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Transcription of *Xanthomonas campestris prt1* gene encoding protease 1 increases during stationary phase and requires global transcription factor Clp

Yi-Min Hsiao[a] and Yi-Hsiung Tseng[b,*]

[a] *Pesticide Chemistry Department, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Wufeng, Taichung 413, Taiwan, Republic of China*

[b] *Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan, Republic of China*

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Abstract

Xanthomonas campestris pv. campestris produces three proteases, Prt1, Prt2, and Prt3, the first two of which are involved in pathogenicity. In this study, nucleotide A 84 nt upstream of the *prt1* start codon, which is 8 nt downstream of the −10 sequence, was determined as the transcription start site by the 5′ RACE (rapid amplification of cDNA ends) method. Using P*prt1-lacZ* transcriptional fusion constructs for assays, several interesting characteristics of *prt1* promoter were revealed. The expression is inducible by LB medium or casein proteins and involves the global transcription factor Clp (cyclic AMP receptor protein-like protein). The region containing bp −392 to −80 relative to the *prt1* translation initiation codon is required for maximal expression, in which bp −392 to −207 responds to the Clp-mediated regulation and the induction. In presence of inducers and the *clp* wild-type background, the levels of expression continue to increase following cell growth until 30 h after the cultures entering stationary phase. Since *prt1* promoter shows no response to stressful conditions and neither growth nor cell viability is affected by *prt1* mutation, Prt1 appears to be a secondary metabolite of *X. campestris* pv. campestris.

Keywords: Cyclic AMP receptor protein-like protein; Promoter-*lacZ* reporter assay; Protease; Transcription; *Xanthomonas campestris*

Xanthomonas campestris pv. campestris is the Gram-negative bacterium causing black rot in crucifers [1]. In a liquid culture, this bacterium starts to accumulate great amounts of an exopolysaccharide (xanthan gum) after entering stationary phase [2]. In addition, it is capable of secreting several extracellular enzymes. These extracellular products have long been considered important virulence determinants in *X. campestris* pv. campestris [3,4], and strong evidence for the role of extracellular enzymes in the virulence has come from mutations in the secretion pathway genes resulting in the failure of enzyme secretion with a concomitant loss of virulence [5,6].

Three proteolytic enzymes have been detected in *X. campestris* pv. campestris: the serine protease PRT1 and two zinc-requiring metalloproteases PRT2 and PRT3 [7,8]. PRT1 is an inducible enzyme consisting of 580 amino acids (57 kDa) [7,9,10]. Its deduced amino acid sequence shares strong homology with the subtilisin family of serine proteases, which require a divalent metal ion for stability and activity [10]. PRT2 protease is similar to the neutral protease from *Aeromonas proteolytica* and the two extracellular metalloproteases from *Erwinia chrysanthemi*, which require Zn^{2+} for activity and Ca^{2+} for stability [7]. PRT3 is a minor protease in *X. campestris* pv. campestris [11]. A mutant lacking PRT1 and PRT2 proteases showed considerable loss of virulence when tested in turnip leaves [7].

Regulation of these protease genes is still unclear. It is only known that the protease level is drastically reduced after mutation of *clp* gene, coding for the global transcription factor Clp (cyclic AMP receptor protein-like protein), which also regulates the secretion of several other extracellular enzymes, pigmentation, xanthan polysaccharide production, pathogenicity, and the

* Corresponding author. Fax: +886-4-2287-4879.
E-mail address: yhtseng@dragon.nchu.edu.tw (Y.-H. Tseng).

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synthesis of cell surface structure required for the infection of filamentous phage ϕLf [12–14]. Therefore, towards an understanding of pathogenicity of *X. campestris* pv. campestris, we chose to study the regulation of *prt1* gene. In this study, we used *lacZ* reporter assay to localize the promoter regions of *prt1*, applied the technique of 5′ RACE (rapid amplification of cDNA ends) to map the 5′ end of the *prt1* mRNA, and showed that expression of *prt1* is inducible by casein proteins in presence of Clp. The reporter assay also showed that *prt1* expression continues to increase after the cultures entering stationary phase.

Materials and methods

Bacterial strains, plasmids, and growth conditions. X. campestris pv. campestris 17 (Xc17) was a wild-type strain isolated in our laboratory from the leaves showing symptoms of black rot [15]. *Escherichia coli* DH5α [16] was the host for DNA cloning. Luria–Bertani (LB) broth and L agar were the general-purpose media for bacterial cultivations [17]. The XOLN medium contained basal salts, 0.0625% tryptone, and 0.0625% yeast extract [18]. Glucose was supplemented at a final concentration of 20 mM. *E. coli* and *X. campestris* pv. campestris strains were grown with at 37 and 28 °C, respectively. Antibiotics were added when necessary: ampicillin (50 μg/ml), kanamycin (50 μg/ml), and tetracycline (15 μg/ml).

DNA techniques. Restriction enzymes, T4 DNA ligase and Taq polymerase were purchased from Promega. All recombinant DNA protocols and other DNA manipulations, including the conditions used for plasmid DNA extractions, restriction endonuclease digestions, agarose gel electrophoresis, and isolation and ligation of DNA fragments have been described elsewhere [19]. *E. coli* DH5α was transformed by standard methods [19] and *X. campestris* pv. campestris strains were transformed by electroporation [20]. The DNA sequences on both DNA strands were determined by the dideoxy method [21].

Mapping the 5′ end of the prt1 mRNA. The 5′ RACE (rapid amplification of cDNA ends) system [22] was used for the determination of transcription initiation site with the kit (Version 2.0) purchased from Life Technologies. Total RNA was isolated from Xc17 (mid-exponential phase) by the Qiagen RNA extraction system (Qiagen, Valencia, CA) according to manufacturer's instructions. The gene-specific primers for reverse transcription and for nested PCRs 1 and 2 were 990R (complementary to *prt1* nt 380–399, 5′-CAGGATCTG GTCGACTTCAA-3′), 950R (complementary to *prt1* nt 340–359, 5′-GCGGCCAATTGCCGCATCAG-3′), and 623xbaR (complementary to *prt1* nt 11–32, 5′-GCTCTAGACCAGTACGCTTGCGGAGAGA CG-3′). The PCR products were directly ligated into pTAdv vector (Clontech) which had the M13 forward and reverse primers for sequence verification.

Preparation of Pprt1-lacZ transcriptional fusion constructs. Nested deletions of the Xc17 *prt1* promoter region were obtained by PCR amplification with the chromosomal DNA as the template. The primers used are listed in Table 1. A *Pst*I site and an *Xba*I site were created at the 5′ ends of the forward and the reverse primers, respectively. PCR was initiated with a hot start (5 min), followed by 30 cycles of reactions, each of which including denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, which were then followed by a final extension time of 7 min. The amplicons were separately ligated into the PCR cloning vector pTAdv (Clontech). Each of the cloned fragments was confirmed to have no point mutation by DNA sequencing. The fragments were each excised from the respective plasmids with *Pst*I and *Xba*I and cloned into the multiple cloning sites upstream of the promoter-less *lacZ* gene in the promoter-probing vector pFY13-9 [23], a broad-host-range RK2 derivative. The resultant plasmids pFY–591–80, pFY–392–80, pFY–207–80, pFY–591–207, pFY–392–207, and pFY–591–392 thus contained the 512-, 313-, 128-, 385-, 186-, and 200-bp upstream regions of *prt1*, respectively (Fig. 1).

Construction of prt1 mutant. A *prt1* mutant was constructed by insertional mutation of Xc17 and designated as Xc17(prt1::pME488). For the insertion, the 488-bp *Mfe*I–*Eco*RI fragment internal to the Xc17 *prt1* gene was cloned into the multiple cloning sites of pOK12, a kanamycin-resistant P15A derivative [24] which could not be maintained in *Xanthomonas*, giving rise to plasmid pME488. pME488 was electroporated into Xc17 allowing for homologous recombination through the identical regions in the chromosome and the plasmid by a single crossover. The resultant strain Xc17(prt1::pME488) was Km^r and exhibited much reduced proteolytic activity on LB plate containing 1% skimmed milk. The residual proteolytic activity was presumably owing to the other proteases such as Prt2 and Prt3. Insertion of pME488 into *prt1* gene via a single crossover event was confirmed by Southern hybridization.

Measurement of β-galactosidase activity. Strains to be assayed for β-galactosidase activity were grown overnight and then inoculated into the same fresh media to obtain an initial OD_{550} of 0.35. Samples were taken in triplicate at intervals and the β-galactosidase activities were assayed according to the previously described methods [17]. The β-galactosidase activity of the P*prt1-lacZ* fusions was expressed as Miller units, taken the means of three independent determinations.

Results and discussion

Mapping the 5′ end of the prt1 transcript

The putative promoter has previously been predicted for the *X. campestris* pv. campestris *prt1* gene coding for protease 1, which contained a −35 sequence (TTGTCC)

Table 1
Oligonucleotide primers used in PCR for amplification of the *prt1* promoter-containing fragments

Primer	Sequence[a]	Positions[b]
1pstF	5′-AACTGCAGCTGGCCCGTGACCGCGGCATGC-3′	−591 to −564
200pstF	5′-AACTGCAGTGTCGCTGCGCCAGGAGCTGAC-3′	−392 to −371
200xbaR	5′-GCTCTAGAATCGTCACCTCGCCCGCCGGTG-3′	−392 to −413
385pstF	5′-AACTGCAGGAGACTGAACGGTGCCGTACGG-3′	−207 to −186
385xbaR	5′-GCTCTAGACGCGATGACATGCGGCGACACC-3′	−207 to −228
512xbaR	5′-GCTCTAGACGGATCGCCCCTGTTATCGATC-3′	−80 to −101

[a] Added restriction sites are underlined.
[b] Positions corresponding (for forward primers, with F) or complementary (for reverse primers, with R) to the regions relative to the translation initiation codon of the Xc17 *prt1* gene.

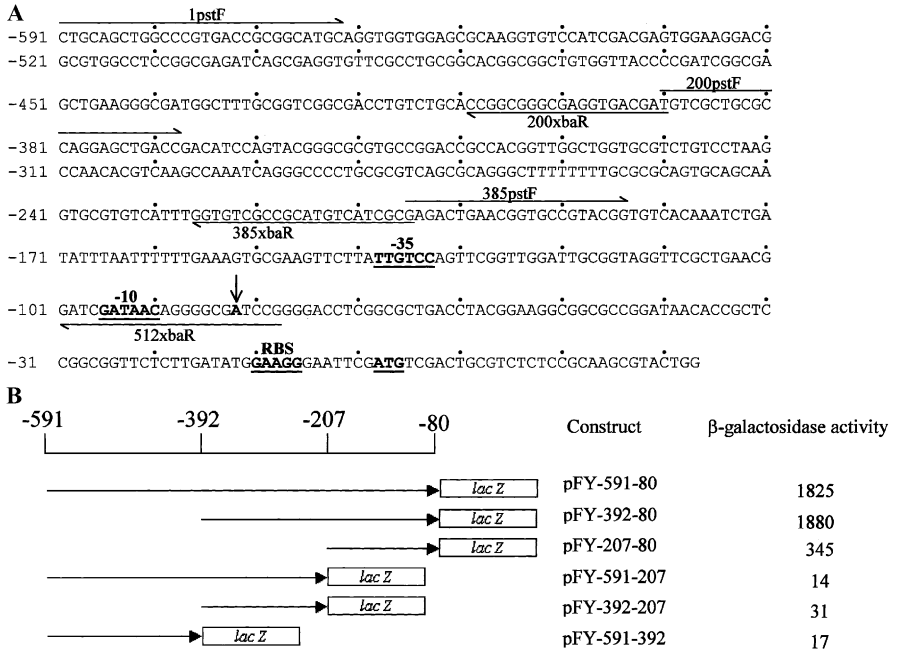

Fig. 1. (A) Sequence of the *X. campestris* pv campestris *prt1* upstream region. The transcription initiation site (+1) is indicated by a vertical arrow. The previously predicted −10 and −35 sequences [9], the start codon (ATG), and the putative ribosomal binding site (RBS) are shown in bold and underlined. Horizontal arrows indicate the positions and directions of the oligonucleotide primers that were used for PCR amplification of DNA fragments for the preparation of P*prt1-lacZ* fusion constructs. The numbering is relative to the translation initiation codon of *prt1* gene. (B) Deletion mapping of the *X. campestris* pv. campestris *prt1* promoter region. Horizontal arrows indicate the regions cloned into the promoter-probing vector pFY13-9, being placed upstream of the promoter-less *lacZ* gene to form the P*prt1-lacZ* fusion constructs. The nucleotide positions of each fragment relative to the *prt1* initiation codon are used for designating the constructs by placing the numbers after pFY. β-Galactosidase activity (Miller units) was measured in Xc17 cells (grown in LB) containing different transcriptional fusion constructs.

at −135 and a −10 sequence (GATAAC) at −93 relative to the translation initiation codon (Fig. 1A, 10). To map the transcription start site of *prt1* gene, the 5′ RACE method was used. The two fragments generated by nested PCR were 152 and 479 bp (Fig. 2). Sequencing results showed that transcription was initiated at the residue A locating 84 nt upstream from the translation initiation codon, which is eight nucleotides downstream of the −10 sequence (Fig. 1A). The distance between the transcription start site and the −10 sequence is similar to the cases normally seen in bacteria, although the 5′ untranslated region is long.

Deletion mapping of the prt1 promoter regions

To locate the promoter region of *prt1*, six overlapping fragments from the *prt1* upstream region were PCR-amplified and cloned into the promoter-probing vector, pFY13-9, forming transcriptional fusion constructs as described in Materials and methods. The longest one of these fragments (the region from −591 to −80 relative to *prt1* translation initiation codon in construct pFY–591–80) contained 4-bp downstream of the transcription start site and over 500 bp of the upstream sequence, so as to ensure that the complete promoter region was included (Fig. 1B). The resultant plasmids were electroporated into the wild-type Xc17, and the transformants were separately grown in LB medium for the measurement of β-galactosidase activity following cell growth.

Fig. 1B shows the β-galactosidase levels measured in the cultures of these clones after growing in the LB medium for 48 h. The highest levels of enzyme were detected in Xc17(pFY–591–80) and Xc17(pFY–392–80), which were 1825 and 1880 U, respectively. These two fragments may represent the regions that are capable of maximal level of *prt1* expression, suggesting that the complete promoter sequences are included in the −392/−80 region. Only a low level of β-galactosidase was expressed by Xc17(pFY–207–80), 345 U (Fig. 1B), confirming that the upstream region from −392 to −207 is also required for maximal level of expression. The remaining three clones, Xc17(pFY–591–207), Xc17(pFY–591–392), and Xc17(pFY–392–207), showed enzyme levels within the range detected in the cells containing the empty plasmid, pFY–13–9, indicating that the –591–207 region is not sufficient for any significant expression.

It was noted that the enzyme levels in the cultures of Xc17(pFY–591–80) and Xc17(pFY–392–80) increased following cell growth, and the levels kept increasing without leveling off at 30 h after entering stationary phase. The courses of increase were similar to those seen in Xc17(pFY–392–80) grown in LB or XOLN with skim-

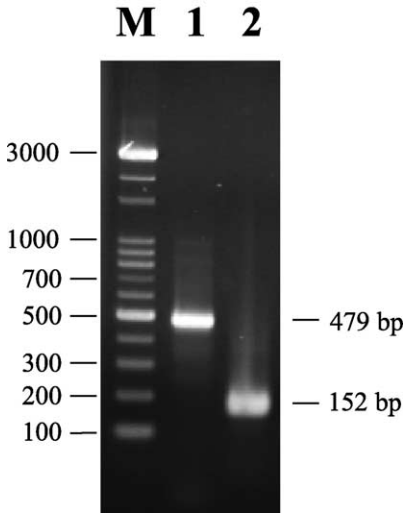

Fig. 2. Mapping of the 5′ of *prt1* transcripts by the 5′ RACE system. Fragments were amplified by PCR using Abridged Universal Amplification Primer (AUAP), supplied by Life Technologies in combination with gene-specific primer 950R (lane 1) or 623xbaR (lane 2). Lane M, molecular weight markers (100-bp DNA ladders).

med milk, as shown in Figs. 3 and 4. This observation is similar to the cases in the production of extracellular products, including among others the extracellular proteases, in some Gram-negative bacteria such as *Pseudomonas fluorescens* and *Erwinia* [25–27]. In these cases, activation of synthesis of the extracellular products involves quorum-sensing mechanism. This similarity suggests that the *X. campestris* pv. campestris *prt1* expression may also be regulated by the quorum-sensing circuit.

The prt1 promoter is induced by casein proteins

The effects of media on the level of protease activity in *X. campestris* pv. campestris have previously been evaluated, and it was shown that considerable induction of protease activity occurs in minimal medium supplemented with skimmed milk [7]. Here, we reexamined the effects of casein proteins on *prt1* gene expression with the use of the *prt1-lacZ* transcriptional fusion constructs pFY–392–80 and pFY–207–80 (Fig. 1B). The cells of Xc17(pFY–392–80) and Xc17(pFY–207–80) were cultured in the XOLN medium with or without 1% skimmed milk and the β-galactosidase activity was measured following cell growth. As shown in Fig. 3, the cultures with skimmed milk gave higher final yields (about 7 U of OD_{550}) than those without (about 3 U of OD_{550}), and the final OD_{550} of different strains were similar in the same media. In presence of casein proteins, the β-galactosidase activity in Xc17(pFY–392–80) increased following growth and reached 1810 U (Fig. 3), the same level as that detected in the same cells grown in LB after 48 h (Fig. 1B). Without skimmed milk, only 411 U of β-galactosidase was produced (Fig. 3). In contrast, cultures of Xc17(pFY–207–80) exhibited low levels of β-galactosidase activity, less than 300 U, with or without skimmed milk (Fig. 3), indicating that this region shows no significant response to the presence of casein proteins.

The above data indicate that (1) *prt1* promoter is indeed inducible by addition of casein proteins, (2) the region in response to the induction is located between −392 and −207, and (3) since the same levels of *prt1* promoter activity were induced by LB medium (containing peptides and amino acids) and skimmed milk (containing proteins and amino acids), it appears that the induction is resulted from peptides, the products generated by protease digestion, instead of a certain specific protein molecule.

Clp is required for transcription of prt1 gene

AU56E is a *clp* mutant isolated by Tn*5* insertion, whose mutation causes pleiotropic effects including, among others, defect in synthesis of xanthan polysaccharide, loss of virulence, and resistance to filamentous phage ϕLf [13,14]. In this study, we tested the hydrolytic

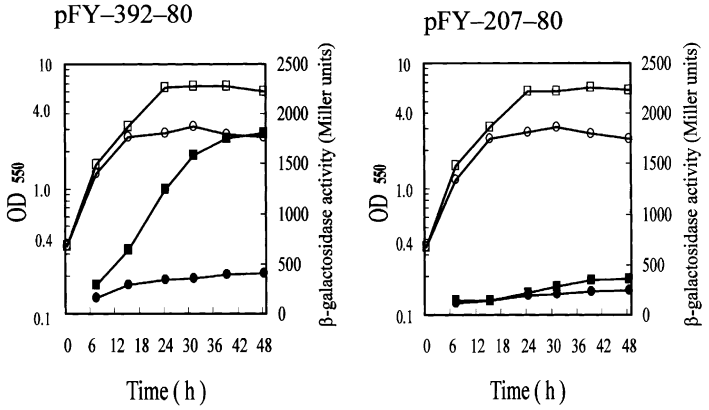

Fig. 3. Levels of β-galactosidase (filled symbols) as a function of cell growth (open symbols) in Xc17(pFY–392–80) and Xc17(pFY–207–80). Cells were grown in the XOLN medium with (□, ■) or without (○, ●) 1% skimmed milk.

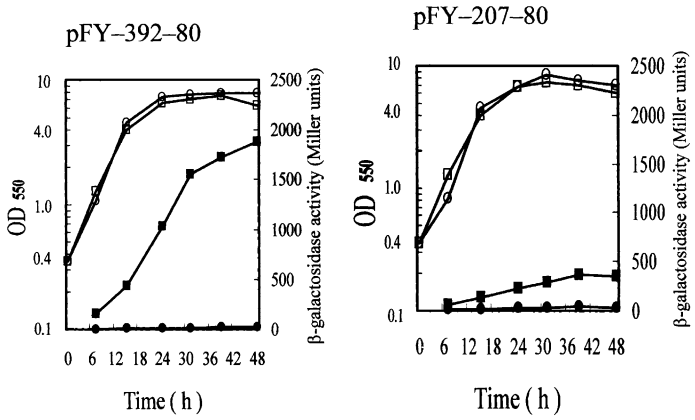

Fig. 4. Levels of β-galactosidase (filled symbols) as a function of cell growth (open symbols) in the wild-type Xc17 (□, ■) and its *clp* mutant AU56E (○, ●) containing the fusion construct pFY–392–80 or pFY–207–80.

ability of AU56E against starch, cellulose, pectin, and casein proteins in plate assays (1% final concentration). While the α-amylase activity was not affected, the other three activities were drastically reduced (data not shown). The latter data, indicating that AU56E exhibited much less proteolytic activity, are contrary to the report by de Crecy-Lagard et al. [12] that mutation in the *X. campestris* pv. campestris *clp* gene causes an elevation of protease activity by 50%.

The DNA sequence for Clp binding has been evaluated and found to be similar to that of the *E. coli* CRP-binding site (5′-AAATGTGATCTAGATCACAT TT-3′) [25]. However, no sequences showing similarity to the conserved CRP-binding site were found in the *prt1* promoter region (Fig. 1A). Therefore, it is reasonable to predict that the *prt1* promoter is regulated by a regulatory protein (a transcription factor) whose expression in turn requires the function of Clp. In other words, the Clp-mediated regulation of *prt1* expression may be achieved in a cascade manner.

We have also assayed the activity of Xc17 *clp* promoter using the *lacZ* reporter system and no differences were found between the cells grown in LB and XOLN, indicating that the level of Clp is not affected by the presence of casein proteins (Tseng et al., unpublished data). These observations suggest that regulatory function of Clp is independent of casein proteins and therefore independent transcription factors may be involved in *prt1* regulation.

To localize the region of *prt1* promoter that is involved in Clp-mediated regulation, plasmids pFY–392–80 and pFY–207–80 were separately electroporated into AU56E, then the cells were grown in LB and the β-galactosidase activity was measured following cell growth with the cells of Xc17(pFY–392–80) and Xc17(pFY–207–80) as controls. As shown in Fig. 4, all strains grew at the same rates and reached the same final yields (6.5–7.0 U of OD_{550}). These data coincide with our previous observations that *clp* mutant retains the ability to utilize different carbon sources [14]. The increases of enzyme level in Xc17(pFY–392–80) followed the same course as when the cells were cultured in XOLN plus skimmed milk, and 1880 U of enzyme activity was detected at 48 h (Fig. 4). In contrast, the β-galactosidase levels in AU56E(pFY–392–80) were as low as those with the empty pFY13-9, only 25 U (Fig. 4). These data agreed with the observation that the protease activity is drastically reduced in *clp* mutant, AU56E, and demonstrated that Clp is indeed required for the transcription of *prt1* gene. The β-galactosidase activity expressed by Xc17(pFY–207–80) was about 350 U, which was about 18 times by AU56E(pFY–207–80), 20 U (Fig. 4), suggesting that the −207/−80 region exhibited a weak response to Clp-mediated regulation. However, because the response is weak, it is possible that the region required for the Clp-mediated regulation is located in the vicinity of position −207, i.e., including also the upstream sequence. Therefore, taken together, it is reasonable to predict that the site responding to the casein proteins-mediated induction locates upstream of the site responding to Clp-mediated regulation.

The prt1 promoter is not induced by stressful conditions

Because the level of *prt1* expression kept increasing after the cells cease growing, it was obvious that the primary role PRT1 played was not in growth. We were therefore interested to know whether PRT1 plays a role which is similar to that played by the stress-inducible ATP-dependent Clp serine proteases in *E. coli* required for repairing or degrading proteins damaged during stressful conditions [29]. Therefore, we tested whether *prt1* promoter was inducible by heat shock. Strains Xc17(pFY–392–80) and Xc17(pFY–207–80) were separately grown at 28 °C to mid-exponential phase in LB (about 6 h). Then each of the cultures was divided into two parts, one was further grown at 28 °C and the other at 35 °C. Samples were taken every 15 min till 60 min for

β-galactosidase assays. At the normal growth temperature, about 126 and 54 U of the enzyme were detected for Xc17(pFY–392–80) and Xc17(pFY–207–80), respectively. After heat shock, the β-galactosidase levels were retained at the same levels, about 116 and 45 U for Xc17(pFY–392–80) and Xc17(pFY–207–80), respectively. These results suggest that the *prt1* promoter is not heat stress inducible.

Mutation in prt1 has no effect on cell viability

We were also interested to know whether the function of PRT1 is required for maintaining cell viability. To test this possibility, we isolated a *prt1* mutant, designated as Xc17(prt1::pME488), by insertional mutation as described in Materials and methods. Xc17(prt1:: pME488) was used for measurements of growth rate and the number of viable cells after the cultures had entered stationary phase. In theses experiments, the wild-type Xc17 was used as the control. The results indicated that Xc17(prt1:: pME488) grew at the same rates as Xc17 in XOLN or LB. The colony forming units counted at 8, 12, and 18 h after the cultures entering stationary phase ranged from 1.12×10^9 to 1.39×10^9 CFU/ml for Xc17(prt1:: pME488) and 1.08×10^9 to 1.28×10^9 CFU/ml for Xc17. These results indicated that neither growth rates nor viability was affected by a mutation in *prt1* gene.

Conclusion

In this study, we have carried out transcriptional analysis of the *X. campestris* pv. campestris 17 *prt1* gene, and several interesting features have been revealed. First, the region containing bp −392 to −80 is required for maximal level of *prt1* expression. Second, the global transcription factor Clp is required for expression of *prt1* in presence of casein proteins or their proteolytic products (in LB) as the inducing substances. The observation that Clp exerts a positive regulation is contrary to the previous finding of de Crecy-Lagard et al. [12] that Clp regulate *prt1* negatively. Since no consensus Clp binding sequence is present in *prt1* promoter, it appears that Clp regulation is exerted indirectly via a regulatory protein, a transcription factor, whose expression requires Clp. Third, induction of *prt1* promoter is equally efficient by either casein proteins or LB medium, suggesting that the induction is not responding to specific peptide molecules. Fourth, continuing increase in *prt1* expression after cultures entering stationary phase is similar to the situation of xanthan gum biosynthesis, in which cultures start to accumulate xanthan after entering stationary phase. It is also similar to the activation of synthesis of extracellular products in Gram-negative bacteria which involve quorum-sensing mechanism. By analogy, it is possible that expression of *prt1* also involves quorum-sensing. Fifth, since PRT1 is neither required for viability nor responding to stressful conditions, this proteolytic enzyme may simply be a secondary metabolite required for pathogenicity.

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