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.

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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 ϕ Lf (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 *prtI* (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;

Abbreviations: AUAP, abridged universal amplification primer; CBS, Clp-binding site; DSF, diffusible signal factor; PGA, polygalacturonic acid; 5' RACE, rapid amplification of cDNA ends; TIS, transcription initiation site; Xcc, *Xanthomonas campestris* pv. *campestris*.

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 *prt1*) 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 methylesterases (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://xccc.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* DH5 α (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 AU56E (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 (Fu & Tseng, 1990). Glycerol (20 g l⁻¹), glucose (20 g l⁻¹) and polygalacturonic acid (PGA, 5 g l⁻¹, Sigma-Aldrich) were supplemented as required. Antibiotics were added when necessary: ampicillin (50 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), gentamicin (15 μ g ml⁻¹) and tetracycline (15 μ g 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

Primer	Sequence*	Site†	Direction‡ and use
–390XhoI	5'-CTCGAGGAGCGCGAGGAGATCGACAA-3'	–390	F, promoter analysis
–144PstI	5'-CTGCAGTAACGCCACGCACTAACTTG-3'	–144	F, promoter analysis, gel retardation and complementation
–74XhoI	5'-CTCGAGTGCTGCTTCGTTTGCACG-3'	–74	F, promoter analysis, gel retardation
+56XbaI	5'-TCTAGAAGAAGAGACGCGTGCCGTTC-3'	+56	R, promoter analysis
+75NdeI	5'-CATATGAACTGCTTGCTGCCGCCAT-3'	+75	F, mutant construction and confirmation
+1472EcoRV	5'-GATATCCAACGCAGGAAAGTGCGGT-3'	+1472	R, mutant construction
+1476XbaI	5'-TCTAGAATCACA ACGCAGGAA AGTGCG-3'	+1476	R, complementation
+906	5'-ACTTGGAGGCGATGGTCTTG-3'	+906	R, mutant confirmation
+56B	5'-AGAAGAGACGCGTGCCGTTC-3'	+56	R, gel retardation
	(5' biotin labelled)		

*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 dideoxy 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 *PstI*–*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^r* cartridge from pUCGM (Schweizer, 1993) was inserted into the unique *HincII* site within the pOKpeh insert. The resultant plasmid, pOKpehG, was electroporated into *Xc17* allowing for double crossover. Successful insertion of a *Gm^r* 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-CGGCAGGCAT-3'), 1614R (complementary to *pehA* nt 300–319, 5'-CCAACGTCACGCCAGTAGGA-3') and 1806R (complementary to

pehA nt 108–127, 5'-CCTGGCAGGTCGCCGAATA-3'), respectively. The PCR products were ligated into the *yT&A* 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.

Gel retardation assay. The DNA probes used for gel retardation were prepared by PCR amplification of the desired *Xc17 pehA* upstream regions, using 5'-end biotinylated synthetic oligonucleotides as the primers (Table 1). The amplicons were purified from agarose gels. The recombinant Clp protein, the binding conditions, and detection procedures were as previously described (Hsiao *et al.*, 2005). Protein contents were determined by the method of Bradford (1976).

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 (*rpfF* mutant) were tested as described in Methods. Consistent results were obtained from three experiments with triplicate samples.

activity retained by MH172 was presumably due to PglA or other unknown polygalacturonase(s). In MH172 with cloned *pehA*, MH172(pRK $_{peh}$), 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-I²²T, 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-polygalacturonase precursors of *Agrobacterium vitis* 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 σ^{70} 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, CGTGGN₆GCACT, with 6/10 matches (underlined bases) to the consensus sequence (Hsiao *et al.*, 2005), was located at -117 to -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, to

Fig. 3. (a) Transcriptional fusion constructs used for deletion mapping of the *Xcc pehA* promoter region. Horizontal lines represent regions cloned to form the constructs; the region containing the putative CBS is shown as a rectangle. Numbers with pFY prefix are nucleotide positions relative to the *pehA* TIS. (b) Gel retardation assay showing dose responses of Clp binding to the *pehA* promoter region. Clp protein (0, 1 or 2 μ g as labelled above the lanes) was incubated with the biotin-labelled *pehA* promoter regions (probe a spanning -144 to $+56$, or probe b encompassing -74 to $+56$). The *engA* promoter region, -81 to $+22$ relative to the TIS, was used as the positive control. Three independent experiments were performed and similar results were obtained.

Table 2. *pehA* promoter activity in different Xcc strains containing pFY-144+56 under different conditions

Cells were grown in glycerol or glucose with or without PGA and in the presence or absence of one type of stress condition.

Mutation	Carbon source	Particular growth condition	OD ₅₅₀	β -Galactosidase activity* (Miller units)
None	Glycerol		0.73	3 472 $\pm$ 142
	Glycerol + PGA		1.02	18 954 $\pm$ 2026
	Glucose		1.45	2 039 $\pm$ 145
	Glucose + PGA		1.36	5 158 $\pm$ 366
<i>clp</i>	Glycerol		0.73	155 $\pm$ 15
	Glycerol + PGA		1.05	810 $\pm$ 75
	Glucose		1.17	79 $\pm$ 10
	Glucose + PGA		1.04	253 $\pm$ 19
<i>rpjF</i>	Glycerol		0.77	1525 $\pm$ 5
	Glycerol + PGA		0.96	7 816 $\pm$ 49
	Glucose		1.55	901 $\pm$ 6
	Glucose + PGA		1.50	2 091 $\pm$ 21
None	Glycerol	Oxygen limitation	0.33	2 194 $\pm$ 243
	Glycerol	Nitrogen starvation	0.27	1 330 $\pm$ 200
	Glycerol	High osmolarity	0.93	3 485 $\pm$ 306
	Glucose	Oxygen limitation	0.35	2 686 $\pm$ 121
	Glucose	Nitrogen starvation	0.43	1 368 $\pm$ 174
	Glucose	High osmolarity	1.39	1 891 $\pm$ 233
	Glycerol + PGA	Oxygen limitation	0.37	2 803 $\pm$ 152
	Glycerol + PGA	Nitrogen starvation	0.33	3 236 $\pm$ 203
	Glycerol + PGA	High osmolarity	1.14	20 845 $\pm$ 860
	Glucose + PGA	Oxygen limitation	0.45	1 939 $\pm$ 191
	Glucose + PGA	Nitrogen starvation	0.49	2 119 $\pm$ 301
	Glucose + PGA	High osmolarity	1.67	6 636 $\pm$ 453

*Measurements were carried out at 8 h. The results are presented as the mean $\pm$ standard deviation ($n=3$).

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 β -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;

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-TCTAGATCACATTT-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 Crombrughe *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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