long, encodes a putative protein of 239 amino acids with a predicted molecular mass of 26.2 kDa and an isoelectric point of 5.57. The predicted amino acid sequence shares up to 77% similarity with products of genes that in some bacteria precede the rpoN gene and that are all homologous to a superfamily of ATP-binding proteins (Table 1; see Thony and Hennecke 1989 for a review). The translation product of ORF3, which is predicted to be RpoN, is 479 amino acids in length, and has a predicted molecular mass of 52.2 kDa and an isoelectric point of 4.95 (Fig. 2). A multiple sequence alignment to other RpoN proteins revealed conservation throughout the entire length of the polypeptide, with two stretches in the C-terminal region that are signatures characteristic for RpoN: a putative helix-turn-helix motif (Fig. 2; amino acids 366 to 385), and a decapeptide, the so-called RpoN-box (Fig. 2; amino acids 456 to 465). The X. campestris pv. vesicatoria protein is most similar (65%) to RpoN proteins of Azotobacter vinelandii (Merrick et al. 1987) and Pseudomonas putida (Kohler et al. 1994), and shares 43% identity and 62% similarity with RpoN of A. eutrophus (Warrelmann et al. 1992). An additional ORF, which is often found downstream of rpoN, appears to also be conserved in X. campestris pv. vesicatoria but is incomplete. Comparison of the first 25 amino acids of the corresponding product revealed up to 62% identity with known sequences.

![Fig. 1: The rpoN region of Xanthomonas campestris pv. vesicatoria. The DNA sequence of the rpoN homologous region (2,715 bp, starting from the EcoRV site of plasmid pB405) was determined for both strands with the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). The sequence was analyzed with the University of Wisconsin GCG 8.0 package (Devereux et al. 1984). Codon preference analysis revealed three open reading frames (ORFs 1 to 3); their location and orientation is indicated by arrows. ORF1: nucleotide position 1 to 354; ORF2: nucleotides 354 to 1073; ORF3, which is the rpoN gene: nucleotides 1124 to 2553. The insertion sites of the omega cassette (Ω) and transposon Tn3-gusA are marked by triangles. Relevant restriction sites are indicated: EcoRV (E), BamHI (B), PstI (P), Smal (S).](image)

Table 1. Amino acid sequence conservation between open reading frame 2 (ORF2) of Xanthomonas campestris pv. vesicatoria and that of other bacteria*

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Protein length (amino acids)</th>
<th>Amino acid identity/similarity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligenes eutrophus</td>
<td>280</td>
<td>64.2/77.1</td>
<td>Warrelmann et al. 1992</td>
</tr>
<tr>
<td>Azorhizobium caeni-nodosans</td>
<td>281</td>
<td>68.4/76.1</td>
<td>Stigter et al. 1993</td>
</tr>
<tr>
<td>Rhizobium meliloti</td>
<td>270</td>
<td>63.2/77.0</td>
<td>Albright et al. 1989</td>
</tr>
<tr>
<td>R. leguminosarum</td>
<td>258</td>
<td>62.0/75.7</td>
<td>GenBank accession no.</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>241</td>
<td>59.2/77.1</td>
<td>Powell et al. 1995</td>
</tr>
</tbody>
</table>

*Database searches were done with TBLAST (Altschul et al. 1990). Proteins were aligned with the GCG program GAP (Devereux et al. 1984).
Since RpoN in a number of bacteria is indispensable for growth under nitrogen-limiting conditions or for use of dicarboxylates as carbon source, growth of our rpoN mutants in liquid minimal medium with different nitrogen (ammonium or nitrate) and carbon sources was examined. As shown in Table 2, the Tn3-gus insertion in ORF2, #4206, had no effect on bacterial growth in the different media tested. In contrast, mutations in rpoN abolished bacterial growth on nitrate, which could be restored by plasmid pS42. The different phenotypes of the Tn3-gus insertion mutants in ORF2 and rpoN suggest that these genes are located in different transcription units, as is also the case for the related genes of Rhizobium mellitii (Albright et al. 1989). We then tested for the utilization of different carbon sources. An A. eutrophus rpoN mutant, which was included as a control, was unable to use succinate as carbon source. However, the X. campestris pv. vescicatoria rpoN mutants grew as the wild type in minimal medium containing glucose, fructose, sucrose, malate, citrate, or succinate as sole carbon source. This suggests that the rpoN gene of X. campestris pv. vescicatoria controls growth on nitrogen but is not involved in transport or catabolism of dicarboxylates, as is the case in rhizobia and a number of other bacteria (Merrick 1993). As some bacteria, e.g., Bradyrhizobium japonicum or Azorhizobium caulinodans, possess two rpoN genes (reviewed by Merrick 1993) one might argue that this could also be the case in X. campestris pv. vescicatoria. Southern hybridizations with the insert of rpoN clone pB405 as a probe and stringent conditions did not reveal a second, homologous copy in the genome of strain 85-10.

The finding that disruption of the X. campestris pv. vescicatoria rpoN gene did not abolish pathogenicity and hrp gene induction is markedly different from results obtained for P. syringae pv. phaseolicola. In P. syringae, RpoN is essential for hrp gene activation, probably in concert with HrpS, which contains a highly conserved domain present in σ44-enhancer-binding proteins (Fellay et al. 1991; Grimm et al. 1995). HrpS is a member of the large family of two-component activators. This is also the case for the recently identified X. campestris pv. vescicatoria HrpG protein, which appears to be at the top of the regulatory cascade (Wengelnik et al. 1996b). However, HrpG and HrpS belong to different subclasses of response regulators since HrpG does not contain the σ44-interacting domain (Wengelnik et al. 1996b). In conclusion, the rpoN independence of X. campestris pv. vescicatoria hrp gene regulation and function together with the nature of the hrp regulators suggest that regulation of hrp genes is fundamentally different in Xanthomonas and Pseudomonas.

ACKNOWLEDGMENTS

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LITERATURE CITED


Table 2. Growth of Xanthomonas campestris pv. vescicatoria open reading frame 2 (ORF2) and rpoN mutants in minimal medium with ammonium or nitrate as sole nitrogen source

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Ammonium</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. campestris pv. vescicatoria strain</td>
<td>Bacterial growth</td>
<td></td>
</tr>
<tr>
<td>85-10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-10:4206</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-10:rpoN-Ω</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>85-10:rpoN-Ω (pS42)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>85-10:rpoN-Ω (pS42Ω)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-10:rpoN-4214</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>85-10:rpoN-4214 (pS42)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* Bacteria were grown overnight at 28°C in liquid minimal medium (XMM) containing ammonium chloride or potassium nitrate. XMM is identical to minimal medium FN (Schlegel et al. 1961), except that 0.15% phosphate buffer (instead of 1.5%) was used. Growth was assessed by measurement of the turbidity at 600 nm.

*b* + = growth; - = no growth.


