

The Early Stages of Filamentous Phage ϕ 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 ϕ Lf, although it retains the capability to support ϕ 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* 17 (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 *rfbCDAB-pmi* gene cluster, *eps3* encodes UDP-glucose dehydrogenase, *eps6* encodes UDP-glucose pyrophosphorylase, and *eps7* carries the gum gene cluster (Tseng *et al.*, 1999).

ϕ 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 ϕ 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 (*gII-gX-gV-gVII-gIX-gVIII-gIII-gVI-gI-gXI*) (Wen, 1992), but it lacks the *gIV* 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, ϕ 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 *gII* (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 *gIIIs* and the encoded proteins (pIIIs) of ϕ Lf, ϕ Xv (a filamentous phage of *X. campestris* pv. *vesicatoria*), and ϕ Xo (a filamentous

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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, *RsrII* was used to digest the AU56E::pOK12Tc chromosome, since pOK12Tc and Tn5(pfm)CmKm did not contain the recognition site for *RsrII*. Therefore, digestion with *RsrII* would cut down the integrated Tn5(pfm)CmKm::pOK12Tc along with the flanking chromosomal sequences (Figure 1B). In Southern hybridization of the *RsrII* 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 *RsrII*, self-ligated by treatment with T4 ligase, and then transformed into *E. coli* DH5 α . 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 *RsrII*-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 gene responsible for AU56E mutation. Third, 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 pPS09 (Figure 1D), was electroporated into Xc17 allowing for integration to generate strain Xc17::pPS09 (Figure 1E). Fourth, the Xc17::pPS09 chromosome was digested with *KpnI* to cut down the integrated pPS09 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 ϕ Lf, 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 *HincII* fragment from the pKN60 insert, cloned in pRK415 to form pRKH19, 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 (Crp)-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 His⁹⁸ into Arg⁹⁸ (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 (*orf264*) on the opposite strand (nt 37-831 in the 2,085-bp sequenced fragment) whose predicted protein had a striking sequence similarity

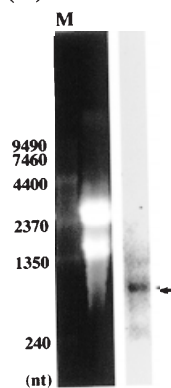
(A)

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CAGAGACCCATACATACCTGTCACTATACCGAGAACAGGTATACGGTGGTGGCCGACGCTCTACTCGAGGAACGGCCCGACGAAG 550
T E P Y T H V T I H S K D M H A V V A D S I L E K G A Q K
AACAGCTAGTGGCCGAGAAGGCTCTAGTCCTAGCAGTGGCTGCGTGGAAACACCTAGCATTAGGACTGGCGCTATAACTCCTACAAC 637
K D I V P E E S I L I T V S A G Q P D Y D Q R A I N L I N
CGCGGCTACTAAAGCCGGTGTAGGCAGTCTAGACCCAGTCTGCCAGGCGTAGCATGACGAGCAGCTACATGAGCTACATCGCGACT 724
A G I I E A V D T L I Q T L R D A D Y Q E D I Y E I Y R Q
GCGAGGAGAAGCCATGCACGTATCGTCTGCAGCATCTACAACCTCGAGTTGCGGAACCACTCCAATAACTGGGGACGTCGGACTCT 811
R E E E T R A Y C V D Y I N F S L A K T L N N F G Q L R L
                                Tss →
      ◀ speD (orf264) TCCTTTGGGAAGACGGGAAAGGGGGCAATTATGGGCCAAAGCTCTCATCAACGAAATG 898
GCTCCGTTGCCGAACCTGGTGGCCGACCGAGGAAACCTTCTGCCCTTCCCCCGTTAATACCCGGTTTCGAGAGTAGTTGCTTAC
R P L P K V M          S/D
                                S/D      clp (orf230) ▶
CCGCGTTGAGAGTGGTTTACCTTGCATGACTCATGCCGTTAAGCTTTCGGGAATTACACGCGGAACTACCATGAGCCTAGGGAACAC 985
GGCCGCAACTCTCACCAAATGGAACGTACTGAGTACGGAATTCGAAGCCCTTAATGTGCGCCTT      M S L G N T
GACGGTTGTGACTACGACGGTACGTAAACCTACCCCTCACTGACGCTGGACGCGGGCACCATTGAGCGATTCTTGGCGCACAGCCA 1072
T V V T T T V R N A T P S L T L D A G T I E R F L A H S H
CCGAGCGCTATCCGACCGGACCGATGTGTTCCGGCCGGGAGACCCCGCTGGCACCTCTACTACGTGATCAGCGCTCGGTGAG 1159
R R R Y P T R T D V F R P G D P A G T L Y Y V I S G S V S

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(B)



(C)

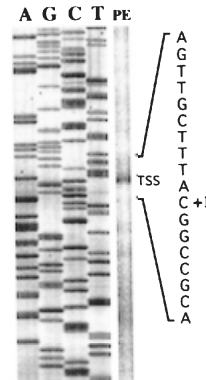


Figure 2. (A) The upstream region of *speD* and *clp* genes in *X. campestris* pv. *campestris* 17. Shown are 368-nt of the *speD* N-terminus (leftward in lower strand), 191-nt of the *clp* N-terminus (rightward in upper strand), and the 137-nt intergenic region. S/D represents the predicted Shine-Dalgarno sequence. Tss is the determined transcription start site for *clp* gene. The bold-faced K and S are the amino acid residues Lys¹¹² and Ser¹¹³ corresponding to the proteolytic cleavage site of the SpeD in *E. coli*. The blocked region is the sequence complementary to the primer used for primer extension. (B) Northern hybridization of the *clp* transcript from Xc17. Left panel is the agarose gel electrophoresis of the total RNA, and the right panel shows the Northern hybridization. Lane M contained RNA markers with the sizes shown on the left. The arrow indicates the transcript of ca. 800 nt. (C) Primer extension mapping of the Xc17 *clp* transcript 5' endpoint. The same RNA prepared for Northern hybridization was used as the template and a synthetic oligonucleotide 5'-GGGTCCGATAGCGCCTGC-3', complementary to nt 107-124 counting from the *clp* start codon, the blocked region in (A), as the primer. Markers (lanes A, G, C, and T) were created by sequencing plasmid pKHC15, containing the *HincII* fragment cloned in pUC18, with the same primer and Sequenase™. Lane PE contained the primer extension product. Tss in (A), the -71 nucleotide G, represents the transcription start site.

(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 Lys¹¹¹ and Ser¹¹² (Tabor and Tabor, 1987). The same amino acid residues were found at positions 112 and 113, Lys¹¹²-Ser¹¹³, 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*I site within *clp* gene, TC917(*speD*::Gm) had an insertion at the *Sac*I site within the *speD* gene, and RVG17 had an insertion at the *Eco*RV site locating 0.5 kb downstream of the *clp* gene (Figure 1F). To construct PSG17(*clp*::Gm), plasmid pOHC19 carrying the 1.9-kb *Hinc*II 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 *Hinc*II-digested Xc17 chromosome, whereas the 1.9-kb fragment was enlarged to 2.8 kb in the *Hinc*II-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 carbon sources, 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 ϕ Lf. 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 *Hinc*II-*Hind*III 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 *Eco*RV site of the pDM12 insert, the 1.8-kb *Mlu*I fragment from pKN60 (Figure 1F), to generate pDM12G that was then double cross-overed 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, xylose, galactose, and glycerol (de Crecy-Lagard *et al.*, 1990). In this study, plasmid pOHC19 (with the 1.9-kb *Hinc*II 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 *Hind*III-*Pst*I fragment (Figure 1F) labeled with 32 P 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

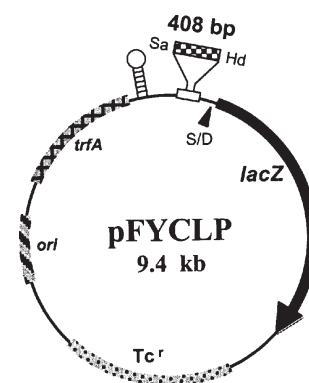


Figure 3. Plasmid pFYCLP. The 408-bp *clp* promoter region (*Sac*I-*Hind*III fragment) was cloned into the multiple cloning sites of the promoter-probing vector pFY13-9, a broad host range plasmid derived from RK2. The stem-loop structure represents the *E. coli thr* terminator placed in front of the 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*; *ori*, origin for RK2 replication; *trfA*, trans-acting replication factor encoding the RepA protein; *Tc^r*, tetracycline cartridge.

2A). 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 *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 μ g/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 ϕ Lf in Conventional Infection but Retains Normal Capability to Support ϕ Lf 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 OD₅₅₀ of 0.2, which were then infected with ϕ Lf at an MOI of approximately 0.01 (ca. 2.0×10^6 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 2.0×10^{10} 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 ϕ Lf-infected AU56E did not increase significantly during the first 6 h and increased to about 3.1×10^7 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 ϕ Lf 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 ϕ Lf propagation. In three independent experiments using Xc17 and AU56E (ca. 1.5×10^9 cells/ml) for electroporation with ca. 0.75 μ g RF DNA, we found that $1.2 - 4.6 \times 10^4$ 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 *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 1.4×10^5 PFU/ml in both cultures within the first hour. Then at 4 h post electroporation, the increases slowed down. At 12 h

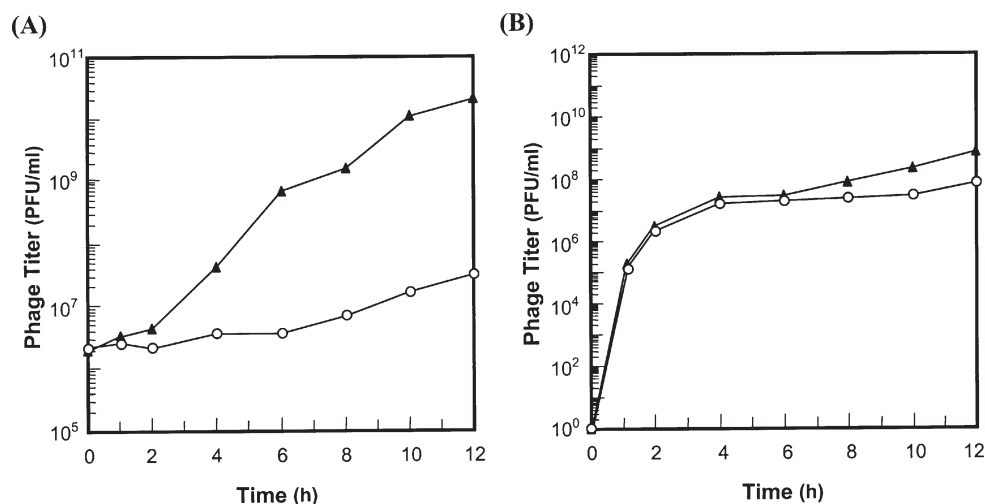


Figure 4. (A) Increase of phage titer in the shaking cultures of *X. campestris* pv. *campestris* strains infected with ϕ Lf. Cells of Xc17 (▲) and AU56E (○) grown overnight were separately diluted into 30 ml of fresh LB broth in a 250-ml flask to obtain an initial concentration of approximately 2.0×10^8 cells/ml. After 30 min, 60 μ l of ϕ Lf suspension (1.1×10^9 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 ϕ Lf RF DNA. Cells of Xc17 (▲) and AU56E (○) subcultured from overnight cultures were grown till an OD₅₅₀ of 0.8. The cells were separately harvested, washed, and resuspended in de-ionized water (1.5×10^9 cells/ml). Three hundred μ l of each cell suspension was mixed with ca. 0.75 μ g of the ϕ Lf 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.

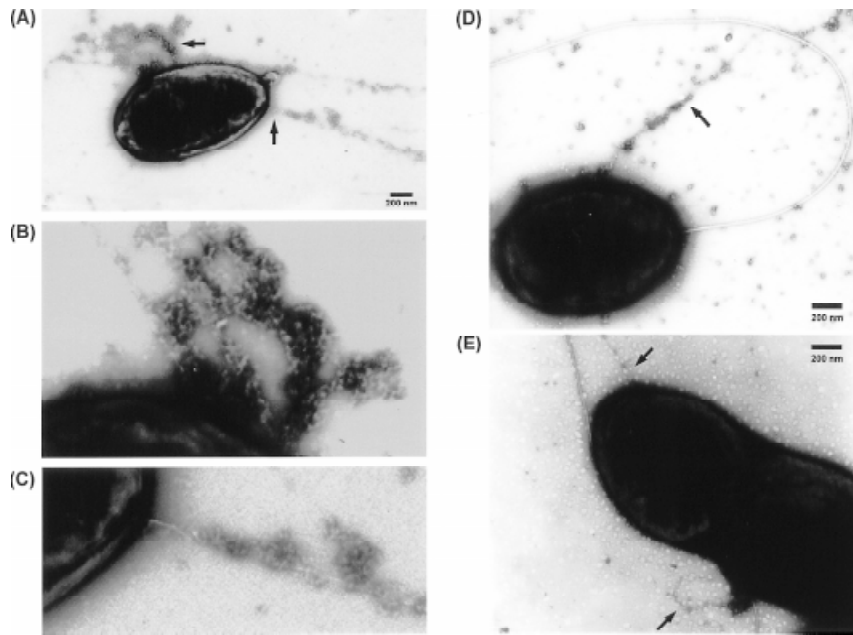


Figure 5. Transmission electron micrograph of *X. campestris* pv. *campestris* cells. The cells were grown in the liquid medium and infected with ϕ Lf at an MOI of 10, and then treated as described in Materials and Methods. (A), Xc17 infected with ϕ Lf. (B), enlargement of the upper left part of (A) indicated by a horizontal arrow, indicating the multiple attachments. (C), enlargement of the lower right part of (A) indicated by a vertical arrow, showing a single attachment. (D), AU56E infected with ϕ Lf, showing the monotrichous flagellum and the attachment of a single ϕ Lf particle (arrow). (E), AU56E(pRKH19) infected with ϕ Lf, showing the flagellum and two attached ϕ Lf particles (arrows).

post electroporation, the phage particles released from the electroporated AU56E reached 7.0×10^7 PFU/ml, which was about 10% of that released from the electroporated Xc17 cultures, 7.0×10^8 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 ϕ 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 ϕ 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 μ m 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 ϕ 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 in the parallel experiments. These efficiencies were surprisingly low comparing to the efficiency of the infection of *E. coli* TG1 with M13, in which an efficiency reaching 100% could be observed (unpublished results). When the ϕ Lf-infected AU56E was examined, only eight out of over 10,000 cells examined were found to have the phage particles attached; and with no exception, all adsorbed cells had only single attachments (Figure 5D). This value was within the range of the efficiencies determined by counting infective centers of AU56E, 6.0×10^{-4} to 3.7×10^{-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, pRKH19, 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 Clp from *X. campestris* pv. *campestris* B-1459 required for pathogenicity and regulation of the synthesis of xanthan, extracellular enzymes, and pigment (de Crecy-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 crp* 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 *eps8* whose mutation is responsible for the pleiotropic effects, because i) mutation by insertional mutagenesis of the *clp* flanking regions does not cause pleiotropic effects, ii) a transcript with a size similar to that of the *clp* coding region is detectable by Northern hybridization, iii) promoter activity is detectable in the *clp* upstream region by promoter probing assays, iv) primer extension has determined the transcriptional start site in the *clp* upstream region, and v) an inverted repeat with the potential to form a stem-loop structure resembling a transcription terminator is present downstream of the *clp* gene, suggesting that transcription is terminated here.

Several important observations were made concerning infection of the *clp* mutant AU56E by ϕ Lf, including i) AU56E is incapable of plaque formation in spot tests with ϕ Lf, exhibiting a phenotype of phage resistance, ii) in conventional infection, AU56E can still produce low titers of ϕ Lf, suggesting that ϕ Lf 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 ϕ Lf infection, iv) ϕ Lf 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 ϕ Lf adsorption. These observations indicate that the *clp* mutation results in the failure to accommodate the early stage of ϕ Lf 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 Ff 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 synthesis, 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 Ff can bypass the pilus and infect F plasmid-free (F^-) *E. coli* strains at a frequency ranging from 10^{-7} to 10^{-5} per cell. Assuming that the process of ϕ Lf infection parallels that of Ff phages, our observation that normal capability for ϕ Lf 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 Ff phages, several filamentous phages have been shown to initiate 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 CTX ϕ (Bradley, 1979; Coetzee *et al.*, 1980; Model and Russel, 1988; Waldor and Mekalanos, 1996). The early stage of ϕ Lf 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 ϕ Lf 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 pili 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 pv. vesicatoria that has bundle-forming type IV fimbriae and pv. hyacinthi that has type IV pili 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 pili 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.

Table 1. Bacterial strains, phages and plasmids used in this study

Strain, phage or plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5 α	F ⁻ <i>supE44</i> Δ <i>lacU169</i> (ϕ 801 <i>acZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan, 1983
IT1201	W3110(Δ <i>cya</i> ::Cm, <i>crp</i> ::Tn5)	Aiba, H.
<i>Xanthomonas campestris</i> pv. <i>campestris</i>		
Xc17	Wild-type strain isolated in Taiwan, Ap ^r	Yang and Tseng, 1988
P20H	Non-mucoid mutant isolated from Xc11A by mutagenesis with nitrous acid, Ap ^r	Yang <i>et al.</i> , 1988
AU56E	<i>clp</i> mutant isolated from Xc17 by Tn5(pfm)CmKm insertion, Ap ^r , Cm ^r , Km ^r	Tseng <i>et al.</i> , 1999
AU56E::pOK12Tc	AU56E-derived mutant with pOK12Tc integrated into the Km cartridge of the Tn5(pfm)CmKm in AU56E chromosome	This study
Xc17::pPS09	Xc17-derived mutant with integrated pPS09	This study
PSG17(<i>clp</i> ::Gm)	Xc17-derived mutant with a Gm cartridge inserted in the <i>Pst</i> I site within <i>clp</i> gene	This study
RVG17	Xc17-derived mutant with a Gm cartridge inserted at 0.5 kb downstream from <i>clp</i> gene	This study
TC917(<i>speD</i> ::Gm)	Xc17-derived mutant with a Gm cartridge inserted in <i>speD</i> gene	This study
Phages		
ϕ Lf	Filamentous phage of <i>X. campestris</i> pv. <i>campestris</i>	Wen, 1992
ϕ L7	Virulent tadpole-shaped phage of <i>X. campestris</i> pv. <i>campestris</i>	Su <i>et al.</i> , 1990
Plasmids		
pUC18/19	<i>E. coli</i> general cloning vector with <i>lacZ</i> α fragment, Ap ^r	Yanisch-Perron <i>et al.</i> , 1985
pUCGm	Small broad-host-range gentamycin cassette contained in pUC19 derivative	Schweizer, 1993
pOK12	<i>E. coli</i> general cloning vector derived from P15A, with <i>lacZ</i> α fragment, Km ^r	Vieira and Messing, 1991
pOK12Tc	pOK12 derivative with a Tc cartridge cloned from mini-Tn5 Tc	This study
pRS154	pOK12 derivative with an insert of 13.3-kb cloned from AU56E::pOK12Tc, including the sequences derived from Tn5(pfm)CmKm which was inserted in the <i>clp</i> gene, a pOK12Tc integrated in the Tn5(pfm)CmKm via the two kanamycin cartridge, the upstream flanking chromosomal sequence of 0.2-kb, and the downstream flanking chromosomal sequence of 7.8-kb	This study
pPS09	pOK12 carrying the 0.9-kb <i>Pst</i> I fragment internal to the pRS154 insert, which was 2.0-kb downstream from <i>clp</i> gene	This study
pKN60	pOK12 derivative with an insert of 6.0 kb, containing <i>speD</i> and <i>clp</i> genes, cloned from Xc17 chromosome	This study
pKHC15	pUC18 carrying an insert of 1.9-kb <i>Hinc</i> II fragment, containing <i>clp</i> , from Xc17 chromosome	This study
pOHC19	pOK12 derivative with an insert of 1.9-kb <i>Hinc</i> II fragment containing the Xc17 <i>clp</i> gene	This study
pOHC19G	pOHC19 derivative with a Gm cartridge inserted in the unique <i>Pst</i> I site of the insert	This study
pDM12	pOK12 derivative containing the 1.8-kb <i>Mlu</i> I fragment, downstream of <i>clp</i> , cloned from pKN60	This study
pDM12G	pDM12 derivative with a Gm cartridge inserted in the unique <i>Eco</i> RV site of the pDM12 insert	This study
pSAM107	pOK12 derivative with an insert of 0.7-kb <i>Hinc</i> II- <i>Hind</i> III fragment cloned from pKN60	This study
pSAM107G	pSAM107 derivative with a Gm cartridge inserted in the unique <i>Sac</i> I site within <i>clp</i> gene	This study
pRK415	Broad-host-range gram-negative cloning vector derived from RK2, with <i>lacZ</i> α fragment, Tc ^r	Keen <i>et al.</i> , 1988
pRKE60	pRK415 derivative carrying the 6.0-kb insert from pKN60	This study
pRKH19	pRK415 derivative with the 1.9-kb <i>Hinc</i> II fragment containing the Xc17 <i>clp</i> gene	This study
pFY13-9	Promoter-probing vector derived from pRK415, using <i>lacZ</i> as the reporter	Yang, 1997
pFYCLP	pFY13-9 derivative with the 408-bp <i>Sac</i> I- <i>Hind</i> III fragment containing the <i>clp</i> promoter cloned in the upstream of the promoter-less <i>lacZ</i> gene	This study

However, based on our sequence analysis of the pv. *campestris pil* gene cluster (Lee and Tseng, 1999), it seems reasonable to predict that ϕ Lf infects through attachment to a type IV pilus, similar to the situation in filamentous phage Cf 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 pili and virulence factors in several bacteria; for examples, the Pap pili and heat-stable enterotoxin of *E. coli* and the virulence-associated pectinolysis 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, 1997b). The results showing that mutation in *clp* causes pleiotropic effects and that the cloned *clp* gene is capable of partially complementing the *E. coli crp cya* double mutant have identified the *X. campestris* pv. *campestris* Clp as a global transcriptional factor. Therefore, our observation that the Xc17 *clp* mutation exhibits drastically reduced efficiency

of ϕ Lf adsorption indicates that synthesis of the primary receptor, i.e., a type IV pilus as predicted above, for ϕ Lf 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 ϕ Lf adsorption in the *clp* mutant is a further indication that type IV pilus biogenesis is affected in the *clp* mutant AU56E.

Upstream of the Xc17 *clp* 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 *clp* 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, whose 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% tryptone (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 [γ -³²P]ATP were purchased from Amersham Life Science. For DNA manipulation, the methods described by Sambrook *et al.* (1989) were used, which included preparation of plasmid 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 × Tris-acetate-EDTA buffer), and transformation of *E. coli*. The RF DNA of ϕ Lf 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 *et al.*, 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 (GCG, 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'-GGGTCGGATAGCGCCTGC-3', complementary to nt 107-124 counting from the Xc17 *clp* 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 ϕ Lf propagation and for titer assays following the double layer method described by Eisenstark (Eisenstark, 1967). ϕ Lf was purified as previously described (Lin *et al.*, 1999). Spot test was carried out by dropping 5 µl of a phage suspension (1.1×10^{11} PFU/ml of ϕ Lf or 2.0×10^{10} PFU/ml of ϕ L7) onto a lawn of the indicator cells (2.5×10^8 cells) from an overnight culture that had been included in the top LB agar.

Electron Microscopy

For observation of pilus, cells of Xc17 and AU56E 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 *E. coli* DH5 α bearing pili that were readily visible as a positive control. For observation of phage adsorption, overnight cultures of Xc17, AU56E, and the complemented strain AU56E(pRKH19) were harvested by centrifugation ($8,000 \times g$ for 5 min at 4°C), inoculated into fresh LB broth (initial OD₅₅₀ of 0.3), and grown for 12 h. The cells were pelleted and then resuspended in LB medium (ca. 1.5×10^9 cells/ml). Aliquots of 1.0 ml were dispensed in the 1.5-ml microcentrifuge tubes and chilled on ice for 5 min, then 0.15 ml of ϕ Lf suspension was added at an MOI of 10. After 30 min on ice, the mixtures were each pelleted by centrifugation at $3,000 \times g$ for 5 min at 4°C, washed twice with cold deionized water, and then resuspended in deionized water (5×10^9 cells/ml). The samples were separately dropped onto grids (300 mesh), which had been treated by coating with Formva and one drop of 0.1% bacitracin. After 1 min, the cells were stained with 2% uranyl acetate for 15 sec, and then examined under a JEOL JEM-1200EX II electron microscope at an operating voltage of 100 kV. The experiments were repeated three times, each time with the samples prepared from fresh cultures. In each experiment, three grids were prepared for each sample and 40 meshes, each containing 180 to 250 cells, of each grid were examined.

Aliquots of the phage ϕ Lf infected samples, prior to dropping onto the grids, were taken and immediately subjected to infective center assays to

determine the numbers of cells that had been infected among the whole population.

Determination of Xanthan Concentration

The cells from overnight cultures were inoculated into fresh XOLN medium containing 80 mM glucose, at an initial OD₅₅₀ of 0.35, and grown for 72 h. The cultures were diluted 2-fold and the cells were removed by centrifugation ($15,000 \times g$, 15 min). The xanthan polysaccharide in the supernatants was precipitated with 70% ethanol in the presence of 40 mM NaCl. The amount of xanthan was measured by the anthrone method as described previously (Fu and Tseng, 1990).

Pathogenicity Test

Overnight cultures (ca. 2×10^9 cells/ml) 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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