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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 *hrp* 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 *hrp* 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 *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity) genes. *hrp* 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 *hrp* 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 *hrp* gene expression has been studied most extensively in *Pseudomonas syringae,* where it involves three regulatory genes: *hrpR*, *hrpS*, and *hrpL*. The HrpR and HrpS proteins are highly similar and belong to the large family of two-component response regulators (Grimm et al. 1995). They positively regulate expression of *hrpL*, which in turn activates all other *hrp* genes (Xiao et al. 1994). A preliminary report indicates that induction of *hrp* 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 *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease of pepper and tomato. In *X. campestris* pv. *vesicatoria*, six structural *hrp* loci, *hrpA* to *hrpF*, 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 hrp* gene function.

Southern hybridization with the 1.6-kb *Pvu*I-*Sma*I 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) contains a single hybridizing sequence on a 5.3-kb *Eco*RV-*Bam*HI fragment (data not shown). Hybridization was performed at 65°C, followed by washing at low stringency—1× SSPE (0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, ph 7.4), 0.1% sodium dodecyl sulfate. *Eco*RV-*Bam*HI 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 *Eco*RV-*Bam*HI 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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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 Thöny 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.

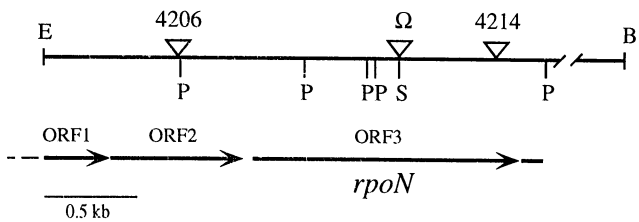

Fig. 1. The *rpoN* region of *Xanthomonas campestris* pv. *vesicatoria*. The DNA sequence of the *rpoN* homologous region (2,715 bp, starting from the *Eco*RV site of plasmid pB405) was determined for both strands with the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). The sequence was 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 1114 to 2553. The insertion sites of the omega cassette (Ω) and transposon Tn*3-gusA* are marked by triangles. Relevant restriction sites are indicated: *Eco*RV (E), *Bam*HI (B), *Pst*I (P), *Sma*I (S).

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  1  MKARLQTSLG QQLVMTPQLR QAIKLLQMST TELEVEIAEA VETNPLLEWA
 51  DEAAHAASDI AVDSSPSAST EQPQREEVAP AERDEDWSQD ELQWTGSGSG
101  GSFDDDESGD AAERVAESET LADHLLWQLH LSPLSPRDRQ IGAMLIDALD
151  EDGYLREPLS AILETLALGA AVDEADVLTV LHQIQRFDPV GVGARTLGEC
201  LALQLGVLDA DTPGRELALQ IVAGPLERLP RSGVAGLAHE LKRSTADVEQ
251  AVQLVRSLDP RPGKQIGDLS QDTYVVPDCV IWRQRGVWRA ALAGRAQPKV
301  TIHRGYENLI RSCGESDAGY LRGQLQEARW LLKSLEARGE TLLRVVRCLL
351  EHQAGFLEFG AQALRPLTLR EIAGELGLHE STISRAIARK YVRTPRGTIA
401  LRAFFASGID TDSGGEASST AIQAMIRRLI DAENPRKPLS DAKLADLLKT
451  SGVPVARRTV AKYREAMNIS ASHERVRIA*
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Fig. 2. Deduced amino acid sequence of the *rpoN* gene. Two motifs highly conserved among all RpoN proteins are underlined: the helix-turn-helix motif (position 366 to 385), and the RpoN-box (position 456 to 465).

To analyze whether the *rpoN* gene plays a role in pathogenicity and *hrp* gene regulation, the 5.3-kb *Eco*RV-*Bam*HI fragment of pB405 was subcloned into pLAFR6 (Bonas et al. 1989) to yield pS42, and mutagenized with Tn*3-gus* as described previously (Bonas et al. 1991). Two transposon insertions were further characterized. Their exact insertion site was determined by sequencing. Insertion #4206 was found to be in ORF2, and insertion #4214 in the *rpoN* gene, thus interrupting translation of the corresponding proteins after amino acids 122 and 438, respectively. An additional, site-specific, mutation in the central region of the *rpoN* gene was generated by insertion of the omega cassette (Prentki and Krisch 1984) into the unique *Sma*I site (pS42Ω). The Tn*3-gus* and Ω insertions were introduced into the *X. campestris* pv. *vesicatoria* 85-10 genome by marker exchange mutagenesis, and verified by Southern analysis as described (Bonas et al. 1991). In this context, it is notable that, unlike the case with *Rhizobium meliloti* (Albright et al. 1989), a mutant was obtained in ORF2. When the three different mutants were inoculated into pepper plants (Bonas et al. 1991) they behaved as the wild type, i.e., produced disease symptoms in the susceptible pepper line ECW and induced the HR in the resistant line ECW-10R. For the *rpoN*::Ω mutant the growth in planta was determined after inoculation of 10^4 CFU per ml and was shown to be identical to that of wild-type strain 85-10 (data not shown). These results suggest that the *X. campestris* pv. *vesicatoria* *rpoN* gene is not involved in bacterial pathogenicity. To determine whether the *rpoN* gene is involved in regulation of *hrp* genes we analyzed the activity of two *hrp* promoters in the *rpoN*::Ω mutant. Plasmid pPB400 (Fenselau and Bonas 1995), which carries a *hrpB* promoter–*gusA* fusion in pL6GUSB, was introduced into wild-type strain 85-10 and the *rpoN*::Ω mutant. β-glucuronidase (GUS) activities were determined after bacterial growth in complex medium NYG (Daniels et al. 1984) or *hrp*-inducing tomato conditioned medium (TCM) as described previously (Schulte and Bonas 1992). As expected, GUS activities in NYG were in the range of background: 0.008 and 0.016 units per 10^{10} CFU for 85-10 and the Ω mutant, respectively. In TCM, the *hrpB* promoter was induced in both strains. GUS activities were 4.6 units per 10^{10} CFU for 85-10 and 4.2 units per 10^{10} CFU for the *rpoN*::Ω mutant. Similarly, a *hrpC* promoter-*gus* fusion was induced in the mutant to the same level as in 85-10 (data not shown).

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

Bacterium	Protein length (amino acids)	Amino acid identity/similarity (%)	Reference
Alcaligenes eutrophus	280	64.2/77.1	Warrelmann et al. 1992
Azorhizobium caulinodans	281	64.8/76.1	Stigter et al. 1993
Rhizobium meliloti	270	63.2/77.0	Albright et al. 1989
R. leguminosarum	258	62.0/75.7	GenBank accession no. U23471
Escherichia coli	241	59.2/77.1	Powell et al. 1995

[a] 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 Tn*3-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 Tn*3-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 meliloti* (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. *vesicatoria 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. *vesicatoria* 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. *vesicatoria*. 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. *vesicatoria 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 σ^{54}-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. *vesicatoria* 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 σ^{54}-interacting domain (Wengelnik et al. 1996b). In conclusion, the *rpoN* independence of *X. campestris* pv. *vesicatoria 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*.

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

	Nitrogen source	
	Ammonium	Nitrate
***X. campestris* pv. *vesicatoria* strain**	**Bacterial growth**	
85-10	+[b]	+
85-10::4206	+	+
85-10::rpoN-Ω	+	−
85-10::rpoN-Ω (pS42)	+	+
85-10::rpoN-Ω (pS42Ω)	+	−
85-10::rpoN-4214	+	−
85-10::rpoN-4214 (pS42)	+	+

[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.

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LITERATURE CITED

Albright, L. M., Ronson, C. W., Nixon, B. T., and Ausubel, F. M. 1989. Identification of a gene linked to *Rhizobium meliloti ntrA* whose product is homologous to a family of ATP-binding proteins. J. Bacteriol. 171:1932-1941.

Altschul, S. F., Gish, W., Miller, W., Meyers, E., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Curr. Top. Microbiol. Immunol. 192:79-98.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Bonas, U., Stall, R. E., and Staskawicz, B. 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. 218:127-136.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W., and Fielding, A. H. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. 3:3323-3328.

Devereux, J., Haeberli, P., and Smithson, O. 1984. A comprehensive set of sequence analysis programmes for the VAX. Nucleic Acids Res. 12:387-395.

Fellay, R, Rahme, L. G., Mindrinos, M. N., Frederick, R. D., Pisi, A., and Panopoulos, N. J. 1991. Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction. Pages 45-52 in: Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. Mol. Plant-Microbe Interact. 8:845-854.

Grimm, C., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. Mol. Microbiol. 15:155-165.

Kohler, T., Alvarez, J. F., and Harayama S. 1994. Regulation of the *rpoN*, *ORF102*, and *ORF154* genes in *Pseudomonas putida*. FEMS Microbiol. Lett. 115:177-184.

Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Merrick, M. 1993. In a class of its own - the RNA polymerase sigma factor s^{54} (s^{N}). Mol. Microbiol. 10:903-909.

Merrick, M., Gibbins, J., and Toukdarian, A. 1987. The nucleotide sequence of the sigma factor gene *ntrA* (*rpoN*) of *Azotobacter vinelandii*: Analysis of conserved sequences in NtrA proteins. Mol. Gen. Genet. 210:323-330.

Powell, B. S., Court, D. L., Inada, T., Nakamura, Y., Michotey, V., Cui, X., Reizer, A., Saier, M. H., and Reizer, J. 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIANtr affects growth on organic nitrogen and the conditional lethality of an erats mutant. J. Biol. Chem. 270:4822-4839.

Prentki, P., and Krisch, H. M. 1984. *In vitro* insertion mutagenesis with a selectable DNA fragment. Gene 29:303-313.

Römermann, D., Warrelmann, J., Bender, R. A., and Friedrich, B. 1989. An *rpoN*-like gene of *Alcaligenes eutrophus* and *Pseudomonas facilis* controls expression of diverse metabolic pathways, including hydrogen oxidation. J. Bacteriol. 171:1093-1099.

Schlegel, H. G., Kaltwasser, H., and Gottschalk, G. 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38:209-222.

Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. J. Bacteriol. 174:815-823.

Stigter, J., Schneider, M., and de Bruijn, F. J. 1993. *Azorhizobium caulinodans* nitrogen fixation (*nif/fix*) gene regulation: Mutagenesis of the *nifA* -24/-12 promoter element, characterization of a *ntrA*(*rpoN*) gene, and derivation of a model. Mol. Plant Microbe Interact. 6:238-252.

Thöny, B., and Hennecke, H. 1989. The -24/-12 promoter comes of age. FEBS Microbiol. Rev. 63:341-358.

Warrelmann, J., Eitinger, M., Schwartz, E., Römermann, D., and Friedrich, B. 1992. Nucleotide sequence of the *rpoN* (*hno*) gene region of *Alcaligenes eutrophus*: Evidence for a conserved gene cluster. Arch. Microbiol. 158:107-114.

Wengelnik, K., Marie, C., Russel, M., and Bonas, U. 1996a. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. 178:1061-1069.

Wengelnik, K., Van den Ackerveken, G., and Bonas, U. 1996b. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. Mol. Plant Microbe Interact. 9:704-712.

Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. 4:132-138.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.