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Characterization and transcriptional analysis of an ECF sigma factor from Xanthomonas campestris pv. campestris

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Abstract
The genomic DNA segment encoding the rpoE gene and its flanking region was cloned from Xanthomonas campestris pv. campestris strain 11 (Xc11). The transcriptional start site of rpoE was located at nucleotide G, which is 33 nucleotides preceding the putative translation initiation codon of rpoE, and a extracytoplasmic function sigma factors (σE)-dependent promoter was identified with −35 (5′-GAACTT-3′) and −10 (5′-TCTCA-3′) consensus sequences. The protein encoded by rpoE gene acted as a sigma (σ) factor and was sufficient to direct core RNA polymerase to the rpoE promoter and to stimulate initiation of transcription in vitro. The specific binding of the reconstituted EcσE holoenzyme with the Xc11 rpoE promoter was demonstrated by gel retardation assay and DNase I footprint analysis. This study clearly demonstrated that the rpoE-rseA-mucD genomic organization of X. campestris is similar to that found in Xylella fastidiosa, however, expression of rpoE in X. campestris is autoregulated by its own σE-dependent promoter.

Introduction

Extracytoplasmic function (ECF) sigma factors (σE) belong to a subgroup of the σ70 family and most of them are involved in the environmental stress responses (Raivio & Silhavy, 2001). The stress responses controlled by σE seem to depend on their host microorganisms. For example, Salmonella typhimurium σE is required for virulence, cold shock, and stationary-phase survival (Testerman et al., 2002; McMeechan et al., 2007), whereas σE in Mycobacterium tuberculosis and Escherichia coli can be induced by sodium dodecyl sulfate (SDS), H2O2, and heat shock (Erickson & Gross, 1989; Raman et al., 2001). Pseudomonas aeruginosa AlgU is involved in alginate biosynthesis (Schurr et al., 1996). Some σE, such as Streptomyces coelicolor σE and Bacillus subtilis σV, are important for protecting cells from damage in the cell envelope (Hong et al., 2002; Butcher & Helmann, 2006).

Xanthomonas campestris is the major species of the genus and includes more than 140 pathovars (pv.), which are distinguished by the plants they infect (Vauterin et al., 2000). The pathovar campestris is the causal agent of black rot disease in many cruciferous plant species and is the primary choice for extracellular polysaccharide slime (EPS) production in industry. The EPS produced by X. campestris pv. campestris (Xcc), called xanthan gum, has a wide range of industrial applications (Sutherland, 2001) and has been reported to be an important virulence factor in plant diseases (Becker et al., 1998). The synthesis of xanthan gum and extracellular enzymes has been extensively studied in Xcc, and this bacterium has been used as a model organism for investigating the mechanism of bacterial pathogenesis (Tang et al., 1991). However, the regulatory networks that control the expression of these related genes in Xcc remain to be explored.

In a previous report, we cloned and sequenced the rpoH gene encoding the σ32 of Xcc11, and the transient increase in the level of σ32 during heat shock was confirmed by Western blot analysis (Huang et al., 1998). Interestingly, a putative σE-type promoter was recognized at the regulatory region of the Xcc11 rpoH gene. It has been demonstrated that the genes encoding the heat-shock proteins of E. coli are arranged in two major regulons controlled by the alternative sigma factors, σ32 and σE (Raivio & Silhavy, 2001). These two stress-induced regulons appear to be interconnected based on the observation that the σ32-encoding gene (rpoH) of E. coli is transcribed by RNA polymerase (RNAP) containing σE. In the present study, we demonstrated the presence of an...
σE in Xcc, and the DNA sequences, −35 (5′-GAACCTT-3′) and −10 (5′-TCTCA-3′), that are recognized by σE to enable transcription from cognate promoters were explored. Moreover, our data suggest that expression of rpoE in X. campestris is autoregulated.

Materials and methods

Disruption of the rpoE gene

To construct an insertion mutant of the Xcc rpoE gene, a unique PstI site (CTGCA) in the coding sequence of rpoE in pBK-CMV1A3 was first created by replacing the C with a G (underlined) at nucleotide position 240 within rpoE via site-directed mutagenesis (Ho et al., 1989). The 1.5-kb EcoRI fragment containing the mutated rpoE gene was then cloned into the EcoRI site of the PET-24b vector (Novagen), generating plasmid PET-M22. A gentamycin-resistant cassette was excised as a 0.85-kb PstI fragment from pUCGM (Schweizer, 1993) and inserted into the internal unique PstI site of the rpoE gene in PET-M22. The resulting plasmid, PETgM22, was linearized with ApaI, electroporated into Xcc, and selected for gentamycin-resistant cells. The rpoE mutant (SY82) was verified by PCR and Southern blot analyses.

RNA extraction and primer-extension analysis

RNA used for mapping the 5′-end of rpoE mRNA was prepared from Xcc and rpoE mutant SY82 cells as described previously (Mehta & Rosato, 2003). The primer rpoE-P, 5′-CGGCCGACCGACTCCACGTCCCTGAGG-3′, which is located from 19 to 50 bp downstream of the putative rpoE translational initiation codon, was radioactively labeled with [γ-32P] ATP (6000 Ci mmol−1, PerkinElmer) at the 5′-terminus. The labeled primer (50–80 nM) was incubated with 1 μg of total RNA, heat denatured at 65 °C for 5 min, and then quickly chilled on ice to allow primer annealing. The SuperScript II Reverse Transcriptase (Invitrogen) was used for an extension reaction that was carried out according to the manufacturer’s instructions. The primer-extension products were dissolved in formamide loading dye (80% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) and analyzed on a 6% denaturing polyacrylamide gel. The gel was visualized by autoradiography using the Phosphoimager Typhoon 9200 (GE Healthcare).

S1 nuclease protection assay

S1 nuclease protection assay was performed as described previously (Sambrook & Russell, 2001). To prepare the DNA probe, we amplified the promoter region of rpoE (−439 to +82) from plasmid pBK-CMV1A3 containing the Xcc rpoE gene and upstream promoter region with 5′-labeled rpoE-P and T3 (5′-ATTAAACCCTCAAAAGGA-3′) primers. Approximately 10 μg total RNA was mixed with 2 pmol labeled DNA probe in 30 μL hybridization buffer [40 mM PIPES (pH 6.4), 0.4M NaCl, and 0.1 mM EDTA (pH 8.0)] RNA and the labeled DNA probe were denatured at 85 °C for 10 min, and then hybridized at 50 °C for 12 h. A total of 20 U of S1 nuclease (Promega) was added to digest the RNA–DNA hybrids at 37 °C for 30 min, and the reaction was stopped by phenol/chloroform extraction. The products were analyzed on a 6% denaturing polyacrylamide gel.

Gel retardation assay

A 208-bp DNA fragment containing the rpoE promoter region from 125 bp upstream to 82 bp downstream of the rpoE transcription start site was generated by PCR using plasmid pBK-CMV1A3 as the template and primers rpoE-GF (5′-GTCGCCACGAGCGTCCGGCGCCAGTG-3′) and rpoE-P. The amplified product was then used for gel retardation, DNase I footprint, and in vitro transcription analyses. Reconstitution of the RNAP holoenzymes was carried out according to the procedures described previously (Tang et al., 1996), with minor modifications. Briefly, the purified Xcc core RNAP was incubated with an equal volume of each purified σ factor (molar ratio, σ/RNAP = 5) in assay buffer [40 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.1 mM dithiothreitol, 7 mM Mg(OAc)2, and 75 μg mL−1 bovine serum albumin] for 20 min on ice. The reconstituted RNAP holoenzymes, designated as Eσ2 and Eσ32, respectively, were then used for in vitro assays. The purified Xcc RNAP holoenzyme (Eσ32) and the reconstituted Eσ2 and Eσ32 (100 nM) were individually incubated with c. 0.3 ng of the radioactively labeled DNA probe for 20 min at 28 °C in a final volume of 20 μL assay buffer. After addition of 1 μL heparin solution (10 μg mL−1), samples were loaded onto a 4% nondenaturing polyacrylamide gel (29:1, acrylamide/bis).

DNase I footprint assay

The one-end labeled DNA templates were generated by PCR with rpoE-GF and rpoE-P primers using a combination of one unlabeled primer and the second 5′-end radioactively labeled primer. Binding reaction was carried out as described previously (Colland et al., 2000). The reconstituted EcoE (12.5–50 nM) and appropriate DNA templates (0.15 ng) were incubated in 20 μL of assay buffer for 20 min at 28 °C. Next, 10 μL of DNase I solution [0.05 U DNase I (Worthington Biochemicals), 10 mM Tris-HCl (pH 7.9), 10 mM Mg(OAc)2, 10 mM CaCl2, and 125 mM KCl] were added and incubated at room temperature for 30 s, or for 25 s when RNAP was not present in the reaction solution. The reactions were quenched by adding 200 μL stop solution.
[0.4 M NaOAc, 2.5 mM EDTA, and salmon sperm DNA (20 μg mL⁻¹)]. Digested products were ethanol-precipitated, dried, resuspended in 6 μL formamide loading dye, and analyzed on a 6% denaturing polyacrylamide gel.

**In vitro run-off transcription assay**

To allow the formation of the RNAP–DNA complex, we first incubated DNA templates (0.1 pmol) with 2 pmol of either reconstituted enzymes or core RNAP alone in 20 μL of assay buffer for 20 min at 28 °C. A single round transcription reaction was performed by adding 5 μL of a prewarmed NTP (nucleoside triphosphate) substrate solution [400 μM ATP, GTP, and UTP each, 100 μM CTP, 10 μCi [α-³²P]CTP (3000 Ci mmol⁻¹, GE Healthcare), and 10 μg mL⁻¹ heparin] and allowed to proceed for 20 min at 28 °C (Colland et al., 2000). Reactions were terminated by the addition of an equal volume of stop solution (40 mM EDTA). The transcripts were precipitated with ethanol, dried, resuspended in 6 μL formamide loading dye, and analyzed on a 6% denaturing polyacrylamide gel.

**Results**

**Sequence analysis of the cloned Xc11 genomic DNA fragment containing rpoE**

Based on conserved amino acid sequences of RpoE from various bacterial species, a set of degenerate primers, SigE-N [5'-GCTTT(C/T)TA(C/T)ACCTGGCTGA(C/T)CG-3'] and SigE-C [5'-ACG(C/G)GTG(G/A)CGCC(G/C)ACG(C/G)GG(G/A)CA(G/C)AT(G/C)CAT-3'], was used in PCR to amplify an internal segment of the rpoE gene from the Xc11 chromosome. The 300-bp PCR product was verified by DNA sequencing and used as a probe in the hybridization analysis to screen a constructed Xc11 genomic library (Huang et al., 1998) for the entire rpoE coding region. Of the 4000 phages examined, two plaques were found to strongly react with the probe. In vivo excisions were performed to excise pBK-CMV phagemids from the two isolated recombinant λ phages. The two pBK-CMV-based phagemid derivatives, pBK-CMV1A3 and pBK-CMV6A3, which contained about 4.0- and 6.0-kb of Xc11 genomic DNA fragments, respectively, were isolated. Restriction mapping and hybridization analysis revealed that these two plasmids contain a 2.4-kb overlapping fragment of Xc11 genomic DNA fragments, respectively, were isolated.

**Primer extension and S1 nuclease protection identify nucleotide G, – 33 relative to rpoE, to be the transcription start site**

As a prelude to promoter characterization, primer extension was performed using RNA isolated from Xc11 cultured at 28 °C or subjected to heat shock at 42 °C for 20 min. As shown in Fig. 2a, the level of mRNA was moderately increased under heat shock (compare lanes 2 and 1). Moreover, the transcription start site was located at nucleotide G, which was 33 nt preceding the putative translation initiation codon of rpoE. The dependence of Xc11 rpoE expression on RpoE was further analyzed by S1 nuclease mapping and a result similar to that of primer-extension analysis was observed (Fig. 2b, lanes 1 and 2). The discrepancy about the position of the transcription start site might be due to the different methodologies used in mapping the 5'-end of rpoE mRNA. No RNA-protected fragment was observed in the experiment with RNA isolated from the SY82 (Fig. 2b, lane 3), indicating that Xc11 RpoE might be involved in the expression of rpoE.

Analysis of the sequences immediately upstream of the transcription start site revealed a putative – 35 and – 10 consensus sequence of σE-dependent promoters. The

![Fig. 1. Schematic representation of organization of the Xc11 rpoE, rseA, and mucD genes. Arrows indicate the orientation and the relative sizes of the three putative ORFs. The bent arrow denotes the position of the identified σE-dependent promoter of the rpoE gene. The positions of the SigE-N and SigE-C primers for PCR amplification are indicated. The sequence of the rpoE promoter region is shown below the map. The – 35/– 10 region, transcription start site (+1), and the putative translation initiation codon ATG are in boldface. The positions of the RBS and the rpoE-P primer for primer-extension analysis are underlined.](image-url)
sequence, 5′-TCTCA-3′, was located 23 nt upstream of the predicted ribosome-binding site (RBS) and was a close match to the 5′-TCTRA-3′ consensus sequence of *E. coli* promoters known to be recognized by the σE factor (Misiakas & Raina, 1998). The 5′-GAACTT-3′ recognition sequence, identical to the canonical sequence recognized by σE of various bacterial species (Fig. 2c). In addition, the 16 bp spacing between the 5′-GAACTT-3′ and 5′-GAACTT-3′ regions was as well conserved as those of other σE-dependent promoters (Rouvière et al., 1995).

**Gel retardation and DNAse I footprinting show that Xc11 rpoE is directed by a σE-dependent promoter and appears to be autoregulated**

The presence of a σE-dependent promoter suggested that transcription of the Xc11 rpoE gene might be autoregulated. Accordingly, a gel retardation assay was performed to investigate further the interaction of Xc11 RpoE and core RNAP with the rpoE promoter DNA. The His-tagged σE (hRpoE) was overexpressed, and purified by passing through a Ni–NTA column (Qiagen). The purity of hRpoE was > 95% as judged by Coomassie Blue staining of the SDS-PAGE gel (Supporting Information, Fig. S1). The results showed that Xc11 core RNAP failed to cause any shifting (Fig. 3, lane 2) and a band with lower mobility was detected when both Xc11 core RNAP and hRpoE were present (Fig. 3, lane 3). However, no detectable binding to the 208-bp DNA probe was observed when the gel shift assay was carried out using purified Xc11 Es or reconstituted Es (Fig. 3, lanes 4 and 5). These results clearly demonstrated the specific binding of the reconstituted Es with the Xc11 rpoE promoter in vitro.

The promoter consensus sequence recognized by Xc11 Es was further identified by DNAse I footprint analysis. As shown in Fig. 4a and b, the reconstituted Xc11 Es protected a region of 45–54 bp on both strands of the DNA template from DNAse I cleavage. Because of the GC content of the promoter region, the border of the DNAse I protection region on the template and the nontemplate strands could not be precisely determined. However, the Xc11 Es was found to encompass the putative σE-type promoter −35 and −10 consensus sequences. The DNA regions from −39 to +6 on the nontemplate strand and −46 to +8 on the template strand were protected. These observations clearly demonstrated that Xc11 rpoE is directed by a σE-dependent promoter and thus autoregulated.

**Fig. 2.** Determination the transcription start site of Xc11 rpoE by primer-extension assay (a) and S1 nuclease mapping (b). The asterisk indicates the nucleotide corresponding to the transcription start sites. (b) The result of S1 nuclease protection assay with RNA prepared from the wild-type Xc11 and the rpoE mutant SY82 strains. The arrow denotes the position of the RNA-protected DNA fragment. Lanes 1 and 2 indicate RNA extracted from Xc11 grown in 28°C or subjected to a heat shock at 42°C for 20 min, respectively. Lane 3 represents RNA extracted from SY82 with a heat shock at 42°C for 20 min. (c) Alignment of the putative σE-dependent promoter sequences. The putative −35 and −10 promoter regions inferred from the transcription start sites (underlined) are highlighted in boldface. The consensus sequences for the σE-dependent promoter in *Xanthomonas campestris* (Xc) and those from *Escherichia coli* (Ec), *P. aeruginosa* (Pa), and *Salmonella enterica* serovar Typhimurium (St) are shown for comparison. R = G/A.

**Fig. 3.** Binding of the reconstituted Es to the Xc11 rpoE promoter. The radioactive-labeled 208-bp DNA fragments were incubated with either Xc11 core RNAP or reconstituted RNAP holoenzyme containing a purified sigma factor as indicated. The positions of unbound probes and DNA–protein complexes are indicated by arrows.
In vitro assay demonstrates Xc11 RpoE to be transcriptionally active

To further demonstrate that the protein encoded by the rpoE gene is transcriptionally active, run-off transcription assays were performed with core RNAP in the presence or absence of purified Xc11 hRpoE protein. Purified Xc11 core RNAP alone produced no specific transcript from the rpoE promoter template (Fig. 5a). However, the RNAP holoenzyme reconstituted by the addition of purified hRpoE to Xc11 core RNAP was able to specifically transcribe the rpoE promoter in vitro. The size of the transcript was estimated to be 82 bases in length, and is consistent with transcription initiating from the transcriptional start site of the rpoE mapped by primer-extension analysis. The promoter specificity of purified hRpoE was further examined using the RNAP holoenzyme reconstituted from purified hRpoE and core RNAP from E. coli in place of Xc11 in the run-off transcriptions. As shown in Fig. 5b, E. coli core RNAP alone gave no specific RNA synthesis. In the presence of purified hRpoE, the reconstituted RNAP holoenzyme was able to specifically recognize the $\sigma^E$ consensus promoter upstream
of the rpoE and resulted in the production of a specific transcript.

Discussion

Since the discovery of the first $\sigma^E$, numerous $\sigma^E$ have been identified in many bacteria (Lonetto et al., 1994; Raivio & Silhavy, 2001). Sequence alignment revealed that bacterial $\sigma^E$ share a high sequence homology. However, the functions of these $\sigma$ factors in response to environmental stress are varied and remain to be explored for most of them. Here, for the first time, DNA sequences that are recognized by the $\sigma^E$ of *X. campestris* were identified.

Genome sequence analysis revealed the presence of a gene cluster containing *rpoE*, *rseA*, and *mucD* genes in *X. campestris* (da Silva et al., 2002). In this study, the *rpoE* gene encoding the putative $\sigma^E$ of Xc11 was cloned and purified. The results of DNAase I footprint and gel retardation analyses revealed that the purified hRpoE protein is capable of reconstituting with Xc11 core RNAP and specifically binds to the $\sigma^E$-type promoter consensus sequences. Furthermore, *in vitro* transcription analysis showed that the purified hRpoE allowed the production of a specific RNA transcript by either *X. campestris* or *E. coli* core RNAP from a DNA template containing the *rpoE* promoter. These results clearly demonstrate that RpoE acts as a $\sigma$ factor, being able to direct core RNAP to the *rpoE* promoter and stimulating the initiation of transcription.

Despite the fact that a similar genomic organization has been found in both *X. campestris* and *X. fastidiosa*, the expression of *rpoE* is surprisingly different. It has been demonstrated that *rpoE*-rseA-mucD (XF2241) of *X. fastidiosa* constitutes a transcription unit, and expression of this operon is driven by the housekeeping $\sigma$ factor (da Silva Neto et al., 2007). In contrast to its counterpart in *X. fastidiosa*, expression of *rpoE* in *X. campestris* is autoregulated by its own $\sigma^E$-dependent promoter just like most of the known orthologues. The dependence of Xc11 *rpoE* expression on RpoE was verified by S1 nuclease mapping using RNA isolated from the *rpoE* mutant SY82. As shown in Fig. 2b, the expression from the *rpoE* promoter was abolished in the RNA sample isolated from SY82.

In *E. coli* and *Salmonella enterica* serovar Typhimurium, expression of the *rpoE* gene has been shown to be directed by an autoregulated $\sigma^E$-dependent promoter and promoters recognized by other $\sigma$ factors (Rouvière et al., 1995; Miticka et al., 2003). Consistent with this view, a consensus for the $\sigma^E$-dependent promoter that shows a very high degree of conservation to *E. coli* $\sigma^E$ and *P. aeruginosa* AlgU-binding sites (Missiakas & Raina, 1998) was identified upstream of the transcription start site of Xc11 *rpoE*. However, no promoter recognized by other $\sigma$ factors was revealed by both primer-extension and S1 nuclease analyses. We have previously proposed a putative $\sigma^E$-dependent promoter consensus upstream of the Xc11 *rpoH* gene (Huang et al., 1998). Both counterpart promoters have identical sequences in their respective $-35$ (5′-GAACCTT-3′) promoter region, and the putative $-10$ region of *rpoH*, 5′-TCCAA-3′, is very similar to that of *rpoE* (5′-TCTCA-3′) (Fig. 4c). Furthermore, the 16-bp spacing between the $-35$ and $-10$ regions of the *rpoH* promoter is also conserved. Previous analysis of the regulatory region of the enteric bacteria *rpoH* genes revealed the presence of at least three promoters, one of which is controlled by $\sigma^E$, while the remaining two promoters are recognized by RNAP containing $\sigma^{70}$ (Ramirez-Santos et al., 2001). In *E. coli*, transcription from the
σE-dependent promoter becomes more prominent and is the only active promoter in response to thermal stress (Erickson et al., 1987). This suggests that the expression of the rpoH is regulated in a complex manner to ensure continuous production of the σE-dependent heat-shock proteins under various metabolic and environmental conditions. The role of Xc11 RpoE in the regulation of rpoH expression is now under investigation. It is very likely that the heat-shock σ factor gene rpoE of Xc11 is subject to complex regulation as those found in E. coli as well as in other bacteria.

The σE of E. coli and several other bacteria are generally thought to be a global regulator and have been demonstrated to be involved in the adaptation to environmental stresses and regulation of the expression of genes related to extracellular enzymes and virulence factors (Helmann, 2002). It was of interest to investigate the physiological role that RpoE played in X. campestris. Comparison of the growth curves of the wild-type Xc11 and rpoE mutant SY82 strains revealed no significant difference in Lennox–Broth (LB) medium at 28 °C (Fig. S2a). This indicated that the rpoE gene is dispensable under normal growth conditions. Both of the Xc11 and SY82 strains exhibited mucoid phenotypes when grown on an LB plate. In accordance with this, the production of exopolysaccharides and pathogeni-

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rpoE (LB) medium at 28
These experiments suggested that rpoE might regulate genes essential for survival under a heat shock response. Similar observations have been reported in X. fastidiosa (da Silva Neto et al., 2007) and Burkholderia cepacia (Devescovi & Venturi, 2006), and it is very likely that rpoE has evolved to play different roles in different bacterial species.

In conclusion, our results showed that the cloned rpoE, rseA, and mucD genes of X. campestris have a genomic organization similar to that found in X. fastidiosa; however, expression of rpoE in X. campestris is autoregulated by its own σE-dependent promoter. The purified hRpoE acts as a σ factor and is sufficient to direct core RNAP to the rpoE promoter and stimulate the initiation of transcription in vitro. In addition, the DNA sequences that are recognized by Xc11 ErE to enable transcription from cognate promoters were identified. Further studies are needed to better understand the roles of rpoE in regulating the expression of the rpoH as well as genes encoding the extracytoplasmic stress response in X. campestris.

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Purification of the overexpressed Xc11 RpoE protein.

Fig. S2. Analysis of the rpoE mutant SY82 strain in response to different environmental stresses.

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