The Early Stages of Filamentous Phage $\phi$Lf Infection Require the Host Transcription Factor, Clp

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Abstract

*Xanthomonas campestris* pv. *campestris* produces great amounts of an exopolysaccharide (EPS), xanthan gum. Eight *eps* loci involved in biosynthesis of the EPS were previously located in the chromosome map of strain Xc17. In this study, the *eps8* region was cloned, sequenced and found to contain a *crp* homologue whose deduced amino acid sequence possesses similarity to that of the cyclic AMP receptor protein of bacteria, with the highest identity (97%) being shared with the *X. campestris* pv. *campestris* B-1459 *clp* gene previously shown to be involved in pathogenicity and regulation of the production of xanthan, extracellular enzymes, and pigment (de Crecy-Lagard V., Glaser P., Lejeune P., Sismeiro O., Barber C.E., Daniels M.J., and Danchin A., *J. Bacteriol.* 172:5877-5883, 1990). Based on sequence identity, pleiotropic effects of the mutation, the ability to complement an *Escherichia coli cya crp* mutant, and Southern hybridization detecting a single copy in the chromosome, we propose this *eps8* gene to be the Xc17 *clp*. In addition to the previously reported properties, a *clp* mutant (AU56E) cannot be plaqued with filamentous phage $\phi$Lf, although it retains the capability to support $\phi$Lf DNA replication and release authentic phage particles upon electroporation of the RF DNA. Infective center assays demonstrated that the frequency of infection is 460- to 7,500-fold lower in AU56E compared to that in the wild-type Xc17. Electron microscopy, which showed no surface appendages other than the monotrichous flagellum, confirmed that AU56E drastically diminishes in the efficiency of phage adsorption. These results suggest Clp to be regulating the biosynthesis of the primary receptor, most likely a type IV pilus. Upstream to *clp* is a homologue of the *E. coli speD* gene required for spermidine synthesis. Mutation of the *clp* flanking regions and transcriptional analyses suggest *clp* to be monocistronic and the only gene contained at the *eps8* locus.

Introduction

The gram-negative, yellow-pigmented *Xanthomonas campestris* pv. *campestris*, a member of the Pseudomonadaceae, is the plant pathogen which causes black rot in crucifers (Williams, 1980). It manifests mucoid colonies due to the production of great amounts of an exopolysaccharide (EPS), xanthan gum, which has a variety of applications in oil drilling, the food industry, cosmetics and agriculture (Sandford and Baird, 1983). Recently, we have isolated non-mucoid and low-mucoid mutants affected in EPS synthesis from *X. campestris* pv. *campestris* Xc17 (Xc17) by transposition with a Tn5 derivative, Tn5(pfm)CmKm, which carried unique sites for several rare-cutting restriction enzymes suitable for pulsed-field gel electrophoresis (PFGE)-based physical mapping (Wong and McClelland, 1992). Based on the data of PFGE and Southern hybridization, these mutants were mapped to eight *eps* (exopolysaccharide synthesis) loci on the circular physical map of Xc17. Through complementation tests, the functions for four of them have been identified: *eps1* contains the *rbCDAB-pmi* gene cluster, *eps3* encodes UDP-glucose dehydrogenase, *eps6* encodes UDP-glucose pyrophosphorylase, and *eps7* carries the gum gene cluster (Tseng et al., 1999).

$\phi$Lf is a filamentous phage specifically infecting *X. campestris* pv. *campestris* (Tseng et al., 1990). Like other filamentous phages, it has a circular single-stranded DNA (ssDNA) genome of 6.0 kb and propagates without lysis of the host cells. Several interesting properties of $\phi$Lf that are different from those of other filamentous phages are known. First, its genome contains ten genes on the viral strand, which has an organization like that of the other filamentous phages (gli-gX-gVI-gVII-gIX-gVIII-gII-gVI-gI-gXII) (Wen, 1992), but it lacks the *glV* homologue required for phage export, a function which can be complemented by the protein secretion gene *xpsD* (Wen et al., 1996). Second, different from the other filamentous phages that contain all genes on the viral strand, $\phi$Lf contains a gene (orf137) on the complementary strand presumably required for phage morphogenesis (Wang, 1993). Third, it has a mechanism to integrate its RF DNA into the host chromosome (Fu et al., 1992). Fourth, its origin of viral strand replication (ori) is contained within the coding region of *glI* (Lin and Tseng, 1996), the gene coding for the replication initiation protein (pII), instead of being contained in the major intergenic region as in other filamentous phages. Fifth, its pII contains sequence domains conserved in the superfamily I replication initiation proteins of the rolling-circle replicating replicons (Lin et al., 1996), a superfamily not including the Rep proteins of other filamentous phages (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993). Recently, we have studied the *glls* and the encoded proteins (pllls) of $\phi$Lf, *X* (a filamentous phage of *X. campestris* pv. vesicatoria), and $\phi$Xo (a filamentous...
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phage from *X. oryzae* pv. *oryzae*). These studies have shown the *glls* to be the genes responsible for phage adsorption to the host receptor (Lin et al., 1999), determining host specificity. However, the nature of the host surface structure serving as receptor remains unknown although a cluster of type IV pilus genes is required for the early steps of φLf infection (Lee and Tseng, 1999).

In this study, the mutation in locus *eps8* of *Xc17* was found to cause pleiotropic effects, including decreased production of xanthan polysaccharide, strongly reduced pathogenicity, and failure to support φLf plaquing due to the loss of the function of adsorption. Sequence analysis showed that locus *eps8* encodes a CRP homologue, belonging to a family of transcriptional factors involved in the global regulation. This CRP homologue of *Xc17* showed 97% identity in amino acid sequence to the *X. campesstri* pv. *campesstri* B1459 CLP previously shown to be involved in xanthan production, pathogenicity, and regulation of the synthesis of pigment and extracellular enzymes (de Crecy-Lagard et al., 1990; Dong and Ebright, 1992). The high degree of identity together with the data of Southern hybridization, showing that only a single copy is present, indicated that this gene is the *Xc17* clp gene. Results of transcriptional analyses suggest this *Xc17* clp gene to be monocistronically. In addition, the strand opposite to ORF1 was found to encode a speD homologue required for the synthesis of spermidine in *E. coli*.

**Results**

**Characteristics of the Non-Mucoid Mutant AU56E with a Tn5(pfpm)CmKm Insertion in Locus *eps8***

The *eps8* mutant AU56E was isolated from the wild-type *Xc17* (Ap) by transposon mutagenesis with a mini-Tn5 derivative, Tn5(pfpm)CmKm, with the gene coding for chloramphenicol acetyl transferase and neomycin phosphotransferase II. AU56E was therefore able to grow in the LB medium containing ampicillin, chloramphenicol, and kanamycin (Tseng *et al.*, 1999). The mutant was stable, since both drug resistance and the mutant phenotype were retained after repeated subculturing for many generations. On LB agar plates, AU56E formed non-mucoid colonies that were smaller in size and darker in yellow color than those of the wild-type *Xc17*. However, growth rates were similar for both strains in the XOLN medium containing sucrose, glucose, fructose, galactose, xylose or succinate, indicating that the ability to utilize various carbon sources was not affected. When the cells were grown in XOLN containing 80 mM glucose for 72 h, AU56E produced approximately 1,100 µg/ml of xanthan polysaccharide, which was about 29% of that produced by *Xc17* (ca. 3,800 µg/ml). In pathogenicity tests on cabbage leaves, *Xc17* caused severe symptoms around the cuttings 5 days after inoculation. In contrast, only very mild yellowing was caused by AU56E at 3 weeks post-inoculation (data not shown).

Phages φLf and φL7 were used routinely in our laboratory for strain verification by spot testing. Dropping with φL7 suspension formed clean clearing zones on the lawn of a susceptible strain; whereas φLf formed clean clearing zones on the lawn of a non-mucoid strain, such as P20H, but formed very turbid clearing zones on a mucoid strain such as *Xc17* due to overflowing of the viscous xanthan gum. In this study, AU56E was tested with these two phages (1.0 × 10⁸ PFU of φL7 and 5.5 × 10⁸ PFU of φLf) using *Xc17* as the control. Surprisingly, while phage sensitivity to both phages was observed in *Xc17* and to φL7 in AU56E, no clearing zone was caused by φLf on the lawn of AU56E (data not shown), suggesting that AU56E might have lost the normal function required for φLf infection.

The above-described results indicated that mutation in AU56E had caused pleiotropic effects.
Cloning of the Gene Responsible for the Mutation in AU56E

A chromosome walking strategy was employed to clone the wild-type Xc17 gene that could complement the mutation in AU56E. The cloning was accomplished in two stages, both including chromosomal integration of a plasmid by homologous recombination via single-crossover, followed by cloning the integrated plasmid along with the flanking chromosomal sequences. The steps are depicted in Figure 1. First, pOK12Tc constructed by cloning the tetracycline cartridge into pOK12, a kanamycin-resistant P15A replicon that cannot be maintained in X. campestris, was electroporated into AU56E. Since the kanamycin cartridge in pOK12Tc and the Tn5(pfm)CmKm of AU56E were of the same source (de Lorenzo et al., 1990), homologous recombination was allowed for integration of the whole plasmid. One of the resultant strains, resistant to Cm, Km, and Tc was designated as AU56E::pOK12Tc (Figure 1B). Second, Rsrl was used to digest the AU56E::pOK12Tc chromosome, since pOK12Tc and Tn5(pfm)CmKm did not contain the recognition site for Rsrl. Therefore, digestion with Rsrl would cut down the integrated Tn5(pfm)CmKm::pOK12Tc along with the flanking chromosomal sequences (Figure 1B). In Southern hybridization of the Rsrl digest using the labeled pOK12 probe, a single signal corresponding to a 15.4 kb fragment was detected (data not shown). This fragment was cut down from the AU56E::pOK12Tc chromosome with Rsrl, self-ligated by treatment with T4 ligase, and then transformed into E. coli DH5α. The recombinant plasmid thus obtained was designated pRS154 (Figure 1C). Data of restriction mapping and Southern hybridization showed that the 15.4-kb pRS154 insert included i) the sequences derived from Tn5(pfm)CmKm, ii) the integrated pOK12Tc, iii) the upstream flanking chromosomal sequence of 0.2-kb, and iv) the downstream flanking chromosomal sequence of 7.8-kb (Figure 1B). Using the probe prepared from pRS154 for hybridization, we detected one 8.0-kb fragment in the Rsrl-digested Xc17 chromosome (data not shown). This size was equal to the sum of the chromosomal sequences flanking the inserted Tn5(pfm)CmKm::pOK12Tc in pRS154. Since only one fragment was detected in the Xc17 chromosome, it appeared that there was a single copy of the cloned plasmid. Therefore, for further chromosome walking, the 0.9-kb PstI fragment at 2.0 kb downstream from the Tn5(pfm)CmKm insertion site was cloned from the pRS154 insert into pOK12 and used as the homologous region for subsequent integration into the Xc17 chromosome. The resultant plasmid, designated as pH590 (Figure 1D), was electroporated into Xc17 allowing for integration to generate strain Xc17::pH590 (Figure 1E). Fourth, the Xc17::pH590 chromosome was digested with KpnI to cut down the integrated pH590 together with the 6.0-kb upstream flanking sequence. This linear DNA molecule was self-ligated, resulting in plasmid pKN60 (Figure 1F). The 6.0-kb insert of pKN60 was subsequently cloned into the broad-host-range vector pRK415, forming pRKE60, for complementation. After electroporation, the resultant strain, AU56E(pRKE60), regained both mucoid phenotype, susceptibility to β-lactam and pathogenicity, although the AU56E(pRKE60)-infected leaves took 2 to 3 more days than those infected by Xc17 to show symptom. These results indicated that the DNA fragment cloned in pRKE60 indeed contained the wild-type gene responsible for the AU56E mutation.

Nucleotide Sequence Analysis

By Southern hybridization, we located the Tn5(pfm)CmKm insertion within the 0.4-kb HindIII-PstI fragment of the AU56E chromosome (Figure 1F). In addition, deletion mapping showed that the 1.9-kb HindIII fragment from the pKN60 insert, cloned in pRK415 to form pRK419, was still capable of complementation. Therefore, this DNA region was subcloned from pKN60 and sequenced. A total of 2,085 bp was revealed. Nucleotide sequence comparison showed that this Xc17 fragment was highly homologous to the 1,718-bp fragment from X. campestris pv. campestris B-1459. It is worth noting that the sequence of the B-1459 DNA region was first determined by de Crecy-Lagard et al. (1990) and later revised by Dong and Ebright (1992). Therefore, our sequence comparison was done with the information in the database (accession number M58745). According to the sequence analysis by de Crecy-Lagard et al. (1990), the B-1459 fragment contains two overlapping coding sequences on the same strand, an incomplete open reading frame (ORF1, nt 1-600) and the clp gene (nt 600-1,292). While no known function has been assigned for ORF1, clp encodes a cyclic AMP receptor protein (Cpr)-like protein called Clp (230 amino acid residues with an MW of 25,625), a global transcriptional factor involved in pathogenicity and regulation of the production of pigment, xanthan, and extracellular enzymes (de Crecy-Lagard et al., 1990; Dong and Ebright, 1992). The Xc17 fragment (2,085 bp) and the B-1459 fragment (1,718 bp) had an overlapping of 1,716 bp, between bp 370-2,085 of the Xc17 fragment and bp 1-1,716 of the B-1459 fragment, with a sequence identity of 98%.

As expected, an ORF homologous to the B-1459 clp gene was found, stretching between nt 969 and 1,661. This ORF, starting with ATG at five nt downstream of a consensus Shine-Dalgarno (S/D) sequence (Figure 2A), was able to encode a polypeptide of 230 amino acids with a calculated MW of 25,686. The deduced amino acid sequence shared 97% identity with that of the B-1459 Clp. At the nucleotide level, the Xc17 gene differed from the B1459 gene at thirteen positions; among which only the changes at nt positions 239 and 416 affected the codons, resulting in the conversion of His66 into Arg66 (CAC to CGC) and Val139 into Ala139 (GTT to GCT), respectively. A lower degree of sequence identity was also shared between the Xc17 gene product and several other global regulators of gene expression, including the Vfr of Pseudomonas aeruginosa (48%, [West et al., 1994]), the Crp of Haemophilus influenzae Rd. (47%, [Chandler, 1992]), and the homologues from several members of Enterobacteriaceae (around 45%, [Cossart and Gicquel-Sanzey, 1982; Schroeder and Dobrogosz, 1986; Reverchon et al., 1997; Skorupski and Taylor, 1997a; Skorupski and Taylor, 1997b]).

Unexpectedly, in the upstream region corresponding to the B-1459 ORF1 with a nucleotide sequence identity of 99%, we identified an ORF (orf254) on the opposite strand (nt 37-831 in the 2,085-bp sequenced fragment) whose predicted protein had a striking sequence similarity...
(65% identity) to the E. coli speD gene product, S-adenosyl methionine (SAM) decarboxylase (Tabor and Tabor, 1987). This speD homologue started with GTG at nine nt downstream of a putative S/D sequence complementary to the 3'-end of the X. campestris pv. campestris 16S rRNA (Lin and Tseng, 1997), and was able to encode a polypeptide of 264 amino acids with a calculated MW of 30,700 (Figure 2A). In E. coli, SpeD and SpeE, the spermidine synthase encoded by speE, are required for the biosynthesis of spermidine from SAM: SpeD catalyzes decarboxylation of SAM, then the decarboxylated SAM is reacted with putrescine by the catalysis of SpeE to form spermidine and methylthioadenosine (Tabor and Tabor, 1987). The E. coli SpeD is a 12.4-kDa protein produced from a 30.4-kDa proenzyme (264 amino acid residues) upon proteolytic cleavage between Lys\(^{111}\) and Ser\(^{112}\) (Tabor and Tabor, 1987). The same amino acid residues were found at positions \(112\) and \(113\), Lys\(^{112}\)-Ser\(^{113}\), of the predicted protein product of orf264 (Figure 2A). Based on the sequence similarity in the deduced product, orf264 was identified to be the speD homologue in X. campestris pv. campestris. Information about the role spermidine plays in bacteria is limited, and searching in database revealed that E. coli speD and speE were the only bacterial genes that had been characterized. Therefore, the X. campestris pv. campestris speD gene appeared to be a case second to...
that of E. coli.

In the spacer between speD and clp genes (137 bp), there should be two promoters running at opposite directions each required for the transcription of one of the genes. However, no sequence resembling the E. coli-type promoter was found (Figure 2A). Ten base pairs downstream from the stop codon of clp, there was an inverted repeat (nt 1,672-1,699 in the 2.1-kb sequenced fragment), which had the potential to form a stem-loop structure resembling a transcriptional termination signal.

Mutation in clp but not the Flanking Regions is Responsible for Pleiotropic Effects in AU56E

To test whether mutations in the regions flanking clp also cause pleiotropic effects, three mutants were constructed by insertional mutagenesis, which involved in vitro insertion of a 0.9-kb Gm cartridge (Schweizer, 1993) into the target gene or DNA fragment cloned in pOK12 followed by exchanging the interrupted fragment with the chromosomal wild-type copy by a double-crossover event. Mutant pSG17(clp::Gm) had an insertion at the Pst site within clp gene, TC917(speD::Gm) had an insertion at the Sac site within the speD gene, and RVG17 had an insertion at the EcoRV site locating 0.5 kb downstream of the clp gene (Figure 1F). To construct PSG17(clp::Gm), plasmid pOHC19 carrying the 1.9-kb HincII fragment (from the pKN60 insert) was used for Gm cartridge insertion to generate pOHC19G, which was electroporated into Xc17 allowing for marker exchange. Two types of transformants were obtained; one having the whole plasmid integrated was resistant to kanamycin and gentamycin, whereas the other having only the Gm cartridge integrated was resistant to gentamycin. In Southern hybridization using labeled pOHC19G as the probe, a 1.9-kb fragment was detected in the HincII-digested Xc17 chromosome, whereas the 1.9-kb fragment was enlarged to 2.8 kb in the HincII-digested PSG17(clp::Gm) chromosome, indicating the insertion of a single copy of Gm cartridge. Like AU56E, PSG17(clp::Gm) manifested non-mucoid colonies that were smaller in size and darker in yellow color than those of the wild-type Xc17, retained the ability to utilize various carbohydrate fermentation patterns, and only caused very mild yellowing after prolonged incubation in pathogenicity testing. In addition, PSG17(clp::Gm) did not form clearing zones in spot test with filamentous phage qLF. These results confirmed that clp was indeed the gene whose mutation caused the pleiotropic effects. This was further confirmed by complementation of PSG17(clp::Gm) with pRKH19 carrying the cloned Xc17 clp gene.

To construct TC917(speD::Gm), plasmid pSAM107 carrying the 0.7-kb HincII-HindIII fragment (from the pKN60 insert) was used for Gm cartridge insertion to generate pSAM107G, which was electroporated into Xc17 for marker exchange. Mutant RVG17 was constructed by inserting the Gm cartridge into the EcoRV site of the pDM12 insert, the 1.8-kb MluI fragment from pKN60 (Figure 1F), to generate pDM12G that was then double cross-overned into the Xc17 chromosome. Digests of the chromosomes from TC917(speD::Gm) and RVG17 were separately Southern-hybridized to verify that insertion of the Gm cartridge was via double-crossover. These two mutants exhibited the same phenotypes as the wild-type Xc17 in colony morphology, pigmentation, pathogenicity and phage sensitivity (data not shown), indicating clp to be the only gene whose mutation was responsible for the pleiotropic effects in AU56E.

Complementation of E. coli cya crp Mutant

It has been demonstrated that after transformation, the cloned B-1459 clp gene can partially restore the carbohydrate fermentation pattern in a cya crp E. coli mutant; fermentation of maltose, lactose, arabinose, gluconate, and ribose is restored but not of melibiose, xylene, galactose, and glycerol (de Crecy-Lagard et al., 1990). In this study, plasmid pOHC19 (with the 1.9-kb HincII fragment containing clp gene) was electroporated into cya crp mutant IT1201 and the resultant transformant was tested for the ability to grow in MacConkey agar plates containing 1% of lactose, maltose, gluconate or glycerol. The results showed that IT1201(pOHC19) regained the ability to utilize the three sugars but not glycerol. In the parallel experiments, pOHC19G with the clp being inactivated by Gm cartridge insertion was incapable of complementing the E. coli mutant, indicating that the cloned Xc17 clp is indeed the gene responsible for the complementation.

Transcriptional Analyses of clp Gene

To detect the clp transcript, we carried out Northern hybridization. Total mRNAs were prepared from a culture of Xc17 grown until the mid-exponential phase. The upstream 384-bp HindIII-PstI fragment (Figure 1F) labeled with 32P was used as the probe. In the hybridization, a transcript of approximately 800 nt was detected (Figure 2B). Since the size of this transcript was similar to that of the coding region, the clp appeared to be monocistronic.

The transcriptional initiation site of clp gene was determined by primer extension using the same mRNA sample prepared for Northern blotting as the template. The oligonucleotides complementary to nt 107-124 counting from the clp start codon was used as the primer (Figure 3). The primer lysed the E. coli chromosome by cutting it with HindIII and EcoRV, the oligonucleotides were synthesized on the 5′-end with 32P and used as the probe. The hybridization of the probe with the chromosomes revealed a transcript of approximately 800 nt. The results confirmed that clp was indeed the gene whose mutation caused the pleiotropic effects. This was further confirmed by complementation of PSG17(clp::Gm) with pRKH19 carrying the cloned Xc17 clp gene.

Figure 3. Plasmid pFYCLP. The 408-bp clp promoter region (Sac-HindIII fragment) was cloned into the multiple cloning sites of the promoter-probing vector pFY13-9, a broad host range plasmid derived from RK2. The stem-loop structure represents the E. coli thr terminator placed in front of the cloned sequence to prevent read-through from upstream. Abbreviations: lacZ, the promoter-less β-galactosidase gene as the reporter; S/D, Shine-Dalgarno sequence of lacZ; orf, origin for RK2 replication; trfA, trans-acting replication factor encoding the RepA protein; Te′, tetracycline cartridge.
Results showed that the primer extension product had a C as its 3’ end (Figure 2C). Therefore, the complementary base G locating 71 nucleotide upstream from the clp initiation codon was determined as the clp transcription start site (Figure 2A).

Promoter-probing vectors, with promoter-less β-galactosidase gene or luxAB genes as the reporter cloned in broad host range RK2 derivatives, have been constructed in our laboratory and used to detect the promoter sequences of \textit{X. campestris pv. campestris} (Weng et al., 1996; Yang, 1997). In this study, to detect the clp promoter activity, the 408-bp SacI-HindIII fragment containing the clp upstream region (Figure 1F, 2A) was cloned into pFY13-9 resulting in plasmid pFYCLP (Figure 3). Since this region presumably contained two promoters in opposite directions, one for the speD homologue and the other for the clp, sequence determination was performed to verify that the clp promoter and the reporter gene were aligned in the same direction. After verification, pFYCLP was electroporated into Xc17 and AU56E. Both strains containing pFYCLP manifested deep blue colonies on LB plates containing X-gal (40 µg/ml), but no color change was observed in strains carrying the vector pFY13-9 only (data not shown). These results indicate the presence of promoter sequences in the upstream region of the clp gene.

**AU56E Gives Extremely Low Yield of φLf in Conventional Infection but Retains Normal Capability to Support φLf Propagation**

To test for the capability of phage production in conventional infection, overnight cultures of AU56E and Xc17 were separately inoculated into the fresh LB and grown to an OD₆₀₀ of 0.2, which were then infected with φLf at an MOI of approximately 0.01 (ca. 2.0 × 10⁶ PFU/ml), a relatively low value that would not interfere with the detection of the increases of phage particles in the AU56E cultures. The phage particles released into the culture supernatants were counted following cell growth by the double-layer plaque assay using P20H as the indicator host. As shown in Figure 4A, the wild-type Xc17 was able to release high titers of phage particles following cell growth; the titer continuously increased and reached 2.0 × 10¹⁰ PFU/ml at 12 h post-infection. In other words, an increase of about 10,000-fold was observed. In contrast, the phage titers in the culture supernatants of the φLf-infected AU56E did not increase significantly during the first 6 h and increased to about 3.1 × 10⁷ PFU at 12 h post-infection, only increased by 15-fold (Figure 4A). These data indicated that although the yield was too low to form clearing zones, AU56E was still capable of phage production.

The φLf RF DNA can propagate in the host cells upon electroporation, a transfection-like process, and authentic progeny phage particles can be released afterward by the electroporated cells (Lin et al., 1994). Since the treatment skips the early steps of infection, electroporation is useful to test whether the mutant has the normal ability to support φLf propagation. In three independent experiments using Xc17 and AU56E (ca. 1.5 × 10⁷ cells/ml) for electroporation with ca. 0.75 µg RF DNA, we found that 1.2 - 4.6 × 10⁴ cells/ml had the entry of at least one RF DNA molecule, as determined by counting the infective centers among the electroporated cells. These values were within the range that we normally obtained in electroporation of \textit{X. campestris} (Wang and Tseng, 1992). As shown in Figure 4B, right after electroporation, practically no infective phage particles were detectable in the culture supernatants. However, the titers increased rapidly to about 1.4 × 10⁵ PFU/ml in both cultures within the first hour. Then at 4 h post electroporation, the increases slowed down. At 12 h

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**Figure 4.** (A) Increase of phage titer in the shaking cultures of \textit{X. campestris pv. campestris} strains infected with φLf. Cells of Xc17 (▲) and AU56E (○) grown overnight were separately diluted into 30 ml of fresh LB broth in a 250-ml flask to obtain an initial concentration of approximately 2.0 × 10⁶ cells/ml. After 30 min, 60 µl of φLf suspension (1.1 × 10⁶ PFU/ml) was added into the cultures and the changes in phage titer in the supernatants of the infected cultures were determined following cell growth. (B) Phage production by X. campestris pv. campestris strains upon electroporation with the φLf RF DNA. Cells of Xc17 (▲) and AU56E (○) subcultured from overnight cultures were grown till an OD₆₀₀ of 0.8. The cells were separately harvested, washed, and resuspended in de-ionized water (1.5 × 10⁶ cells/ml). Three hundred µl of each cell suspension was mixed with ca. 0.75 µg of the φLf RF DNA. The mixtures were then subjected to electroporation. Titers of the phage released into the culture supernatants were determined following growth of the electroporated cells.
post electroporation, the phage particles released from the electroporated AU56E reached $7.0 \times 10^7$ PFU/ml, which was about 10% of that released from the electroporated Xc17 cultures, $7.0 \times 10^8$ PFU/ml (Figure 4B). In the electroporated AU56E, RF DNA with the original size (6 kb) was detectable by the alkaline lysis method of plasmid extraction. In addition, the phage particles thus released were infective and contained ssDNA of the same size as the native phage $\phi$Lf genome (data not shown). The results that the same titers of phage were produced at 1 h post electroporation indicated that AU56E retained the normal functions for phage DNA replication, morphogenesis and export. This in turn suggested that the incapability to support the normal phage life cycle by AU56E had resulted from a defect in the early steps of infection. With this defect, none or a very low frequency of subsequent infection of the non-transfected AU56E cells by the released progeny phages could have occurred as in the wild-type cells. Presumably, for the same reason, the phage release from AU56E slowed down 1 h after electroporation, and this might explain why the lawn of AU56E could not form clearing zones in the spot test.

**Electron Microscopy Showed AU56E to Have Drastically Reduced Efficiency in Adsorbing $\phi$Lf**

It is known that *X. campestris* pv. *campestris* has monotrichous flagellum (Bradbury, 1984). The same type of flagellation was also observed in Xc17 and AU56E. In both strains, the flagellum of 0.02 mm in diameter and variable lengths was visible in approximately 80% of the cells grown on plates or in static broth, whereas only 10% of the cells grown with agitation had a flagellum (data not shown). And, no appendages other than flagellum were observed even at a 100,000-fold magnification. The result that flagellum was the only visible appendage was the same as our previous observation on Xc17 (Yang and Tseng, 1988), but was different from the cases in *X. campestris* pv. vesicatoria and *X. campestris* pv. hyacinthi, the pathovars closely related to pv. *campestris*. A bundle-forming type IV pilus has been visualized in pv. vesicatoria cells cultured with shaking until the early stationary phase (Ojanen-Reuhs et al., 1997), and a type IV pilus capable of mediating attachment to the stomata of hyacinth leaves has been observed in pv. hyacinthi cells from 4-day old static cultures (van Doorn et al., 1994).

When the $\phi$Lf-infected Xc17 was examined, about 28% of the cells were found to have phage particles adsorbed. Since the samples were washed twice during the process of sample preparation for microscopy, very few non-adsorbed phage particles were visualized on the background. On most of the adsorbed cells, multiple attachments (3-8 particles per adsorbed cell) were observed (Figure 5A, B). The frequency of adsorption observed by electron microscopy was similar to that (24-32%) determined by counting the infective centers of AU56E, $6.0 \times 10^{-4}$ to $3.7 \times 10^{-5}$ per cell, which was 460-
to 7,500-fold less efficient than that of Xc17. A frequency of phage adsorption close to the wild-type level was restored to AU56E upon introducing the clp-carrying plasmid, pRKH119, as determined by counting the infective centers (data not shown). The numbers of phage particles attached to the individual cells were less than those in Xc17 as observed in electron microscopy (Figure 5E).

**Discussion**

In this study, the gene from the Xc17 chromosomal locus eps8 previously found to be involved in xanthan biosynthesis was cloned and sequenced. Sequence analysis of the cloned DNA fragment revealed a gene that encodes a product showing similarity to the members of the CRP (cyclic AMP receptor protein) family, with the highest identity (97%) being shared with that of Cip from *X. campestris* pv. *campestris* B-1459 required for pathogenicity and regulation of the synthesis of xanthan, extracellular enzymes, and pigment (de Kreyc-Lagard et al., 1990; Dong and Ebright, 1992). Based on (1) sequence identity, (2) similar pleiotropic effects caused by the mutation, (3) the Southern hybridization data showing that a single copy is present in the Xc17 chromosome, and (4) the ability to complement an *E. coli* cya *clp* mutant, we show this gene to be the Xc17 clp. Since our work of chromosome mapping in which eight eps loci were localized (Tseng et al., 1999), this is the fifth eps locus identified for *X. campestris* pv. *campestris*. This clp gene appears to be monocistronic and the only gene in the chromosome mapping in which eight eps loci were localized. Several important observations were made concerning infection of the clp mutant AU56E by CLF, including i) AU56E is incapable of plaque formation in spot tests with CLF, exhibiting a phenotype of plage resistance, ii) in conventional infection, AU56E can still produce low titers of CLF, suggesting that CLF infection occurs but is not efficient enough for plaque formation in which multiple rounds of infection are required, iii) normal ability of plaque formation and phage production can be restored by cloned wild-type clp gene, confirming this gene to be involved in CLF infection, iv) CLF RF DNA can replicate upon electroporation into AU56E, and the electroporated cells are capable of releasing infective progeny phage particles into the culture supernatants, and v) electron microscopy and infective center assays revealed that AU56E is 460- to 7,500-fold less efficient in CLF adsorption. These observations indicate that the clp mutation results in the failure to accommodate the early stage of CLF infection, most likely due to a lack of the receptor. In addition, since the mutation causes drastic reduction in but not complete loss of the ability to adsorb phage, the role of the clp gene is characteristic of a regulatory gene rather than a structural gene encoding the receptor.

In FI phages (M13, f1, and fd), the best studied among filamentous phages, infection is a multistep process initiated by binding of the phage particle to the tip of the pilus, the primary receptor, via an interaction with the phage-encoded gene III protein (pIII) located at one end of the phage particle (Model and Russel, 1988). The tip of the phage particle is then brought to the surface of the bacterium following depolymerization of the pilus into the membrane. There the particle interacts with proteins encoded by the host gene tolQ, tolR, and tolA so as to mobilize the viral DNA into the cytoplasm. After these steps, viral DNA replication, coat proteins, and morphogenesis, phage assembly and export can take place (Model and Russel, 1988). Interestingly, Russel et al. (1988) has shown that depending on the tol functions, filamentous transducing particles of FI can bypass the pilus and infect F plasmid-free (F-) *E. coli* strains at a frequency ranging from 10⁻⁷ to 10⁻⁶ per cell. Assuming that the process of CLF infection parallels that of FI phages, our observation that normal capability for CLF propagation can be restored by electroporation of the phage RF DNA suggests that all steps from DNA replication to phage export are normally operative in AU56E. Therefore, the low phage yield suggests a defect in the primary receptor necessary for the early steps of normal infection. This was confirmed by electron microscopy, in which about 28% of the wild-type cells had phage particles adsorbed in a manner of multiple attachments, whereas only a few among 10,000 mutant cells were found to have single phage attachments. Since the infection frequency of AU56E is still much higher than that observed for tolQ-dependent M13 infection of the F- *E. coli* strain, it is possible that very limited amounts of the protein components necessary for the formation of the primary receptor can be synthesized in some of the clp mutant cells.

In addition to FI phages, several filamentous phages have been shown to have initiated their infection by attaching to a specific pilus, e.g., I pilus for If1, N pilus for IKe, and toxin-coregulated pilus of *Vibrio cholerae* for CTXφ (Bradley, 1979; Coetzee et al., 1980; Model and Russel, 1988; Waldor and Mekalanos, 1996). The early stage of CLF infection is not well studied; only the phage pIII required for adsorption has been identified (Lin et al., 1999), while the host genes required remain largely unknown. Recently, we have cloned and sequenced a cluster of seven pil genes required for CLF infection, and sequence comparison has revealed that the amino acid sequences deduced from these genes possess homology to those of the genes required for biogenesis of type IV pilis in gram-negative bacteria (Lee and Tseng, 1999), although no appendage similar to a pilus is visible on the cell surface of Xc17 by electron microscopy. This is different from the cases in *Vibrio* species that have bundle-forming type IV fimbriae and pv. *hyacinti* that has type IV pilis capable of mediating cell attachment to stomata of *hyacinth leaves* (van Doorn et al., 1994; Ojanen-Reuhs et al., 1997). Explanations for the reason that we failed to observe the pilus by electron microscopy, include i) the pv. *campestris* pilus may not be prominent enough to be visible, and ii) it is maybe too fragile to be kept intact during the process of sample preparation.
However, based on our sequence analysis of the pv. campestris pil gene cluster (Lee and Tseng, 1999), it seems reasonable to predict that ðL is used for attachment to a type IV pilus, similar to the situation in filamentous phage CI in which a type IV fimbrial gene is involved in infection although the presence of pili has not been demonstrated (Su et al., 1999).

The complex formed by cyclic AMP and CRP is a global transcriptional factor that regulates the expression of a great number of genes in Enterobacteriaceae, most notably the genes involved in carbon source utilization whose expression is subjected to catabolite repression (Botsford and Harman, 1992; Kolb et al., 1993). The cAMP-CRP system also regulates expression of pil and virulence factors in several bacteria; for examples, the Pap pil and heat-stable enterotoxin of E. coli and the virulence-associated pectinolytic genes of Erwinia chrysanthemi are positively regulated by cAMP-CRP (Goransson et al., 1989; Reverchon et al., 1997), whereas the I-sex pilus of E. coli and the cholera toxin and toxin-coregulated pilus of V. cholerae are negatively regulated by cAMP-CRP (Harwood and Meynell, 1975; Skorupski and Taylor, 1979b). The results showing that mutation in pil causes pleiotropic effects and that the cloned pil gene is capable of partially complementing the E. coli crr cya double mutant have identified the X. campestris pv. campestris Cip as a global transcriptional factor. Therefore, our observation that the Xc17 cip mutation exhibits drastically reduced efficiency of ðL adsorption indicates that synthesis of the primary receptor, i.e., a type IV pilus as predicted above, for ðL adsorption is positively regulated by Clp. Furthermore, since the general involvement of type IV pili, mediating adhesion to host tissues, in pathogenicity has been well established (Strom and Lory, 1993), concomitant impairment of pathogenicity and ðL adsorption in the cip mutant is a further indication that type IV pilus biogenesis is affected in the cip mutant AU56E.

Upstream of the Xc17 cip gene on the opposite strand, we found a gene with a high degree of identity to E. coli speD involved in spermidine synthesis. Although mutation in this speD homologue caused no effects on any of the phenotypes evaluated, our finding has documented a second speD gene for bacteria. In E. coli, speE and speD are organized in an operon under the control of a promoter located upstream of the speE initiation codon (Tabr and Tabor, 1987). Thus a different genome organization is found in X. campestris pv. campestris, since upstream to the speD homologue is the cip gene residing on the opposite strand, and no gene homologous to speE is found. It is worth noting that, in the X. campestris pv. campestris B-1459 region, corresponding to this Xc17, speD was previously proposed to be ORF1 on the opposite strand, whereas its function was unknown (de Crecy-Lagard et al., 1990). Our open reading frame prediction has revealed a true gene in this chromosomal region.
Experimental Procedures

Bacterial Strains, Phage, Plasmids and Culture Conditions

The bacterial strains, phages, and plasmids used in this study are listed in Table 1. Unless otherwise indicated, LB and L agar (Miller, 1972) were used as the general-purpose media to grow X. campestris pv. campestris (28°C) and E. coli (37°C). XOLN was a basal salt medium containing 0.0625% casein hydrolysate and 0.0625% trypine (Fu and Tseng, 1990). MacConkey agar (Miller, 1972) supplemented with 1% of a sugar was used to test the fermentation ability of E. coli. Carbon sources were autoclaved separately and added prior to inoculation at a final concentration of 20 mM. Antibiotics were added as required: ampicillin (50 mg/ml), chloramphenicol (36 µg/ml), gentamycin (15 mg/ml), kanamycin (50 µg/ml) and tetracycline (15 µg/ml).

DNA Techniques

Restriction endonucleases, Klenow enzyme, T4 polynucleotide kinase were the products of New England Biolabs. T4 DNA ligase and SuperScript™ II RNaseH reverse transcriptase were purchased from Gibco Bethesda Research Laboratories, Inc. S1 nuclease and RNase-free DNase were obtained from Promega. All enzymes were used by following the instructions accompanied. Hybond-N membrane and (32P)PATP were purchased from Amersham Life Science. For DNA manipulation, the methods described by Sambrook, 1989 were used. The 1.5 M potassium acetate/and cesium chloride precipitation and chromosomal DNA, restriction digestion, DNA ligation. (32P)-labeled probe preparation by random priming, Southern hybridization, agarose gel electrophoresis (0.8% agarose in 0.5 x Tris-acetate-EDTA buffer), and transformation of E. coli. The RF DNA of ϕLf was prepared by the alkaline lysis method of Birnboim and Doly (1979). X. campestris pv. campestris was transformed by electroporation (Wang and Tseng, 1992). Single-stranded DNA sequencing was performed by the dideoxy-chain termination method (Sanger, 1977) using a Sequenase 2.0 sequencing kit (United States Biochemical Corp.). Lasergene from DNASTAR, Inc. (Madison, Wis.) was used for DNA sequence analysis. Multiple amino acid sequence alignments were performed using the Genetics Computer Group (GGC, Madison, Wis.) package.

RNA Preparation, Northern Hybridization, and Primer Extension

The methods for RNA preparation, Northern hybridization, and primer extension have been described (Lin et al., 1999). The synthetic oligonucleotide used as the primer was 5′-GGGTCGGATAGCGCCTGCG-3′, complementary to nt 107-112 counting from the Xcl1 5′ end start codon. For comparison, a sequencing reaction was performed using plasmid pKHC15 as the template with the same primer for extension reactions.

Phage Techniques

The non-mucoid mutant P20H was used as the host for phage ϕL propagation and for titer assays following the double layer method described by Eisenstadt (Eisenstadt, 1967). ϕL was purified as previously described (Lin et al., 1999). Spot test was carried out by dropping 5 µl of a phage suspension (1012 PFU/ml of ϕL or 2.0 × 1010 PFU/ml of ϕLf) onto a lawn of the indicator cells (2.5 × 109 cells/ml) from an overnight culture that had been included in the top LB agar.

Electron Microscopy

For observation of pili, cells of Xc17 and AU566E were spread on agar plates and grown for four days or were cultured in liquid medium with or without shaking, from which cells were taken at intervals of 6 h till 30 h. Each of the samples was subjected to electron microscopy. To ensure the observation of cell appendages, we used a cell lysis method of Birnboim and Doly (1979). E. coli cells were readily visible as a positive control. For observation of cell appendages, we used electron microscopy.

Pathogenicity Test

Overnight cultures (ca. 2 × 109 cells/ml) were grown in LB broth were used as the inocula for the pathogenicity test following the procedures described previously, using 2-week old potted cabbage seedlings (Yang and Tseng, 1988).

Nucleotide Sequence Accession Number

The nucleotide sequence determined in this study has been submitted to GenBank under accession no. AF111840.

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References

Plasmid 2: 632-636.