Activation of the *Erwinia carotovora* subsp. *carotovora* pectin lyase structural gene *pnlA*: a role for RdgB

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The activation of pectin lyase (Pnl) production in *Erwinia carotovora* subsp. *carotovora* strain 71 occurs upon DNA damage via a unique regulatory circuit involving *recA*, *rdgA* and *rdgB*. In a similar Pnl-inducible system reconstituted in *Escherichia coli*, the *rdgB* product was found to activate the expression of *pnlA*, the structural gene for pectin lyase. The kinetic data presented here also show that transcription of *pnlA* followed that of *rdgB* in *Er. carotovora* subsp. *carotovora*, indicating a temporal order of gene expression. By deletion analysis we have localized the promoter/regulatory region within a 66 bp DNA segment upstream of the *pnlA* transcriptional start site. This region contains the –10 consensus sequence but not the sequences corresponding to the *E. coli* –35 region. For DNA-binding studies, *rdgB* was overexpressed in *E. coli* and a 14 kDa polypeptide was identified as the gene product. RdgB from crude extracts or a purified preparation caused an identical gel mobility shift of a 164 bp DNA segment containing the *pnlA* promoter/regulatory region. Utilizing DNase I protection assay the RdgB-binding site was localized between nucleotides –29 and –56, i.e. overlapping the position of the putative –35 box. The findings reported here, taken along with our previous observation that the *rdgB* product is required for *pnlA* expression, establishes that *rdgB* encodes a transcriptional factor which specifically interacts with the *pnlA* promoter/regulatory region.

Keywords: cell-wall-degrading enzyme, gene expression, damage-inducible traits, virulence factor, plant-pathogenic bacteria

INTRODUCTION

The production of soft rot in plants or plant organs by *Erwinia* species depends to a large extent upon the ability of the organisms to produce extracellular enzymes, especially pectinases such as pectate lyase (Pel), polygalacturonase (Peh) and pectin lyase (Pnl). Pnl, like Pel and Peh, causes tissue maceration by degrading plant cell wall components. Therefore, it was not surprising that a Pnl-deficient mutant was found to have attenuated pathogenicity (Pirhonen *et al.*, 1991).

A unique feature of pectin lyase (Pnl) production in soft-rotting *Erwinia* is that it is activated by DNA-damaging agents such as mitomycin C (MC), nalidixic acid or UV

light (Barras *et al.*, 1994; Itoh *et al.*, 1980; Kamimiya *et al.*, 1972; McEvoy *et al.*, 1992; Tomizawa & Takahashi, 1971; Tsuyumu & Chatterjee, 1984; Tsuyumu *et al.*, 1985). In addition, in *Erwinia carotovora* subsp. *carotovora* strain 71, Pnl production is co-induced with carotovoricin synthesis (Ctv) and cell lysis (Lss) (Tsuyumu & Chatterjee, 1984; Zink *et al.*, 1985). The nucleotide sequence and transcriptional start site of *pnlA*, the structural gene for Pnl of strain 71, have been determined (Chatterjee *et al.*, 1991). The absence of sequences similar to the consensus catabolite-activating protein (CAP)-binding site and LexA-binding site of *Escherichia coli* was consistent with the observations that the *pnlA* promoter was not susceptible to catabolite repression, and expression of *pnlA* was not substantially affected in an *E. coli* LexA[–] mutant (McEvoy *et al.*, 1987, 1990). The *pnlA* promoter was predicted to consist of a –10 region (TACAAT) similar to the *E. coli* σ^{70} promoter sequence (TATAAT). By contrast, the cor-

Abbreviations: CAT, chloramphenicol acetyltransferase; Kdg, 2-keto-3-deoxygluconate; MC, mitomycin C; Peh, polygalacturonase; Pel, pectate lyase; Pnl, pectin lyase.

responding -35 region (TAAGCG) separated by a 17 bp spacer, shared little similarity to the *E. coli* TTGACA consensus sequence. The absence of a well-matched -35 sequence raised the possibility that the expression of *pnlA* may require a positive activator. Subsequently, two regulatory genes, *rdgA* and *rdgB*, were identified that control Pnl production in *Er. carotovora* subsp. *carotovora* strain 71 (McEvoy *et al.*, 1992; Liu *et al.*, 1994). While the putative *rdgA* product has high similarity with several bacteriophage and bacterial repressors such as ϕ 80 cI, 434 cI, lambda cI, PrtR, and LexA, the predicted *rdgB* product shares substantial homology with the bacteriophage Mu transcriptional activators, the Mor and C proteins. Based upon these and other findings, we had proposed a cascade-type of regulation wherein RdgA was postulated to control *rdgB* expression, and RdgB was presumed to activate the transcription of *pnlA* and other DNA-damage-inducible genes such as *ctv* and *lss*. This model is supported by our subsequent findings that RdgA functions as a repressor of both *rdgA* and *rdgB*, and that the derepression of *rdgA* and *rdgB* expression results from the RecA*-mediated cleavage of RdgA (Liu & Chatterjee, 1996; Liu *et al.*, 1996). However, RdgA does not interact with *pnlA* promoter/regulatory region *in vitro* (Liu *et al.*, 1996). Instead, the overexpression of *rdgB* from a *tac-rdgB* construct activates Pnl production in *E. coli* RecA⁻ strain (Liu *et al.*, 1994), supporting the idea that *rdgB* may encode a positive regulatory factor for the expression of *pnlA*. In this report, we (i) provide evidence for a temporal order of *pnlA* and *rdgB* expression following MC treatment; (ii) present data which for the first time define the *pnlA* promoter; (iii) identify the *rdgB* product and describe its purification; (iv) demonstrate that RdgB is a DNA-binding protein; and (v) specifically describe the location of the RdgB-binding site within the *pnlA* promoter.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. *Er. carotovora* subsp. *carotovora* strain 71 was grown in Luria-Bertani (LB) medium at 28 °C. *E. coli* cultures were grown in LB medium or in NZYM broth (Sambrook *et al.*, 1989) at 37 °C with ampicillin (Ap; 50 or 100 µg ml⁻¹), spectinomycin (Spc; 50 µg ml⁻¹) or streptomycin (Sm; 50 µg ml⁻¹) as required.

RNA assays. *Er. carotovora* subsp. *carotovora* strain 71 was grown in LB at 28 °C. When the cultures attained a OD₆₀₀ value of 1.0 (Beckman DU 70 spectrophotometer), MC was added at a final concentration of 500 ng ml⁻¹. Incubation was continued, and cells were harvested at desired intervals by centrifugation at 4000 g for 10 min at 4 °C. Total RNA was isolated according to the method of Aiba *et al.* (1981), and primer extension analysis was carried out as described previously by Liu *et al.* (1994). The primers 5'-GCGAA-ATTGTGGCTCAG-3' and 5'-TCCAGTTACATTTGCCG-C-3' were used for the assay of *rdgB* and *pnlA* transcripts, respectively. Ten micrograms total RNA was used for each reaction. The labelled *rdgB* and *pnlA* primers were mixed together in the primer extension reactions.

Construction of transcriptional *pnlA-cat* fusions. The 496 bp

EcoRV-MboII fragment, containing the *pnlA* upstream sequence from -453 nt to +43 nt, was treated with the Klenow fragment and cloned into the *SmaI* site of the promoter probe vector, pKK232-8 or the *EcoRV* site of pBluescript SK(+) to produce the plasmids pAKC752 and pAKC742, respectively. Deletions at the 5' end of the 496 bp *EcoRV-MboII* fragment were made in pAKC742 by the Erase-a-Base system (Promega), and the extent of deletions were confirmed by sequencing assays. Sequencing reactions were carried out using Sequenase version 2.0 (USB). Plasmids carrying the desired deletions were digested by *Bss*HII, treated with Klenow fragment, and then digested by *Hind*III. The purified insert DNAs were cloned into pKK232-8 digested by *SmaI* and *Hind*III to produce a series of transcriptional *pnlA-cat* fusions (see Table 1).

Chloramphenicol acetyltransferase (CAT) assay. *E. coli* MC4100 derivatives carrying pAKC740 and each of the *pnlA-cat* plasmids (Table 1) were grown at 28 °C in 20 ml LB supplemented with Ap and Spc to OD₆₀₀ 1.0. Cultures were divided, and half received MC at a final concentration of 2.5 µg ml⁻¹ and was incubated for an additional 8 h. Cells were harvested by centrifugation at 4000 g for 10 min at 4 °C and pellets were washed once with 5 ml CAT buffer (0.1 M Tris/HCl, pH 7.9, 0.1 mM DTT). Cells were subsequently resuspended in 1 ml CAT buffer containing 0.75 mg PMSF ml⁻¹ and 1 mg lysozyme ml⁻¹ and incubated at 37 °C for 30 min. Cells were kept on ice for 30 min, lysed by sonication, and centrifuged at 10000 g for 15 min at 4 °C. The supernatants were assayed spectrophotometrically for CAT activity according to Shaw (1975).

Construction of an *rdgB*-overexpressing plasmid and overproduction and purification of RdgB. The coding region of *rdgB* was amplified by PCR using 5'-GCACATATGACTGAGCCACAATTTTCGCAG-3' as the 5' primer, 5'-GCAAGCTTGCAATTATGCCTGAGTGTGCTG-3' as the 3' primer, and pAKC730 as the template DNA. PCR products were digested with *NdeI* and *Hind*III and cloned into the expression plasmid pT7-7 to produce pAKC826. The fidelity of the amplified DNA was confirmed by sequencing. *E. coli* strain BL21 carrying pAKC826 or pT7-7 was grown at 37 °C in 500 ml of NZYM broth containing Ap. When the OD₆₀₀ value of the culture reached 0.6, lambda CE6 was added at a multiplicity of infection of 5-10 particles per cell. Following a 3 h incubation, the cells were harvested by centrifugation at 5000 g for 10 min, washed once with 0.9 M NaCl, and resuspended in 10 ml binding buffer A [50 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 50 mM KCl, 1.0 mM DTT, 0.1 mM EDTA, 5% (v/v) glycerol] plus 0.5 mM PMSF and 200 mM NaCl. Resuspended cells were lysed in a French press (American Instrument) by two cycles at 1000 p.s.i. (6.9 MPa), and unbroken cells were removed by low-speed centrifugation. Streptomycin sulfate (1 mg ml⁻¹) was added to the cell lysate, and the mixture was centrifuged at 40000 g for 1 h. Solid (NH₄)₂SO₄ was added to the supernatant to yield a 20% (w/v) solution, the mixture was gently stirred for 30 min on ice, and centrifuged at 12000 g for 10 min. The (NH₄)₂SO₄ concentration of the supernatant was increased to 60% (w/v), the mixture was gently stirred for 30 min on ice, and centrifuged as above. The (NH₄)₂SO₄ precipitate containing RdgB was solubilized in buffer A and dialysed for 1 h. The dialysed sample was loaded on a heparin/agarose column (Affi-Gel Heparin, Bio-Rad), and fractions step-eluted with buffer A containing 0.1, 0.3, 0.5, 0.7 and 0.9 M NaCl, respectively. The 0.3 M fraction, which contained RdgB, was concentrated by precipitation with (NH₄)₂SO₄, resuspended in buffer A containing 0.2 M NaCl and dialysed in the same buffer for 1 h. The dialysed sample was loaded onto a

Table 1. Bacterial strains, plasmids and bacteriophage

Strain/plasmid/ bacteriophage	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i>		
DH5 α	ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169 <i>recA1 thi-1</i>	BRL
MC4100	<i>araD139</i> Δ (lacIPOZYA) U169 <i>recA⁺ thi</i> , Str ^r	Liu <i>et al.</i> (1994)
JM109(DE3)	<i>endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi</i> Δ (lac-pro) [F' <i>traD36 proAB⁺ lacI^q lacZ</i> Δ M15] λ I857 <i>ind1 sam-7 lacUV5-T7 gene-1</i>	Promega
BL21	F ⁻ <i>ompT</i> r _B ⁻ m _B ⁻	Stratagene
<i>Er. carotovora</i> subsp. <i>carotovora</i> 71	Wild-type	Zink <i>et al.</i> (1984)
Bacteriophage		
CE6		Stratagene
Plasmids		
pBluescript SK(+)	Ap ^r	Stratagene
pCR II	PCR product cloning vector, Ap ^r	Invitrogen
pKK232-8	Ap ^r	Brosius (1984)
pT7-7	T7 expression vector, Ap ^r	Tabor & Richardson (1985)
pAKC730	<i>rdgA⁺, rdgB⁺</i> , Ap ^r	Liu <i>et al.</i> (1994)
pAKC740	<i>rdgA⁺, rdgB⁺</i> , Spc ^r	Liu <i>et al.</i> (1994)
pAKC742	-453 to +43 <i>pnlA</i> DNA segment in pBluescript SK(+), Ap ^r	This work
pAKC752	<i>pnlA-cat</i> , -453 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D1	<i>pnlA-cat</i> , -366 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D2	<i>pnlA-cat</i> , -216 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D3	<i>pnlA-cat</i> , -144 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D4	<i>pnlA-cat</i> , -124 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D5	<i>pnlA-cat</i> , -100 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D6	<i>pnlA-cat</i> , -66 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC752D7	<i>pnlA-cat</i> , -27 to +43 <i>pnlA</i> DNA segment in pKK232-8, Ap ^r	This work
pAKC826	<i>rdgB</i> under transcriptional control of T7 promoter in pT7-7, Ap ^r	This study
pAKC827	164 bp PCR amplified <i>pnlA</i> DNA segment cloned in pCR II, Ap ^r	This work

Sephacryl S100HR (Sigma) column and eluted with buffer A containing 0.2 M NaCl. Glycerol (50%, v/v) was added to the fractions containing RdgB, and samples were stored at -20 °C. The enrichment of RdgB by the purification steps was followed by SDS-PAGE. Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's specifications.

Gel mobility shift assay. The 164 bp *pnlA* DNA, containing the sequences from -110 to +54 nt (Chatterjee *et al.*, 1991), was amplified by PCR using primers 5'-ACTAAAAGATG-GCAGCGTA-3' and 5'-ATAATAATGTCTTCCAACG-3'. The PCR product was cloned into pCR II (Invitrogen) to produce plasmid pAKC827. The fidelity of the amplified DNA was confirmed by sequencing. The *Eco*RI fragment of pAKC827 was eluted from agarose gel, end-labelled, and purified on Sephadex G-50. DNA-protein interaction was assayed in 20 μ l binding buffer A containing 1 μ g salmon sperm DNA, 2 μ g BSA, and differing amounts of RdgB preparations. After incubating for 20 min at room temperature, the reaction mixture was subjected to electrophoresis in a 5% (w/v) polyacrylamide gel. The gel was dried and exposed to an X-ray film.

DNase I protection analysis. Ten picomole of primers 5'-ACTAAAAGATGGCAGCGTA-3' or 5'-ATAATAATGTCTTCCAACG-3' was end-labelled by the polynucleotide

kinase (Promega) and [γ -³²P]ATP (DuPont). For PCR amplification of single (5' or 3') end-labelled probe, the reaction mixture contained one labelled primer and the opposing unlabelled primer. Using 50 ng purified *Eco*RI fragment of pAKC827 as the template, PCR was carried out as follows: 20 cycles at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min; followed by one cycle at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 7 min. After removal of the free nucleotides by ethanol precipitation, end-labelled DNA (20000 c.p.m.) was mixed with RdgB in binding buffer A containing 2.5 μ g salmon sperm DNA and 5 μ g BSA in a final volume of 50 μ l. DNA-protein complexes were allowed to form on ice for 20 min. The mixtures were treated with 50 μ l Ca²⁺/Mg²⁺ (5 mM CaCl₂, 10 mM MgCl₂) for 1 min at room temperature, followed by 3 μ l DNase I (0.15 U, Promega) for 1 min at 37 °C. The reactions were terminated by adding 100 μ l stop buffer (200 mM NaCl, 20 mM EDTA, 1.0% SDS, 40 μ g yeast tRNA ml⁻¹). The DNA was purified by extraction with phenol/chloroform, ethanol precipitation, washing with 80% (v/v) ethanol, followed by resuspension in 4.5 μ l formamide dyes [95% (v/v) formamide, 20 mM EDTA, 0.1% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol]. Sequencing gel (8% acrylamide) was used to analyse the products along with a G + A ladder produced by pyridine formate and piperidine digests of the same labelled fragments as described by Leblanc & Moss (1994).

RESULTS AND DISCUSSION

Kinetics of accumulation of *rdgB* and *pnlA* transcripts

In a previous study we reported that, while there is a basal level of *rdgA* transcription, expression of *pnlA* and *rdgB* is strictly dependent upon MC treatment of RecA⁺ bacteria (Liu *et al.*, 1994). The cascade-type regulatory model (Liu *et al.*, 1994) led us to postulate that *rdgB* expression, which requires a RecA function, might precede *pnlA* transcription. This hypothesis was tested by determining the appearance of *rdgB* and *pnlA* transcripts upon MC treatment of *Er. carotovora* subsp. *carotovora* strain 71. In cells growing in the presence of MC for up to 30 min, there were no detectable *pnlA* or *rdgB* mRNA species (Fig. 1, lanes 1 and 2). However, the *rdgB* mRNA, but not the *pnlA* mRNA, was detected in cells grown for 50 min in the presence of MC (Fig. 1, lane 3). By 60 min, the level of *rdgB* mRNA was significantly higher yet the *pnlA* mRNA species was barely detectable. The *rdgB* mRNA level continued to increase, but it was not until after 80 min of MC treatment that the *pnlA* transcripts showed significant accumulation (Fig. 1, lane 5). These patterns of transcript accumulation support our model that *rdgB* expression is activated upon MC treatment, and as the pool of RdgB polypeptides increases, *pnlA* transcription is initiated.

Deletion analysis of the *pnlA* promoter

To assay the *pnlA* promoter activity, a transcriptional *pnlA*-*cat* fusion in the plasmid pAKC752 (Fig. 2a, b) was transferred by electroporation into *E. coli* RecA⁺ strain MC4100 carrying pAKC740, which contains the *rdgA* and *rdgB* genes (Table 1). CAT production was then assayed in this construct. In the absence of MC, the construct produced only a low level of CAT activity, but expression of the *pnlA*-*cat* fusion was induced upon MC treatment (Fig. 2c). This induction of *pnlA*-*cat*

Fig. 1. Time-course of *pnlA* and *rdgB* transcript accumulation following MC treatment. Ten micrograms RNA was used in each reaction in the primer extension assays. Lanes: 1, 0 min; 2, 30 min; 3, 50 min; 4, 60 min; 5, 80 min.

Fig. 2. (a) Nucleotide sequence of the 5' end of *pnlA* gene of *Er. carotovora* subsp. *carotovora* strain 71. The transcriptional start site is indicated by an asterisk. The palindromic sequence between nucleotides -119 and -95 is underscored with arrows. The putative KdgR-binding site and the deduced -10 region are indicated. The deletion derivatives (D1-D7) used for the assay of *pnlA*-*cat* expression are indicated by vertical arrows. (b) Plasmids carrying deletions within the promoter region of *pnlA*. CAT denotes the reporter gene product of the promoter probe vector pKK232-8, and +1 indicates the transcriptional start site. The number of bases extending upstream of the transcriptional start site in each construct is also shown. (c) Effects of various *pnlA* promoter deletions on the production of CAT in the absence (filled bars) and the presence (open bars) of MC as indicated. Specific activity of CAT is defined as μmol chloramphenicol acetylated min^{-1} (mg protein) $^{-1}$. Numbers on x-axis of graph correspond to those in (b).

Fig. 3. Overproduction of RdgB. Crude extracts were analysed by SDS-PAGE using a 15% (w/v) polyacrylamide gel. Lane 1, 3 µg crude extract from BL21 cells carrying pT7-7; lane 2, 3 µg crude extract from BL21 cells carrying pAKC826. The position of RdgB is indicated by an arrow. Protein markers (BRL low-range) are indicated.

expression demonstrates that the entire promoter/regulatory region resides within the 496 bp *pnlA* fragment cloned into pAKC752.

Subsequently, the deletion derivatives of pAKC752 (D1–D7; Fig. 2a, b) were transferred by electroporation into MC4100 carrying pAKC740. The effect of *pnlA* promoter deletions on *pnlA*–*cat* expression was examined by monitoring CAT production in the absence and presence of MC. The results shown in Fig. 2(c) indicate that deletions from –453 to –66 nt did not significantly affect activation of the *pnlA* promoter. However, a further deletion of 39 bp downstream in pAKC752D7 resulted in loss of the *pnlA* promoter activity. Since pAKC752D6 containing 66 bp sequences upstream of the transcriptional start site responded to Rdg-mediated activation of *pnlA* transcription upon treatment with MC, we concluded that the *pnlA* promoter/regulatory region is localized within this DNA segment. These results exclude the potential role of a palindromic sequence within –119 to –95 nt segment (Fig. 2a; see also Chatterjee *et al.*, 1991) in the activation of *pnlA* expression.

Overproduction and purification of RdgB

We previously reported that in *E. coli* overexpression of *rdgB* driven by the *tac* promoter resulted in the production of Pnl in the absence of *recA*, *rdgA* and MC (Liu *et al.*, 1994). This finding suggested that the *rdgB* product might be required for the activation of *pnlA* expression. To strengthen this observation, we examined binding of RdgB to *pnlA* DNA. The coding region of *rdgB* was amplified by PCR and cloned into the

Fig. 4. Purification of RdgB. Protein samples (3 µg) from each purification step were analysed by SDS-PAGE using a 15% (w/v) polyacrylamide gel. Lane 1, *E. coli* cell extract prepared by disruption in a French press; lane 2, supernatant after 5m treatment; lane 3, 20–60% (w/v) $(\text{NH}_4)_2\text{SO}_4$ fraction; lane 4, eluate from heparin/agarose column; lane 5, eluate from Sephacryl S100HR column. The position of RdgB is indicated by an arrow. Protein markers (BRL low-range) are indicated.

T7 expression vector pT7-7 to produce plasmid pAKC826. When pAKC826 was transferred by electroporation into *E. coli* strain JM109(DE3), no overproduction of RdgB was detected. It is possible that a basal level of RdgB produced in this construct causes toxicity by activating a set of normally silent damage-inducible genes or by inducing ‘translation crashes’ (Kurland & Dong, 1996). Similarly, our inability to clone the entire *rdgB* in a high-copy vector, pBluescript SK, could also be attributed to an apparent toxicity of RdgB (data not shown). To alleviate this problem, pAKC826 was transferred by electroporation into *E. coli* strain BL21 that does not contain the T7 RNA polymerase gene. The induction of *rdgB* was then effected by infecting with lambda CE6 phage which provides the gene for T7 RNA polymerase. Using this approach, a protein of approximately 14 kDa was detected in cells of BL21 carrying pAKC826, but not in cells of BL21 carrying the vector pT7-7 (Fig. 3). Under these inducing conditions, RdgB was the major protein species produced by BL21 carrying pAKC826. The apparent molecular mass of 14 kDa agrees well with the mass of 13.7 kDa of the polypeptide previously deduced from the *rdgB* sequence (Liu *et al.*, 1994), further indicating that this protein is encoded by *rdgB*. The induced 14 kDa protein was purified from a soluble extract of *E. coli*. The RdgB preparation (Fig. 4, lane 5) appeared to be greater than 95% pure on SDS-PAGE.

A crude extract containing RdgB and purified RdgB were compared for their DNA-binding properties. Essentially identical results were obtained with these preparations and for brevity, unless noted otherwise, we present below the data obtained with the crude extract that constituted the starting material for the purification of RdgB.

Fig. 5. Gel mobility shift assays. Lane 1, 2 ng ^{32} P-labelled *pnlA* DNA; lanes 2–7, 2 ng ^{32} P-labelled *pnlA* DNA and 10, 25, 50, 100, 250 and 500 ng crude extract of BL21 carrying pAKC826; lane 8, 500 ng crude extract of BL21 carrying cloning vector pT7-7; lanes 9 and 10, 2 ng ^{32} P-labelled *pnlA* DNA with 42 and 84 ng pure RdgB, respectively. C, complex; F, free DNA.

Identification of the RdgB-binding site

Gel mobility shift assays with the *pnlA* DNA segment from –110 to +54 nt revealed one band with retarded mobility. This mobility shift was detected with 100 ng crude extract containing RdgB (Fig. 5, lane 5) or 42 ng purified RdgB (Fig. 5; lane 9). The efficiency of band shift was directly proportional to the concentration of RdgB in the crude extract (Fig. 5, lanes 2–7). By contrast, proteins present in 500 ng crude extract of BL21 carrying the vector pT7-7 did not cause a band shift (Fig. 5, lane 8).

In DNase I protection assays with crude extract containing RdgB as well as purified RdgB, one protected region was identified between –110 and +54 nt of *pnlA*. The protected sequence mapped within –52 to –29 nt on the sense strand, and within –33 to –56 nt on the antisense strand relative to the *pnlA* transcriptional start site (Fig. 6). Like the gel mobility shift assay, the protective effect was dependent upon RdgB concentration. While 25 ng crude extract failed to protect DNA, higher levels of protein produced a clearer RdgB-protected region (Fig. 6). The finding that 500 ng crude extract of BL21 carrying vector pT7-7 did not prevent DNA from the DNase I digestion (Fig. 6, lane 6) confirms that RdgB binds specifically to the *pnlA* DNA and protects its binding site from DNase I digestion.

Structural features of the *pnlA* promoter

Based upon the findings presented here and in previous reports, we now consider some of the salient features of the *pnlA* promoter/regulatory region. After mapping the 5' end of the *pnlA* mRNA by primer extension (Chatterjee *et al.*, 1991), we had tentatively identified TACAAT as the –10 region (Fig. 7), which closely resembles the consensus sequence for *E. coli* promoters (Harley & Reynolds, 1987; Reznikoff & McClure, 1986). A corresponding *E. coli* consensus –35 region (TTGACA) was not detected in *pnlA*. However, the data presented above show that upstream sequences including the –35 region were protected by RdgB. In

this region, a TTATTAA motif occurs twice separated by a 6 bp spacer. These sequences can form a palindromic structure within the RdgB-binding site (Fig. 7), and may be potentially important in RdgB recognition. The palindromic structure within this site also raises the possibility that RdgB may bind the *pnlA* DNA as a dimer (Bölker *et al.*, 1989).

It should be noted that the RdgB protected region is localized immediately upstream of a putative KdgR-binding site (Kdg, 2-keto-3-deoxygluconate; KdgR, general repressor of genes involved in pectinolysis) (Fig. 7). At this juncture, there is no direct evidence that KdgR interacts with *pnlA* DNA in *Er. carotovora* subsp. *carotovora*. However, in light of its repressive effects on several other pectinase and pectate catabolic genes in *Erwinia chrysanthemi* (Reverchon *et al.*, 1989), the possibility must be considered that KdgR would normally bind to this site in *pnlA*, and that this binding could seriously interfere with RNA polymerase binding to the *pnlA* promoter. Thus, KdgR could block *pnlA* transcription under normal growth conditions, i.e. in the absence of RdgB.

C and Mor proteins, the activators for Mu late or middle promoters, are the only two proteins similar to RdgB (Liu *et al.*, 1994). Margolin *et al.* (1989) proposed the following consensus sequence for four major Mu late promoters: ccATAAcCcCA/GG/CacN₁₆tA..CT. In these instances, the putative –10 and –35 regions are separated by 16 bp, and furthermore there is no similarity between the Mu and *E. coli* –35 regions. By footprinting analysis, Bölker *et al.* (1989) determined that the C protein protected region overlapped the Mu late consensus sequence, but the C-binding site was positioned 22 bp upstream of the –10 region. As also shown in Fig. 7, the *pnlA* promoter contains sequences similar to the Mu late promoter consensus sequence and the C-binding site. However, there are significant differences in size and constitution of the spacer sequence between the –10 and –35 regions. While the C-binding site is localized immediately upstream of the –35 region (Bölker *et al.*, 1989), our data show that the RdgB-binding site extends into the –35 region. Besides, an ATTAT motif was identified within the C-protected region in the *mom* and *mod* promoters (Bölker *et al.*, 1989) that is different from the *pnlA* TTATTAA motif. Such differences in structural characteristics of the binding sites could explain the differences in promoter recognition and activation by RdgB and C proteins, although their overall binding activities categorizes them within the same family of *trans*-acting factors.

In summary, the observed DNA-binding activity of RdgB demonstrates that RdgB functions as an accessory factor for the initiation of *pnlA* transcription. In this regard, the action of RdgB probably is very similar to other activator proteins, such as lambda cI and cII proteins (Ho *et al.*, 1983; Hochschild *et al.*, 1983) as well as *E. coli* CAP, AraC, MalT and OmpR (Lee *et al.*, 1981; Raibaud & Schwartz, 1984). The latter proteins are known to bind sequences within or upstream of the

Fig. 6. DNase I protection analysis of the *pnIA* promoter by RdgB. Prior to digestion with DNase I, about 5 ng end-labelled DNA was incubated with: lanes 1, no RdgB protein; lanes 2–5, 25, 100, 250 and 500 ng crude extract of BL21 carrying pAKC826; lane 6, 500 ng crude extract of BL21 carrying vector pT7-7. The products were fractionated in a 8% (w/v) acrylamide sequencing gel. 5' and 3' refer to the ^{32}P -end-labelled portion of *pnIA* DNA. The G + A chemical sequence of the same labelled DNA fragment is shown in the leftmost lanes. The transcriptional start site is indicated by arrows. Brackets indicate the nucleotide positions relative to transcriptional start site, which are protected from DNase I digestion by RdgB.

Fig. 7. Comparison of RdgB-binding sequence in *pnIA* with the consensus of C-binding sites (C-consensus, Böcker *et al.*, 1989) and the Mu late promoter consensus sequence (Margolin *et al.*, 1989). The transcriptional start site of *pnIA* is mapped at the G residue as +1 (Chatterjee *et al.*, 1991). The deduced –10 (TACAAT) and –35 (TAAGCG) sequences of *pnIA* promoter are shown in italic. The RdgB-protected regions on each strand are indicated by solid horizontal lines. The inverted repeats are indicated by head-to-head arrows. The putative KdgR-binding site is indicated by double underlines.

nonconsensus –35 regions of specific promoters, allowing their recognition and activation by RNA polymerase.

It is important to note that in *Er. carotovora* subsp. *carotovora* strain 71, Pnl production is co-induced with carotovoricin synthesis and cell lysis (Tsuyumu & Chatterjee, 1984; Zink *et al.*, 1985). In a *rdgB*–MudI mutant, the production of Pnl and Ctv and the induction

of cell lysis did not occur after MC treatment (unpublished results). Thus, RdgB functions as a transcriptional activator not only for *pnIA*, but possibly also for other genes of the Rdg regulon, including *ctv* and *lss*. Studies have been initiated to identify these promoter sequences and to determine if a consensus RdgB-binding site exists within these promoters.

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