Regulation of the pehA gene encoding the major polygalacturonase of Xanthomonas campestris by Clp and RpfF

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Exopolysaccharide and several extracellular enzymes of Xanthomonas campestris pv. campestris (Xcc), the causative agent of black rot in crucifers, are virulence factors. In this study, sequence and mutational analysis has demonstrated that Xcc pehA encodes the major polygalacturonase, a member of family 28 of the glycosyl hydrolases. Using the 5'RACE (rapid amplification of cDNA ends) method, the pehA transcription initiation site was mapped at 102 nt downstream of a Clp (cAMP receptor protein-like protein)-binding site. Transcriptional fusion assays showed that pehA transcription is greatly induced by polygalacturonic acid, positively regulated by Clp and RpfF (an enoyl-CoA hydratase homologue which is required for the synthesis of cis-11-methyl-2-dodecenoic acid, a low-molecular-mass diffusible signal factor), subjected to catabolite repression, which is independent of Clp or RpfF, and repressed under conditions of oxygen limitation or nitrogen starvation. Our findings extend previous work on Clp and RpfF regulation to show that they both influence the expression of pehA in Xcc.

INTRODUCTION

Xanthomonas campestris pv. campestris (Xcc) is a Gram-negative plant-pathogenic bacterium that causes black rot in crucifers, resulting in tremendous losses in agriculture (Williams, 1980). This organism is capable of producing large amounts of an exopolysaccharide and an array of extracellular enzymes including proteases, endoglucanases and pectinases. These extracellular products have long been considered important virulence determinants (Chan & Goodwin, 1999; Chang et al., 2001; Chou et al., 1997; Dow et al., 1987; Dums et al., 1991; Soby & Daniels, 1996; Yang & Tseng, 1988). Production of these determinants is regulated by the global transcription factor Clp (cAMP receptor protein-like protein) and the rpf (regulator of pathogenicity factors) gene cluster (Barber et al., 1997; de Crecy-Lagard et al., 1990; Dow et al., 2000; He et al., 2006, 2007; Tang et al., 1991; Tseng et al., 1999; Wilson et al., 1998).

Mutation in clp causes pleiotropic effects, including drastic reduction in the production of exopolysaccharide and extracellular enzymes as well as loss of virulence and sensitivity to filamentous phage fLf (de Crecy-Lagard et al., 1990; He et al., 2007; Lee et al., 2001; Tseng et al., 1999). Gel retardation and transcriptional fusion assays have shown that Clp exerts a positive control over expression of engA (encoding endoglucanase A) by direct binding to the upstream Clp-binding sites, while prt1 (encoding the major protease) without a Clp-binding site is controlled in an indirect manner (Hsiao & Tseng, 2002; Hsiao et al., 2005). Recent microarray analyses have identified 299 Clp-regulated genes in Xcc (He et al., 2007), demonstrating the nature of global control of this transcription factor.

The rpf gene cluster comprises nine genes (rpfA–I), mutations in most of which give similar phenotypes and cause a coordinate downregulation of synthesis of the extracellular products (Barber et al., 1997; Dow et al., 2000;
Tang et al., 1991; Wilson et al., 1998). RpfF (an enoyl-CoA hydratase homologue) is required for the synthesis of DSF (a low-molecular-mass diffusible signal factor identified as cis-11-methyl-2-dodecenoic acid), while RpfB (a long-chain fatty acyl-CoA ligase) plays a minor role in the synthesis (Barber et al., 1997; Wang et al., 2004). Addition of DSF to an rpfF mutant, but not to an rpfB mutant, can phenotypically restore the production of extracellular endoglucanase and pectate lyase (Barber et al., 1997). DSF perception and signal transduction require the two-component system, consisting of the hybrid sensor kinase RpfC and the response regulator RpfG, and mutation in rpfC or rpfG also causes reduction in the extracellular products (Slater et al., 2000; Tang et al., 1991). Other rpf genes are not involved in DSF signalling and have complex regulatory effects. For example, rpfA is known to regulate pathogenicity factor production (Wilson et al., 1998). So far, only two extracellular enzyme genes (engA and pti1) have been demonstrated to be affected by a rpfA mutation (Wilson et al., 1998). Although details of regulatory circuits remain to be elucidated, recent microarray analyses have identified 165 Xcc genes that are regulated by RpfF/DSF (He et al., 2006).

Pectinases are enzymes responsible for the degradation of pectin and can be classified according to their preferential substrates, pectin or polygalacturonic acid (PGA) (Hugouvieux-Cotte-Pattat et al., 1996). Pectin lyases (EC 4.2.2.10) cleave natural pectin and highly methyl-esterified PGA through β-elimination. Pectin methyltransferases (EC 3.1.1.11) catalyse de-esterification of pectin to make substrates available for subsequent actions by the two types of PGA-degrading enzyme, pectate lyases (EC 4.2.2.2), which cleave the glycosidic bonds by β-elimination, and polygalacturonases (EC 3.2.1.15), which catalyse a hydrolytic cleavage (Hugouvieux-Cotte-Pattat et al., 1996; Nasser et al., 1999). Two polygalacturonase genes and five pectate lyase genes have been annotated in the fully sequenced genomes of Xcc strains ATCC 33913 and 8004 (da Silva et al., 2002; Qian et al., 2005) as well as in strain Xc17 (http://xcc.life.nthu.edu.tw), whose genome sequence is almost complete. The five pectate lyase genes for Xcc strains ATCC 33913 and 8004, respectively, are XCC0644 and XC_3591 for pelA1, XCC0645 and XC_3590 for pelA2, XCC2815 and XC_1298 for pelB, XCC1219 and XC_3023 for hrpW, and XCC0122 and XC_0126 for pelE (da Silva et al., 2002; Qian et al., 2005). The two polygalacturonase genes are XCC3459 and XC_0705 for pehA and XCC2266 and XC_1849 for pglA (da Silva et al., 2002; Qian et al., 2005). None of these genes have been studied, although three forms of pectate lyases have been reported (Dow et al., 1987, 1989). Recently, it was found that a mutation in pglA only slightly reduced the level of polygalacturonase activity (Chien, 2006), indicating that PglA is not the major polygalacturonase in Xcc. In this study, we have examined pehA and the results indicated that this gene codes for the major polygalacturonase and its expression is upregulated by Clp in a direct manner, requires RpfF, and is affected by stress conditions.

**METHODS**

**Bacterial strains, media and growth conditions.** Escherichia coli DH5α (Hanahan, 1983) was the host for DNA cloning. The Xcc strain Xc17 was a virulent wild-type strain isolated in Taiwan (Yang & Tseng, 1988). Strains derived from Xc17 by marker exchange included the pehA mutant MH172 isolated in this study, clp mutant AUS56 (Tseng et al., 1999) and rpfF mutant RM17F (Chiang, 2004). Luria–Bertani (LB) broth and L agar (Miller, 1972) were the general-purpose media for cultivating E. coli and Xcc at 37 and 28 °C, respectively. XOLN was a basal salt medium containing 0.625 g tryptone l⁻¹ and 0.625 g yeast extract l⁻¹ (Pu & Tseng, 1990). Glycerol (20 g l⁻¹) and polygalacturonic acid (PGA, 5 g l⁻¹) and polygalacturonic acid (PGA, 5 g l⁻¹, Sigma–Aldrich) were supplemented as required. Antibiotics were added when necessary: ampicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹), gentamicin (15 μg ml⁻¹) and tetracycline (15 μg ml⁻¹).

**DNA techniques.** Enzymes were purchased from Promega, Takara and Roche. Standard protocols were as described by Sambrook et al. (1989). PCR was carried out as previously described (Hsiao et al., 2005) using the primers listed in Table 1. DNA sequences on both

### Table 1. Primers used in this study

<table>
<thead>
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<th>Primer</th>
<th>Sequence*</th>
<th>Site†</th>
<th>Direction‡ and use</th>
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<td>5’-CTCGAGGACCCGAGAGATCGACAA-3’</td>
<td>-390</td>
<td>F, promoter analysis</td>
</tr>
<tr>
<td>-144Prtl</td>
<td>5’-CTGCAATACGGCAGCAGCACTAATTG-3’</td>
<td>-144</td>
<td>F, promoter analysis, gel retardation and complementation</td>
</tr>
<tr>
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<td>5’-CTGAGTGCGCTCTGTTTGGCAG-3’</td>
<td>-74</td>
<td>F, promoter analysis</td>
</tr>
<tr>
<td>+56Xbal</td>
<td>5’-TCTTGAAGAGGACGCGTGCCCTGTC-3’</td>
<td>+56</td>
<td>R, promoter analysis</td>
</tr>
<tr>
<td>+75Ndel</td>
<td>5’-CATATGAAAGCTGGTGTGCGGCAT-3’</td>
<td>+75</td>
<td>F, mutant construction and confirmation</td>
</tr>
<tr>
<td>+1472EcoRV</td>
<td>5’-GATATCCACGCGAGAAATGGCCGCT-3’</td>
<td>+1472</td>
<td>R, mutant construction</td>
</tr>
<tr>
<td>+1476Xbal</td>
<td>5’-TCTTGAATCTACAAGCGGAGAGTGGC-3’</td>
<td>+1476</td>
<td>R, complementation</td>
</tr>
<tr>
<td>+906</td>
<td>5’-ACTTTGAGGGGATGCTCTTG-3’</td>
<td>+906</td>
<td>R, mutant confirmation</td>
</tr>
<tr>
<td>+56B</td>
<td>5’-GAAGGAGACCGGTGCGCTT-3’</td>
<td>+56</td>
<td>R, gel retardation</td>
</tr>
</tbody>
</table>

*Added restriction sites are underlined.
†Position of the 5’ end of the oligonucleotides relative to the transcription initiation site of pehA gene.
‡F, forward direction; R, reverse direction.
strands were determined by the diodeoxy chain-termination method (Sanger et al., 1977). Transformation of E. coli was performed by the standard method (Sambrook et al., 1989) and that of Xcc by electroporation (Wang & Tseng, 1992).

Construction of PehA-expression plasmid pRKpeh and pehA mutant MH172. The PehA-expression plasmid was generated by cloning the 1620 bp DNA fragment encompassing the upstream 218 bp plus the entire coding region of the Xc17 pehA into the Psrl–XbaI sites of the broad-host-range vector pRK415 (Keen et al., 1988). The resultant plasmid, pRKpeh, contained the pehA gene in the downstream, orientated in the same direction as the lac promoter.

Procedures for construction of MH172 were as follows. The 1376 bp NdeI–XhoI fragment containing the Xc17 pehA gene was cloned in the E. coli vector pOK12 (Vieira & Messing, 1991), which contained P15A ori and could not be maintained in Xcc, giving pOKpeh. A Gm’ cartridge from pUCGM (Schweizer, 1993) was inserted into the unique HindIII site within the pOKpeh insert. The resultant plasmid, pOKpehG, was electroporated into Xc17 allowing for double crossover. Successful insertion of a Gm’ cartridge into pehA was confirmed by PCR.

Plate assay for polygalacturonase activity and pathogenicity test. Strains to be assayed were grown overnight in LB medium, diluted with sterile distilled water to an OD550 of 1, and 3 μl was deposited onto the surface of the XOLN plates containing glycerol (20 g l−1) and PGA (5 g l−1), adjusted to pH 5.0 with HCl. After 5 days of incubation, the plates were stained with ruthenium red (2 g l−1) for 1 h and destained with distilled water for 30 min. Polymer degradation by polygalacturonase depleted the plate of stain-binding material, forming clearing zones.

To test for pathogenicity, cells from an overnight culture were diluted with sterile distilled water to OD550 of 1.0, and 3 μl was used as the inoculum for the pathogenicity tests on 2-week-old potted cabbage seedlings according to previously described procedures (Yang & Tseng, 1988). The testing was carried out in three independent experiments with six replicates.

Mapping the 5′ end of pehA mRNA. The 5′ RACE (rapid amplification of cDNA ends) system (Frohman, 1993) was used to determine the transcription initiation site using the Invitrogen Version 2.0 kit. Total RNA was isolated from Xc17 (mid-exponential phase) by the Qiagen RNA extraction system. The Abridged Anchor Primer (AAP) and Abridged Universal Amplification Primer (AUAP) were used in combination with the gene-specific primers. The gene-specific primers for RT-PCR, nested PCR1 and nested PCR2 were 1506R (complementary to pehA nt 408–427, 5′-CGCTGATCAA-3′), 1614R (complementary to pehA nt 300–319, 5′-CCACGTCAGCAGTACGA-3′) and 1806R (complementary to pehA nt 108–127, 5′-CCTGGCAGGTCGCCGGAATA-3′), respectively. The PCR products were ligated into the ytA vector (Yeastern), which contained the M13 forward and reverse primers for sequence verification.

Transcriptional fusion assay. Four PpehA–lacZ transcriptional fusion constructs were generated by cloning PCR fragments into the broad-host-range promoter-probing vector pFY13–9, which used lacZ as the reporter (Lee et al., 2001). Constructs pFY–390 + 56, pFY–144 + 56, pFY–74 + 56 and pFY–25 + 56 carried nt −390/+56, −144/+56, −74/+56 and −25/+56 regions relative to the pehA transcription initiation site, respectively. Strains harbouring these constructs were grown overnight and inoculated into fresh media to obtain an initial OD550 of 0.35, after which growth was allowed to continue. Samples were taken in triplicate at intervals and the β-galactosidase activity was assayed as described by Miller (1972), with the enzyme activity expressed in Miller units.

RESULTS AND DISCUSSION

PehA is the major but not the sole polygalacturonase in Xcc

Pectate lyase and polygalacturonase can be distinguished by altering pH of the PGA-containing overlays in in-gel detection experiments (Ried & Collmer, 1985). In preliminary experiments, we deposited Xcc cells on PGA-containing plates of different pH and found that, while the wild-type Xc17 formed clearing zones at both pH 7.0 and 5.0, the pehA mutant (MH172) showed hydrolytic activities only at pH 7.0, indicating that PehA has a pH optimum near 5.0. Therefore, to detect polygalacturonase activity, Xcc cells were deposited onto XOLN plates containing PGA with the pH adjusted to 5.0. The diameters of the colonies formed by the different cells were similar (±0.6 cm), whereas the diameters of the clearing zones including the colony formed by Xc17 and MH172 were 1.5 and 0.7 cm, respectively (Fig. 1). The residual hydrolytic

![Fig. 1. Detection of pectolytic activity in different Xcc strains. Cells of Xc17 (wild-type), MH172 (pehA mutant), MH172(pRKpeh) (complemented), AU56E (clp mutant) and RM17F (pPf mutant) were tested as described in Methods. Consistent results were obtained from three experiments with triplicate samples.](http://mic.sgmjournals.org)
activity retained by MH172 was presumably due to PglA or other unknown polygalacturonase(s). In MH172 with cloned pehA, MH172(pRKpeh), wild-type-level polygalacturonase activity was restored (Fig. 1). These results indicated that PehA is the major polygalacturonase of Xcc but not the sole enzyme for metabolizing PGA. In parallel experiments, the clp mutant (AU56E) formed no significant clearing zones, whereas the rpfF mutant (RM17F) gave significantly smaller clearing zones (~1.1 cm) (Fig. 1). These results indicated that Clp and RpfF are required for expression of the pehA gene in Xcc.

Strains MH172 and Xc17 grew at comparable rates and reached similar final OD550 values in LB (6.20 vs 6.90) and XOLN (1.10 vs 1.17 with glycerol, 2.20 vs 2.27 with xylose, 2.77 vs 3.22 with glucose, and 1.19 vs 1.22 with PGA as the carbon sources). These results indicated that growth was not affected by a mutation in pehA and confirmed that it is not the sole gene for metabolizing PGA in Xcc.

To test whether the pehA gene is required for pathogenicity in Xcc, MH172 was used to infect cabbage seedlings. Symptoms appeared 7–8 days post-infection, which was about 1 day more than with Xc17 (data not shown). This suggested that pehA plays a minor role in Xcc virulence.

PehA is similar to other polygalacturonases belonging to family 28 of the glycosyl hydrolases

Xcc pehA encoded a protein of 466 aa (including signal peptide) with a calculated molecular mass of 47,452 Da and a pI of 8.31. As a secreted protein, it had an N-terminal signal sequence of 21 aa with a possible cleavage site, ASA21-122T, as predicted by Signal P software (Nielsen et al., 1997). It showed 80 and 79% identities with the X. campestris pv. vesicatoria strain 85-10 Pgl (Thieme et al., 2005) and Xanthomonas axonopodis pv. citri strain 306 Peh-1 (da Silva et al., 2002), respectively. It also shared over 30% identities with the homologous members of family 28 of the glycosyl hydrolases (Davies & Henrissat, 1995) from other bacteria including the endo-polylgalacturonase precursors of Agrobacterium viti and Erwinia carotovora subsp. carotovora, the polygalacturonase precursor of Ralstonia solanacearum, and the putative polygalacturonases of Erwinia chrysanthemi and Leifsonia xyli subsp. xyli (Herlache et al., 1997; Huang & Schell, 1990; Hugouvieux-Cotte-Pattat et al., 2002; Liu et al., 1994; Monteiro-Vitorello et al., 2004). Four amino acid groups (NTD, DD, HG and RIK), presumably involved in catalysis (Bussink et al., 1991), are conserved in these polygalacturonases and are situated at aa 243–245, 266–267, 292–293 and 331–333 in Xcc PehA, respectively.

The pehA gene possesses its own promoter and is likely to be monocistronic

The pehA gene was flanked by the upstream Xcc3460 (180 bp encoding a hypothetical protein) and the downstream Xcc3458 (1140 bp encoding the membrane-bound lytic transglycosylase), both in the same direction as pehA, with intergenic regions of 356 and 354 bp, respectively (da Silva et al., 2002). As it shares long intergenic regions with the flanking genes, Xcc pehA is likely to be a monocistronic gene.

In 5′ RACE experiments, sequencing of the fragment generated by nested PCR (237 bp) showed that nucleotide T, 74 nt upstream from the start codon, was the transcription initiation site of pehA (Fig. 2a, b). A putative

Fig. 2. (a) Mapping of the 5′ end of the Xcc pehA transcript by the 5′ RACE method. The pehA upstream region (~74 to +127 relative to the pehA start codon) was PCR-amplified using AUAP in combination with gene-specific primer 1806R (lane 2). Lane 1 contained molecular size markers. (b) Sequence of the pehA upstream region. Shown are: putative Clp-binding site (CBS), +1 (the determined transcription initiation site, TIS), predicted −10/−35 sequences, putative ribosome-binding site (RBS), start codon (ATG), and position of 1806R.
ribosome-binding site (GGTG) was present 9 nt upstream of the start codon (Fig. 2b). A possible σ70 promoter with a –10 box (TAGAGT) and a –35 box (CTGCCA) was located at –8 and –35 (with a spacer of 21 nt) relative to the transcription initiation site, respectively. A predicted Clp-binding site, CGTGNNnGCACT, with 6/10 matches (underlined bases) to the consensus sequence (Hsiao et al., 2005), was located at –117/–102 relative to the pehA transcription initiation site (Fig. 2b). Note that although the degree of identity to the consensus Clp-binding site sequence was low, the GTG motif essential for Clp binding (Hsiao et al., 2005; see below) was conserved.

Expression of pehA is induced by PGA, requires Clp and RpfF, and is subject to catabolite repression

In preliminary experiments, the four PpehA–lacZ transcriptional fusion constructs (Fig. 3a) were used to assay for pehA promoter activity in LB and samples were taken at 8, 24 and 48 h to measure β-galactosidase activity. As shown in Fig. 3(a), (i) the activities detected were between 1511 and 1755 U in Xc17(pFY–390 + 56) and between 1526 and 1841 U in Xc17(pFY–144 + 56), (ii) the activities (605–694 U) expressed from –74/+56, which included –10 and –35 boxes but not the putative Clp-binding site, were only 36% of that from –144/+56, and (iii) the activities expressed by Xc17(pFY–22 + 56) were around 20 U, which was the same as that by Xc17 carrying vector pFY13–9. These results suggested that the –144/+56 region contains the complete promoter sequence and is capable of maximal-level expression.

To evaluate regulation of pehA transcription, we measured the β-galactosidase levels in different Xcc strains carrying pFY–144 + 56 grown in XOLN containing glycerol or glucose with or without PGA. No significant differences in growth rates or final yields were observed among the strains under the same conditions (Table 2). With glycerol and PGA, the β-galactosidase levels expressed by pFY–144 + 56 reached maxima at 8 h, which were 18 954, 810 and 7816 U in Xc17, AU56E and RM17F, respectively, exhibiting 5.5-, 5.2- and 5.1-fold induction by PGA (Table 2). Since significant elevations were observed irrespective of the mutations, it appears that an independent pathway other than the Clp and RpfF systems is responsible for PGA-mediated induction. It has also been observed that considerable induction of pectate lyase activity occurs in Xcc grown in minimal medium supplemented with PGA, although the mechanisms involved have not been studied (Dow et al., 1987). In Er. chrysanthemi, the PGA-mediated induction requires an intracellular inducer, a product of PGA catabolism,
inactivate the repressor KdgR (Hugouvieux-Cotte-Pattat et al., 1996). Whether a similar regulator(s) is involved in PGA-mediated induction in Xcc will be an interesting topic for further study. With glucose and PGA, Xc17(pFY–144 + 56), AU56E(pFY–144 + 56) and RM17F(pFY–144 + 56) expressed 5158, 253 and 2091 U of  \( \beta \)-galactosidase, which represented increases of 2.5-, 3.2- and 2.3-fold over the same strains grown without PGA, but only 27.2, 31.2 and 26.8 % of that grown in glycerol plus PGA, respectively (Table 2). These observations, showing repression by glucose in all strains, suggest that expression of pehA in Xcc is subject to catabolite repression and that the repression is independent of Clp and RpfF.

The pehA promoter activity in Xc17(pFY–144 + 56) was also determined when cells were grown in XOLN containing glycerol or glucose, with or without PGA, and under a single stress condition for 8 h (Table 2). For oxygen limitation, the cultures were supplemented with fumarate (2.5 %) as an electron acceptor, overlaid with paraffin oil, and left to stand; for nitrogen starvation, tryptone and yeast extract were omitted; for high osmolarity, 0.3 M NaCl was added. Growth measurements indicated that, when compared with those under normal conditions, (i) oxygen limitation or nitrogen starvation both caused reductions in growth rate, and (ii) under high osmolarity, all strains grew at similar rates to those under normal osmolarity (Table 2). As shown in Table 2, in the absence of PGA, (i) with glycerol for growth, the pehA promoter activity at high osmolarity was the same as in normal conditions (3485 vs 3472 U) but the activity was repressed under either of the other stress conditions, and (ii) with glucose for growth, catabolite repression was observed under high osmolarity, but not the other stress conditions, as compared with the cells grown in glycerol. The results in Table 2 also show that, in the presence of PGA, (i) with glycerol for growth, the promoter under high osmolarity exhibited the same level of activity as in normal conditions (20 845 vs 18 954 U), but was repressed under either of the other stress conditions (retained only about 15 % in both cases), (ii) with glucose for growth, oxygen limitation and nitrogen starvation caused repression (38 and 41 % that under normal conditions), while the promoter activity increased 1.3-fold under high osmolarity, and (iii) catabolite repression by glucose was observed under each of the stress conditions and only 69, 66 and 32 % of the activity was retained under oxygen limitation, nitrogen starvation and high osmolarity, respectively.

Regulation of polygalacturonase genes has been studied in Er. chrysanthemi (Hugouvieux-Cotte-Pattat et al., 2002;

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Carbon source</th>
<th>Particular growth condition</th>
<th>OD&lt;sub&gt;550&lt;/sub&gt;</th>
<th>( \beta )-Galactosidase activity* (Miller units)</th>
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<tr>
<td>Glucose  + PGA</td>
<td>High osmolarity</td>
<td></td>
<td>1.67</td>
<td>6 636 ± 453</td>
</tr>
</tbody>
</table>

*Measurements were carried out at 8 h. The results are presented as the mean ± standard deviation (n=3).
Nasser et al., 1999). In glycerol-grown cells, transcription of pehV, pehW, pehX and pehN was stimulated 3- to 15-fold in the presence of PGA. This is similar to the situation in the Xcc pehA gene studied here. In these growth conditions, oxygen limitation slightly increased transcription of pehV, pehW, pehX and pehN (1.6- to 1.8-fold), while nitrogen starvation and high osmolarity reduced their expression. These observations are different from our findings in the Xcc pehA promoter. In glucose-grown cells, induction of Er. chrysanthemi pehN by PGA was 3.3- and 1.5-fold when compared with the wild-type and the crp mutant, respectively. This is different from the situation in Xcc, where the PGA-mediated induction of pehA is independent of Clp or RpfF. Taking these results together, it is apparent that the mechanisms of regulation of the bacterial genes encoding pectolytic enzymes involve complicated circuits that can differ from one organism to another.

**Gel retardation assay shows that Clp binds to the pehA promoter**

The E. coli CRP-binding site (5’-AAATGTGA-TCTAGA-TCACATT-3’) is 22 bp in length and exhibits perfect twofold sequence symmetry, with the bold bases representing the left and right arms each for the binding of one subunit of the active CRP dimer (Berg & von Hippel, 1988; de Crombrugghe et al., 1984). Using mutant sequences of the E. coli lac promoter as substrates, Clp has been shown to have the same DNA-binding specificity as CRP at positions 5, 6 and 7 (GTG motif) of the DNA half-site (Dong & Ebright, 1992). In our previous studies with the Xcc engA promoter, site-directed mutagenesis also indicated that the GTG motif of the proposed Clp core consensus sequence is essential for both DNA–protein complex formation in vitro and engA gene expression in vivo (Hsiao et al., 2005).

The finding that pehA transcription is reduced in the clp mutant (Table 2) and the presence of a predicted Clp-binding site upstream of pehA (Fig. 2b), suggested that Clp directly binds to the Clp-binding site to activate pehA transcription. To demonstrate the binding, gel retardation assays were performed using biotinylated probes, namely regions −144/+56 (probe a) and −74/+56 (probe b) relative to the pehA transcription initiation site. As shown in Fig. 3(b), Clp bound to probe a, but not to probe b, indicating that region −144−73 possesses the complete sequence for Clp to bind directly. In these experiments, it was found that 0.5 μg Clp was sufficient to form a DNA–protein complex and to retard about 50% of the engA probe (Fig. 3b, lanes 1 and 2). In contrast, no −144/+56 probe was bound using the same amount of protein (data not shown) and retardation was detectable only when more than 1.0 μg Clp was added (Fig. 3b, lane 4). This binding affinity is lower than that for the engA region and Clp. A possible explanation for this discrepancy is that the lower degree of identity shared between the Clp-binding site sequence and the consensus sequence (60% in pehA vs 90% in engA) causes a lowered binding affinity. The low binding affinity of the pehA promoter to Clp in vitro is consistent with the lower expression levels during pehA transcription (1841 U) when compared with engA in wild-type cells (8149 U) under the same conditions (Hsiao et al., 2005). It is worth noting that although the Clp-binding site matches only 60% of the consensus sequence, the GTG motif is conserved. This may explain why the pehA Clp-binding site is still capable of binding to Clp.

Recently, it has been shown by microarray analysis in another Xcc strain, XC1, that (i) deletion of clp changes expression of 299 genes, among which 86 possess a predicted Clp-binding site in the upstream region (He et al., 2007), and (ii) expression of 165 genes is affected by a mutation in rpfF (He et al., 2006). However, pehA was not included in their lists. Thus, our findings that both Clp and RpfF positively regulate pehA transcription and that the pehA promoter has a Clp-binding site have extended these previous findings. One possibility for this apparent absence is the genetic variations between strains, similar to the previously observed discrepancy in cell aggregation, i.e. it was found that a mutation in the rpfF gene causes the formation of prominent aggregates in Xcc strain 8400 but not in XC1 (He et al., 2006).

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