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A *Xanthomonas campestris* pv. campestris Protein Similar to Catabolite Activation Factor Is Involved in Regulation of Phytopathogenicity

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A DNA fragment from *Xanthomonas campestris* pv. campestris that partially restored the carbohydrate fermentation pattern of a *cya crp Escherichia coli* strain was cloned and expressed in *E. coli*. The nucleotide sequence of this fragment revealed the presence of a 700-base-pair open reading frame that coded for a protein highly similar to the catabolite activation factor (CAP) of *E. coli* (accordingly named CLP for CAP-like protein). An *X. campestris* pv. campestris *clp* mutant was constructed by reverse genetics. This strain was not affected in the utilization of various carbon sources but had strongly reduced pathogenicity. Production of xanthan gum, pigment, and extracellular enzymes was either increased or decreased, suggesting that CLP plays a role in the regulation of phytopathogenicity.

Xanthomonas campestris pv. campestris is the causal agent of black rot of crucifers, one of the most serious worldwide diseases of cruciferous crops (47). It produces an extracellular polysaccharide, xanthan gum (23), used in a variety of food and industrial applications as a viscosifying, thickening, stabilizing, or suspending agent (40). *X. campestris* pv. campestris has been studied in some detail as a model organism for investigation of the genetics of bacterial phytopathogenicity. Use of molecular genetic tools (9, 44) has led to the cloning of genes essential for xanthan synthesis (20, 43) and production of protease (42), cellulase (17), and polygalacturonate lyase (11), as well as genes that code for positive and negative regulators of extracellular enzyme production and secretion (9). Mutations in any of these genes affect virulence, apparently without affecting bacterial growth ex planta. The precise role of the factors in the disease process is not understood. There is much interest in the regulation of pathogenicity gene expression.

In procaryotes, gene activity is controlled mainly at the level of RNA transcription. In *Escherichia coli* K-12, cyclic AMP (cAMP) and its receptor protein (CRP), also named catabolic activator protein (CAP), are known to modulate transcription initiation or termination for numerous genes (45). The cAMP-CAP complex regulates a variety of functions in *E. coli*, such as induction of synthesis of many catabolic enzymes, repression of several biosynthetic and regulatory operons, and synthesis of structural components of the cell envelope (45). Cross-reacting proteins have been detected by immunological tests in a great variety of gram-negative bacteria (3). The gene that encodes CAP (*crp*) has been cloned and sequenced in three enteric species, i.e., *E. coli* (1, 5), *Salmonella typhimurium* (6), and *Shigella flexneri* (6), and the respective sequences are nearly identical.

An attempt to identify a cAMP-CAP regulatory control factor in *X. campestris* pv. campestris led us to the cloning and sequencing of an *X. campestris* pv. campestris gene that codes for a protein highly similar to CAP in sequence. A strain of *X. campestris* pv. campestris unable to synthesize this protein was constructed by reverse genetics and appeared to be severely reduced in phytopathogenicity, probably as a result of reduction in the synthesis of xanthan, protease, and polygalacturonate lyase, which are known to be necessary for full expression of pathogenicity (11, 17).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *E. coli* and *X. campestris* pv. campestris strains and plasmids used in this work are listed in Table 1. *E. coli* growth medium was either complete LB medium or synthetic M63 medium supplemented with appropriate carbon sources (0.2% [wt/vol]), thiamine (10 μg/ml), and the required amino acids (40 μg/ml). *E. coli* fermentation tests were performed on MacConkey agar plates containing a 1% concentration of the appropriate carbohydrate (29). *X. campestris* pv. campestris growth medium was either complete LB medium or synthetic MM1 medium (33) supplemented with appropriate carbon sources (0.2% [wt/vol]). Agarose (10 g/liter) was used in minimal solid medium since residual growth occurred on agar solid medium with no added carbon source. *X. campestris* pv. campestris was grown at 30°C. Antibiotics were used when required, and their respective concentrations were 100 μg/ml for kanamycin, 100 μg/ml for ampicillin, and 20 μg/ml for tetracycline. For β-galactosidase assays, cells were grown in M63 medium containing 1 mM isopropyl-β-D-thiogalactopyranoside as the inducer and 0.2% pyruvate as the carbon source. When indicated, cAMP and cyclic GMP were added at a 1 mM concentration. Samples of growing cultures were withdrawn at different densities (A_{600}, 0.1 to 0.5), and β-galactosidase activity was determined as described by Pardee et al. (31).

Sequencing. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (35). Single-stranded DNA templates were derivatives of the vectors pTZ18 (27) and tg131 (24) carrying the 1.7-kilobase (kb) cloned insert. Nested deletions extending into the DNA insert from the end proximal to the sequencing primer-

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
C600SF8	F⁻ *thr-1 leuB6 lacY1 rfbD1 thi-1 tonA21 hsdR hsdM recBC lop-11 lig*⁺	39
TP610	C600SF8 *cya-610*	22
TP2339	F⁻ *xyl argH1 lacX74 cya-854 crp-39*	33
X. campestris pv. campestris strains		
NRRLB1459	Sm^r	23
XC1501	NRRLB1459 *clp* Kan^r	This work
Plasmids		
pTZ18	Amp^r	27
pBR322	Tet^r Amp^r	4
pBScrp2	pBR322 *crp* Amp^r	5
pDIA5100	pTZ18 *clp*	This work
pDIA4807	pTZ18 *clp*::Kan^r	This work

binding site (either the universal primer for tg131 derivatives or the reverse primer for pTZ18 derivatives) and generating overlapping clones were performed as described by Dale et al. (7).

Transformation and electroporation. *E. coli* was transformed as described by Mandel and Higa (26). Electroporation of *X. campestris* pv. campestris was performed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) essentially as described for *E. coli* electroporation by Dower et al. (12), with the following modifications. The cells were harvested at an A_{600} of 1.0 and grown for at least 2 h at 30°C after electroporation before plating. The transformant yield was dependent on the sensitivity to *X. campestris* pv. campestris DNA restriction of the plasmid used. The optimum yield of 10^6 CFU/μg of DNA obtained with pSUP106 (37) fell to 5×10^2 CFU/μg with pSA206 (10).

***X. campestris* pv. campestris pigment and xanthan extraction and characterization.** The acetone crude-pigment extraction procedure described by Starr (38) was used. The culture medium for xanthan production estimation was LB medium supplemented with 0.2% glucose. Xanthan was extracted after 9 days of culture. The procedures for extraction and determination of the viscosity and composition (glucuronic, acetic, and pyruvic acid contents) of xanthan were performed as described by Tait et al. (41).

Virulence tests. Bacterial growth in seedlings and pathogenicity for turnips (*Brassica campestris*) and radishes (*Raphanus sativus*) were tested as described by Daniels et al. (8, 9) and Gough and al. (17).

Extracellular enzyme detection. Proteolytic activity was detected on milk agar plates as described by Tang et al. (42). Cup-plate assays for polygalacturonate lyase and cellulase were performed on NYGA-PG and CMC plates as described by Dow et al. (11) and Gough et al. (17), respectively. Amylase was assayed on LB plates containing 0.1% starch developed with iodine fumes after 24 h of incubation at 37°C.

RESULTS

Cloning of an *X. campestris* pv. campestris DNA fragment that confers a Mal⁺ phenotype on a *cya crp E. coli* strain. The adenylate cyclase (*cya*)-deficient strain *E. coli* TP610 is unable to catabolize several carbohydrates (maltose, lactose, and melibiose; for a complete list, see reference 45). To clone a putative *cya* gene, restoration of the ability of TP610 to utilize maltose by transformation with a library of *X. campestris* pv. campestris DNA was attempted. Chromosomal DNA fragments of *X. campestris* pv. campestris resulting from partial *Sau*3A digestion were ligated into the unique *Bam*HI site of pTZ18 and transformed into strain TP610. Transformed bacteria were then plated onto MacConkey plates containing maltose as the indicator carbon source and ampicillin as the selecting agent. Among 50,000 transformants, two clones able to catabolize maltose (red on this medium) were obtained. Each harbored a plasmid which was extracted and characterized. One plasmid contained a 6-kb segment of *X. campestris* pv. campestris DNA, and the other contained a 1.7-kb DNA fragment which derived from the 6-kb segment. This plasmid was kept for further study and named pDIA5100. To assess whether this clone harbored a *cya* gene, it was transformed into a *crp cya*-defective strain (a *cya* gene would be unable to restore maltose fermentation in such a background). pDIA5100 partially complemented the carbohydrate-negative phenotype of the *crp cya* strain *E. coli* TP2339 (restored fermentation of maltose, lactose, arabinose, gluconate, and ribose and not of melibiose, xylose, galactose, and glycerol). These observations indicated that the cloned gene did not encode adenylate cyclase but could encode an activator of transcription. To quantify the effect of this activator on the CAP-cAMP-dependent *lac* operon, β-galactosidase assays were performed. The presence of pDIA5100 in *cya* strain TP610 resulted in a 10-fold increase of β-galactosidase synthesis, thus restoring 25% of the activity present in isogenic *cya*⁺ parental strain C600SF8 (Table 2). No activation of β-galactosidase synthesis by cAMP or cyclic GMP was detected in *cya* strain TP610, which harbored plasmid pDIA5100 (no difference in the rate of β-galactosidase synthesis whether pDIA5100 was present or not when cAMP was added to the external medium).

TABLE 2. β-Galactosidase activity of a *cya E. coli* strain containing the *clp* gene

Strain	Plasmid	β-Galactosidase activity[a] with addition of:		
		Nothing	cAMP	cyclic GMP
C600SF8[b]	None	2,200	2,830	2,030
TP610[c]	None	50	1,134	49
TP610	pBSCrp2[d]	48	3,865	93
TP610	pDIA5100[e]	533	1,203	483

[a] Expressed in Miller units (29).
[b] Wild-type *E. coli* strain.
[c] *cya E. coli* strain.
[d] *crp* gene on a multicopy plasmid.
[e] *clp* gene on a multicopy plasmid.

Nucleotide sequence of the cloned gene: identification of a CAP-like protein. The nucleotide sequence of the cloned 1,718-base-pair DNA fragment was determined (Fig. 1). Two overlapping coding sequences were identified on one strand, the first one, named open reading frame 1 (ORF1), started at the beginning of the fragment and ended at position 603, the second one, named ORF2, started at position 600 and ended at position 1292. A putative ATG start codon was identified for ORF2 at position 600 to 602 overlapping the stop codon of ORF1 (601 to 603). No similarity with known promoter consensus sequences of *E. coli* was detected. It is, however,

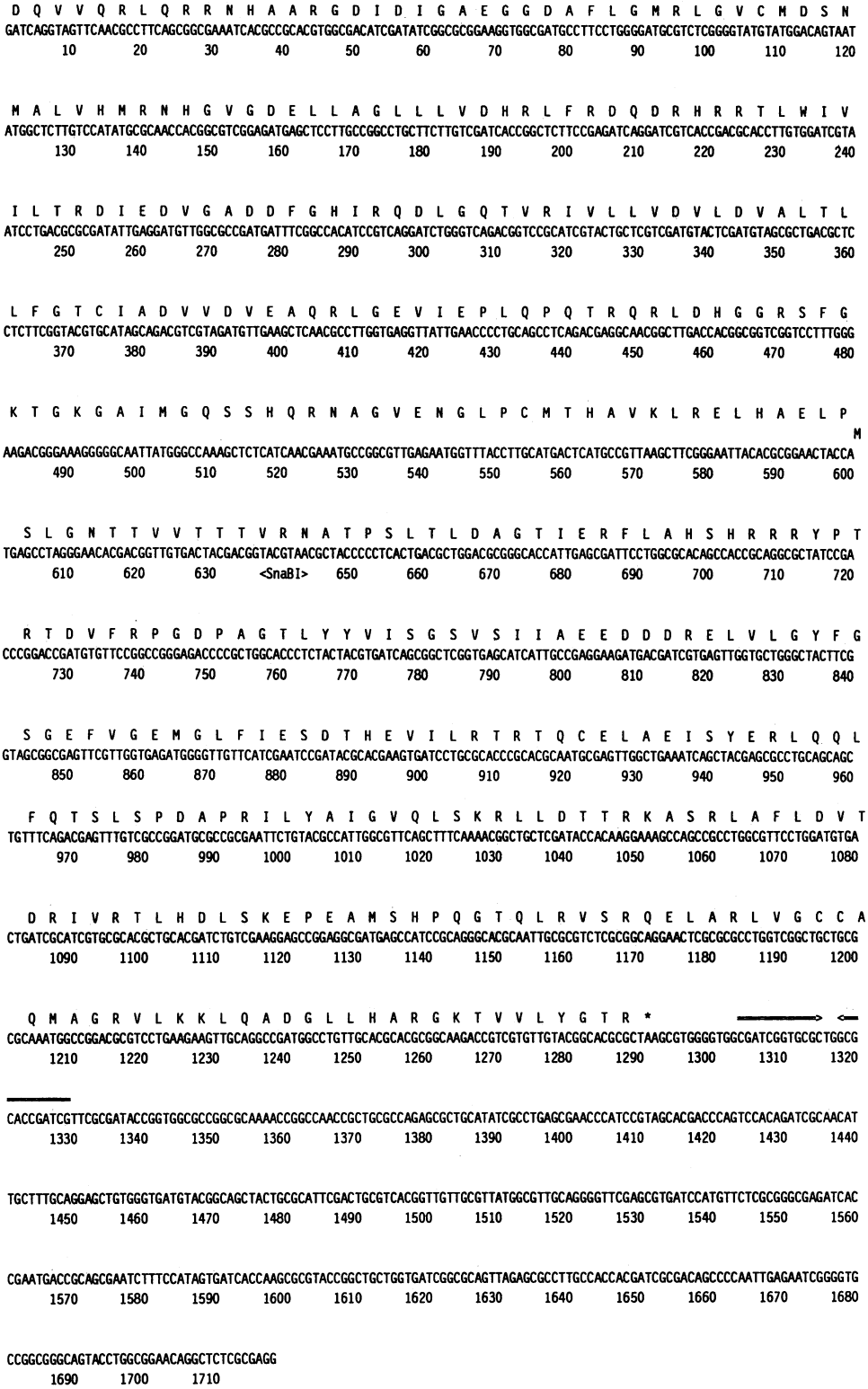

FIG. 1. Nucleotide sequence of a 1,718-base-pair region containing the unknown CDS1 and the gene *clp*. Deduced amino acid sequences are indicated. The putative end codon is shown by an asterisk. Nucleotides showing palindromic symmetry are indicated by opposing arrows. Restriction sites are indicated between brackets.

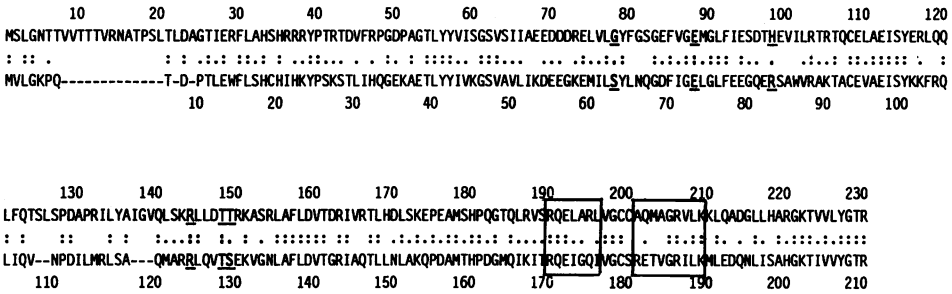

FIG. 2. Comparison of amino acid sequence of CLP and *E. coli* CAP deduced from nucleotide sequences. Identical and similar amino acids are indicated by colons and periods, respectively. The amino acid residues which are supposed to be implicated in the cAMP-binding site in CAP are underlined. The helix-turn-helix motif is boxed.

likely that these two genes constitute the last genes of an operon. Their expression in *E. coli* must be under the control of the *lac* promoter, for ORF1 is in frame with the *lacZ* gene, thus allowing coupled translation of the two genes. To check this hypothesis, we cloned the entire 1.7-kb fragment in the *Sca*I site of pBR322. This plasmid no longer complemented *cya* strain TP610. Finally, 10 base pairs downstream from the ORF2 stop codon lay a sequence (a long inverted repeat) which could correspond to a transcription termination signal.

The deduced amino acid sequence of the corresponding gene products is shown in Fig. 1. These sequences were used to scan the protein libraries at the Unité d'Informatique Scientifique by using FASTP software (25). No similarities were found between the ORF1-encoded protein and any known protein sequence. On the other hand, a striking similarity between the ORF2-encoded protein and the *E. coli* CAP was revealed by this analysis. The comparison (Fig. 2) revealed 45% identical residues and 75% conservative replacements between the two proteins (on the basis of the criteria of Schwartz and Dayhoff [36]). This protein was therefore named CLP (CAP-like protein), and the corresponding gene was named *clp*. A model of the cAMP-binding pocket of CAP has been proposed by Gronenborn et al. (18). All of the amino acid residues involved in the binding interactions are conserved (Fig. 2). The CAP DNA-binding domain has been studied extensively (30). The helix-turn-helix motif is conserved in CLP (Fig. 2). Specific contacts between CAP and the DNA sites have been identified (30), i.e., Arg-181, Glu-182, Lys-188, and Arg-186. The last two residues are conserved in CLP (Lys-209 and Arg-206). The first arginine is changed into alanine in CLP, and it has been showed that substitution of Arg-181 with alanine in CAP eliminates specificity at base pair 5 of the DNA half-site (48). Glu-182, which is known to be implicated in specificity at base pair 7 (13), is replaced by a glutamine in CLP. Many mutations in the *crp* gene (*crp** and *crp*^{i}) which allow CAP to act in the absence of cAMP have been characterized and sequenced (2, 15, 19, 21, 28). Several differences between CAP and CLP can be found at amino acid residues which, when mutated in CAP give rise to a cAMP independence phenotype. One particular change can be noted: Gly-142 of CAP is changed to aspartic acid in CLP, and this very mutation is known to confer a Crp* phenotype on CAP (2).

Construction of an *X. campestris* pv. campestris CLP-deficient strain by reverse genetics. To study the function of CLP in *X. campestris* pv. campestris, a *clp*-deficient mutant was constructed by reverse genetics. In the first step, the *clp* gene cloned in *E. coli* was interrupted by an antibiotic resistance marker. A kanamycin resistance cartridge (from pUC4K [46]) digested by *Hin*cII was inserted into the unique *Sna*BI restriction site present in the *clp* gene (Fig. 2). As expected, the resulting plasmid (pDIA4807) could no longer restore the growth of *cya crp* strain TP2339 on maltose. *X. campestris* pv. campestris was then electrotransformed (see Materials and Methods) by 1 μg of pDIA4811 DNA and plated on LB plates containing kanamycin as the selecting agent. Being a narrow-host-range plasmid, pTZ18 cannot replicate in *X. campestris* pv. campestris. Stable transformants were obtained only if a recombination event had occurred. Thirty-three clones were thus obtained; 4 were still Amp^{r}, whereas 29 were Amp^{s}. This suggested that 88% of the transformants had integrated the Kan^{r}-encoding gene only by a double-crossover event leading to disruption of the gene, whereas 12% had integrated the whole plasmid by a single recombination event. This was confirmed by Southern blotting experiments (data not shown). One Kan^{r} Amp^{s} *X. campestris* pv. campestris mutant (XCV1501) was thus characterized.

Characterization of strain XCV1501. The growth of strain XCV1501 was tested on MM1 plates supplemented with glucose, fructose, mannose, xylose, maltose, mannitol, cellobiose, galactose, or succinate. No differences from wild-type strain NRRLB1459 were noticed. Growth rates were similar for both strains, but XCV1501 gave rise to smaller and more pigmented colonies on LB plates (data not shown). The Clp^{-} phenotype of *X. campestris* pv. campestris thus did not parallel the Crp^{-} phenotype of an *E. coli* strain. Since *X. campestris* pv. campestris is a phytopathogenic bacterium, we compared production of virulence-associated factors and virulence properties in the mutant and wild-type strains.

Pigment extraction from pyruvate-grown cells by acetone revealed a 50% increase of pigment production per cell in the mutant strain. Xanthan production (amount and quality) of the two strains is compared in Table 3. XC1501 produced 30% less xanthan than its parent. The viscosity of the xanthan produced by the mutant strain and its pyruvic acid content (with glucuronic acid content as the standard) decreased by 65 and 50%, respectively. No difference in acetic acid content was found. Extracellular enzyme production was tested by cup-plate assays (Table 4). Protease production increased by 50% in the mutant strain, but cellulase and polygalacturonate lyase production decreased by 85 and 70%, respectively. Amylase production was unchanged. These results indicate that all of the pathogenicity factors tested seem to be positively or negatively affected by CLP mutation. Whether this is direct or indirect is not known.

Requirement of CLP for pathogenesis for plants. Pathogenicity tests were performed on *clp* strain XCV1501 and

TABLE 3. Comparison of xanthan characteristics[a] in the wild-type and Clp⁻ strains

Strain	Polymer concn (g/kg)[b]	Final viscosity (centipoise)	Viscosifying ability (centipoise/g per kg)	Pyruvic acid content (%)	Acetic acid content (%)
NRRLB1459	12.2	365	30	3.3	3.65
XC1501	8.6	90	10.5	1.5	3.25

[a] The different parameters were determined as described in Materials and Methods.
[b] Grams per kilogram (wet weight) of packed cells.

wild-type strain NRRLB1459. The strains were inoculated into turnip seedlings at 10^4 to 10^6 bacteria per seedling. Seedlings inoculated with XCV1501 showed no symptoms of damage (Table 5).

Radish seeds were soaked in a suspension of either XC1501 or NRRLB1459. Scored after 7 days on the arbitrary scale (described in the footnote to Table 6), the mutant strain was found to be much less aggressive than its wild-type parent strain. The pathogenicity of both strains on mature turnip leaves was also tested. Bacteria were allowed to infiltrate the intercellular spaces of leaves at different inoculum levels. Rotting and tissue chlorosis were caused by infiltration of 10^9 to 10^7 CFU of the wild-type strain per ml, with local damage at lower bacterial concentrations. The mutant strain induced only local damage at all of the concentrations tested (data not shown). Thus, with all of these assays, the wild-type strain was found to be significantly more aggressive than the mutant.

To check whether the phenotype of strain XC1501 was due to disruption of the *clp* gene and not to another event leading to reduced virulence of the wild-type strain, the marker exchange experiment was reproduced several times. Twenty Amp^r Kan^r and 20 Amp^s Kan^r strains were isolated. All of the Amp^r strains had a wild-type phenotype, whereas all of the Amp^s strains had a mutant phenotype (small yellow colonies, reduced production of cellulase and polygalacturonate lyase). These results confirm that the phenotype of strain XC1501 is due to disruption of the *clp* gene and not to secondary mutations.

DISCUSSION

An *X. campestris* pv. campestris gene able to restore catabolism of several carbohydrates by a *cya crp* strain of *E. coli* was cloned. Sequencing revealed a high percentage of identity (45%) between the protein encoded by this gene and the catabolite activator protein (CAP) of *E. coli* encoded by gene *crp*. In *E. coli*, the complex between CAP and cAMP is a global regulatory element which coordinately controls the expression of operons scattered on the chromosome (16). Particular mutations in gene *crp* (*crp** and *crp*^{i} mutations) (2, 15, 19, 21, 28) have been shown to produce modified CAPs which no longer need cAMP to activate some of the CAP-sensitive operons. The *clp* gene which was isolated from *X. campestris* pv. campestris directs synthesis of a protein which behaves like CAP mutants of the latter class in that it is able to restore growth on several carbon sources (such as lactose, maltose, ribose, arabinose, and gluconate). CLP activates only part of the catabolic operons, and this could be due to changes in the protein structure and changes in the specificity of recognition of its DNA target site. CLP is very similar to CAP; we failed, however, to detect either a significant cAMP concentration in *X. campestris* pv. campestris or any effect of cAMP on growth (data not shown). Furthermore, binding of radioactive cAMP to CLP was also found to be very weak ($K_a > 15$ mM). This raises the question of CLP descent. Has it recently evolved from a protein able to bind cAMP, or are there other binding substrates which would modulate its activity? It may be significant in this respect to recall the effects on catabolic operons expression in *E. coli* (activation of the arabinose operon) of indoleacetic acid described by Ebright and Wong (14), especially if one remembers that these molecules are universal in plant physiology.

To characterize the CLP effects further, a Clp⁻ strain of *X. campestris* pv. campestris was obtained by using an efficient method of insertional mutagenesis similar to that described by Ruvkun and Ausubel (34). Surprisingly, the Clp⁻ strain was not impaired in the utilization of any carbon source assayed. A search for other phenotypic alterations was therefore undertaken, and we tested the pathogenicity of the *X. campestris* pv. campestris *clp* mutant. The strain was pleiotropically affected in production of all of the phytopathogenicity factors tested: extracellular enzymes, pigment, and xanthan gum. Virulence, quantified by the radish seed inoculation test (a good all-round test for pathogenicity, since it reproduces the natural mode of infection

TABLE 4. Relative extracellular enzyme activities of the wild-type and Clp⁻ strains

Strain	Relative activity of:			
	Amylase[b]	Protease[c]	Cellulase[b]	Pectinase[b]
NRRLB1459	1	1	1	1
XC1501	0.9	1.5	0.15	0.28

[a] Enzyme activities were tested by radial diffusion assays (see Materials and Methods for plate composition). The diameters of hydrolysis areas were measured and related to the numbers of bacteria at the ends of the cultures.
[b] For amylase, cellulase, and pectinase activities, samples of overnight culture supernatants were deposited on the appropriate plate and incubated for 18 h at 30°C.
[c] For protease activity, samples of overnight culture supernatants were used and incubation was at 30°C for 48 h.

TABLE 5. Inoculation of aseptic turnip seedlings with a needle carrying bacteria

Strain and no. of CFU inoculated/seedling	No. of seedlings showing symptoms[a]		
	++	+	−
NRRLB1459			
10^6	1	5	1
10^5	4	1	1
10^4	0	0	7
XC1501			
10^6	0	0	6
10^5	0	0	6
10^4	0	0	9

[a] The seedlings were scored after 2 to 7 days on the following scale; ++, complete collapse and rotting; +, some local damage at the inoculation site but no spread; −, no symptoms.

TABLE 6. Test of pathogenicity of *X. campestris* pv. campestris strains by inoculation of radish seeds

Strain	No. of seedlings showing a score[a] of:			
	0	1	2	3
NRRLB1459	0	2	6	13
XC1501	6	7	12	0

[a] Scored after 7 days on the following scale: 0, no symptoms; 1, black spots on leaves or cotyledons; 2, some rotting of leaves or cotyledons; 3, rotting spreading down stem. The mean scores were as follows: NRRLB1459, 2.52; XC1501, 1.24.

[17]), was reduced in the mutant strain. It therefore seems that a protein very similar in sequence to CAP directly or indirectly regulates a set of genes implicated not in carbon source catabolism but in phytopathogenicity, as though the structure of the regulator, but not the target, had been conserved.

The altered pathogenic powers of the *clp* mutant may be due to the altered characteristics of xanthan gum, as a close correlation has been described between gum viscosity and virulence (32). Synthesis of all of the extracellular enzymes together with xanthan gum production is activated by a cluster of genes that have been cloned in a plasmid (9). At least seven genes seem to be involved in the process. Those which have been sequenced have the features of two-component regulatory systems, and some of the genes appear to regulate the synthesis of others in the cluster (M. J. Daniels, A. E. Osbourn, and J. L Tang, *in* B. J. J. Lugtenberg, ed., *Molecular Signals in Microbe-Plant Symbiotic and Pathogenic Systems*, in press; J. L. Tang, Ph.D. thesis, University of East Anglia, Norwich, United Kingdom, 1989). An unlinked set of genes negatively regulates the synthesis of enzymes and xanthan gum. It interacts in a still poorly understood manner with the positive regulators (Tang, Ph.D. thesis). In spite of this already complex organization, there is circumstantial evidence derived from complementation experiments suggesting the presence of another, unrelated regulatory system (M. K. Sawczyc and M. J. Daniels, unpublished data). CLP could be part of the latter regulatory network, since it seems to be a regulator of a higher level: it controls not only extracellular enzyme production but xanthan gum and pigment production, and it seems to regulate both positively and negatively.

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