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The Cyclic AMP Receptor Protein Is the Main Activator of Pectinolysis Genes in *Erwinia chrysanthemi*

SYLVIE REVERCHON,[1] DOMINIQUE EXPERT,[2] JANINE ROBERT-BAUDOUY,[1] AND WILLIAM NASSER[1]*

Laboratoire de Génétique Moléculaire des Microorganismes et des Interactions Cellulaires, CNRS UMR 5577, INSA Bat 406, 69621 Villeurbanne cedex,[1] and Laboratoire de Pathologie Végétale, INRA, 75231 Paris cedex 05,[2] France

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The main virulence factors of the phytopathogenic bacterium *Erwinia chrysanthemi* are pectinases that cleave pectin, a major constituent of the plant cell wall. Although physiological studies suggested that pectinase production in *Erwinia* species is subjected to catabolite repression, the direct implication of the cyclic AMP receptor protein (CRP) in this regulation has never been demonstrated. To investigate the role of CRP in pectin catabolism, we cloned the *E. chrysanthemi crp* gene by complementation of an *Escherichia coli crp* mutation and then constructed *E. chrysanthemi crp* mutants by reverse genetics. The carbohydrate fermentation phenotype of the *E. chrysanthemi crp* mutants is similar to that of an *E. coli crp* mutant. Furthermore, these mutants are unable to grow on pectin or polygalacturonate as the sole carbon source. Analysis of the nucleotide sequence of the *E. chrysanthemi crp* gene revealed the presence of a 630-bp open reading frame (ORF) that codes for a protein highly similar to the CRP of *E. coli*. Using a *crp::uidA* transcriptional fusion, we demonstrated that the *E. chrysanthemi* CRP represses its own expression, probably via a mechanism similar to that described for the *E. coli crp* gene. Moreover, in the *E. chrysanthemi crp* mutants, expression of pectinase genes (*pemA*, *pelB*, *pelC*, *pelD*, and *pelE*) and of genes of the intracellular part of the pectin degradation pathway (*ogl*, *kduI*, and *kdgT*), which are important for inducer formation and transport, is dramatically reduced in induced conditions. In contrast, expression of *pelA*, which encodes a pectate lyase important for *E. chrysanthemi* pathogenicity, seems to be negatively regulated by CRP. The *E. chrysanthemi crp* mutants have greatly decreased maceration capacity in potato tubers, chicory leaves, and celery petioles as well as highly diminished virulence on saintpaulia plants. These findings demonstrate that CRP plays a crucial role in expression of the pectinolysis genes and in the pathogenicity of *E. chrysanthemi*.

The phytopathogenicity of the pectinolytic erwiniae is chiefly due to their capacity to synthesize and secrete depolymerizing enzymes which degrade the major components of plant cell walls. Among these enzymes, pectate lyases (Pel) play a major role since, when purified, they provoke plant tissue maceration (13).

Erwinia chrysanthemi 3937 synthesizes multiple isoforms of pectinases, including two pectin methylesterases (encoded by the *pemA* and *pemB* genes) (31, 53), five major isoenzymes of pectate lyases (encoded by the *pelA*, *pelB*, *pelC*, *pelD*, and *pelE* genes), and a set of minor pectate lyases (encoded by, e.g., *pelL* and *pelZ*) (34, 45). These pectinases cleave pectin and mainly generate unsaturated digalacturonates which are transported into the bacterium, where they are catabolized by the products of the genes *ogl*, *kduI*, *kduD*, *kdgK*, and *kdgA* (14, 28). In addition, *E. chrysanthemi* produces two cellulases, EGZ and EGY, encoded by the *celZ* and *celY* genes, respectively.

Genetic and physiological studies indicate that pectinase synthesis is regulated by a wide range of environmental conditions (26): presence of pectin-degradative products or plant extracts, anaerobiosis, temperature, nitrogen starvation, osmolarity, iron availability, and growth phase. Therefore, it appears that the regulation of *E. chrysanthemi pel* genes is very complex and may require various regulatory genes (25). In contrast, expression of the genes of the intracellular part of the pectin catabolism pathway is modulated only by pectin degradation products (16).

To identify the regulatory components controlling production of depolymerizing enzymes in *E. chrysanthemi* 3937, a search for regulatory mutants has been initiated. This approach allowed for the identification of three loci involved in the regulation of pectinase genes: *kdgR*, *pecS-pecM*, and *pecT*. The *kdgR* gene product represses expression of all genes involved in pectin catabolism (16, 49). In vitro experiments showed that the KdgR repressor specifically binds to a 17-bp motif present in front of the regulated genes. Moreover, specific binding of the KdgR protein to its operators is inhibited in the presence of 2-keto-3-deoxygluconate (KDG), an inducer of pectinolysis (41–43). The *pecS-pecM* locus controls the synthesis of the pectinases, the cellulase EGZ, and some Out proteins involved in pectinase and cellulase secretion and the production of a blue pigment (46, 50). Subcellular fractionation revealed that PecS is located in the cytoplasm whereas PecM is anchored in the inner membrane. Based on this result, sensor and regulator functions were assigned to PecM and PecS, respectively (50). PecS is a member of the MarR family, which consists of bacterial regulatory proteins involved in sensing phenolic compounds (54). The *pecT* product is a protein of the LysR family, which negatively regulates some *pel* genes. The precise mechanism by which PecT exerts its control has not been elucidated, but this protein probably acts independently of KdgR and PecS (55).

Pectate lyase production in *Erwinia* species is also subjected to cyclic AMP (cAMP)-controlled catabolite repression (24).

* Corresponding author. Mailing address: Laboratoire de Génétique Moléculaire des Microorganismes et des Interactions Cellulaires, CNRS UMR 5577, INSA Bat 406, 20 Avenue Albert Einstein, 69621 Villeurbanne cedex, France. Phone: (33) 04 72 43 80 88. Fax: (33) 04 72 43 87 14. E-mail: lgmm@cismibm.univ-lyon1.fr.

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Genotype or description[a]	Source or reference
E. coli		
NM522	Δ(*lac-proAB*) *thi hsd-5 supE* [F′ *proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15]	Stratagene
M182Δ*crp*	Δ*lacX74 galK galU rpsL* Δ*crp*	8
DC1282	*crp*::Cm^{r}	D. Clark
K38	HfrC	56
E. chrysanthemi		
3937	Wild-type strain isolated from *Saintpaulia ionanthia*	Laboratory collection
A350	*lmrT*^{c} *lacZ2*	Laboratory collection
A837	*lmrT*^{c} *lacZ2*, *kdgR*	16
A2494	*lmrT*^{c} *lacZ2*, *crp*::*uidA*-Km^{r}	This work
A2507	*lmrT*^{c} *lacZ2*, *crp*::Cm^{r}	This work
A2518	*lmrT*^{c} *lacZ2*, *kdgR*, *crp*::Cm^{r}	This work
Plasmids		
pBR322	Ap^{r} Tc^{r}	Laboratory collection
pBluescript	Ap^{r} *lacZ′*	Stratagene
pT7-5, pT7-6	Ap^{r}	56
pWN2155	pT7-6 derivative harboring the 0.9-kb *Ava*I-*Mam*I fragment containing the *E. chrysanthemi crp* gene under the T7 promoter	This work
pUIDK1	pBR322 derivative harboring a *uidA*-Km^{r} cassette	5
pWN1989	pBR322 with the 8.4-kb *Sau*3A fragment harboring the *E. chrysanthemi crp* gene	This work
pWN1990	pBR322 with the 2.5-kb *Nsi*I-*Sal*I fragment harboring the *E. chrysanthemi crp* gene	This work
pWN1991	pBR322 with the 0.9-kb *Ava*I-*Mam*I fragment harboring the *E. chrysanthemi crp* gene	This work
pULB110	RP4::Mu3A, Ap^{r} Tc^{r}	59
Phage		
φEC2	*E. chrysanthemi* generalized transducing phage	47

[a] Genotype symbols are according to Bachmann (4). *lmrT*^{c} indicates that the transport system encoded by the gene *lmrT*, which mediates entry of lactose, melibiose, and raffinose into the cells, is constitutively expressed. *lacZ′* indicates that the 3′ end of this gene is truncated.

Catabolite repression can be observed during growth in the presence of glucose and also in the presence of pectin catabolic products. For instance, high concentration of unsaturated digalacturonate exerts cAMP-reversible self-catabolite repression on pectate lyase production (12, 58). In addition, a *cya* mutant of *Erwinia carotovora* that cannot produce cAMP is defective in pectate lyase synthesis (39). These physiological studies have suggested that, in addition to the specific regulators, the expression of the pectinase genes is also modulated by some global regulatory proteins, such as the cAMP receptor protein (CRP). The direct implication of CRP in the expression of the pectinolysis genes was also suggested by the identification of a putative CRP binding site in the regulatory region of various pectinolysis genes (15, 21, 48). Moreover, modification by site-directed mutagenesis of the putative CRP operator located in the *E. chrysanthemi* EC16 *pelE* regulatory region dramatically reduced *pelE* expression (21). However, these observations represented indirect evidence and did not firmly establish whether CRP is required for expression of the pectinolysis genes. Therefore, we decided to investigate in more detail the role of CRP in *E. chrysanthemi* 3937, which is an important model for studies on the regulation of the complex pectinolytic system and for studies on bacterial pathogenicity.

In this paper, we report the cloning of the *E. chrysanthemi crp* gene and the construction of *E. chrysanthemi crp* mutants by reverse genetics. These *E. chrysanthemi crp* mutants were analyzed for pectinolysis gene expression and pathogenicity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are described in Table 1. *E. chrysanthemi* and *E. coli* cells were grown at 30 and 37°C, respectively, in LB medium or M63 minimal medium (37) supplemented with a carbon source (0.2% except for polygalacturonate and pectin [0.4%]) and, when required, with amino acids (40 μg/ml) and antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin and chloramphenicol, 50 μg/ml; streptomycin, 20 μg/ml; and tetracycline, 20 μg/ml. Fermentation tests were performed on MacConkey agar plates containing a 1% concentration of the appropriate carbohydrate (37).

Genetics techniques. Transduction with phage φEC2 was performed as described by Resibois et al. (47). Marker exchange recombinations were obtained after growth in a low-phosphate-concentration medium as described by Roeder and Collmer (51). Chromosomal localization was performed by using RP4::Mu3A derivatives as described by Hugouvieux-Cotte-Pattat et al. (27).

Expression of the *E. chrysanthemi crp* gene in *E. coli*. To selectively label *E. chrysanthemi* CRP, we used the phage T7-based expression system of Tabor and Richardson (56).

Plate tests and enzyme assays. Clones producing pectate lyases were detected on medium containing polygalacturonate. After growth, plates were flooded with a solution of copper acetate (10%), which forms a blue complex with the polymer, leaving clear haloes around clones producing pectate lyases. Detection of cellulase activity was performed by using Congo red as described by Teather and Wood (57). Assays of pectate lyase, β-glucuronidase, and β-galactosidase were performed on toluenized cell extracts. Pectate lyase activity was determined by the degradation of polygalacturonate to unsaturated products that absorb at 235 nm (38). Specific activity is expressed as micromoles of unsaturated products liberated per minute per milligram (dry weight) of bacteria. β-Glucuronidase activity was measured by following the degradation of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol that absorbs at 405 nm (5). Specific activity is expressed as nanomoles of products liberated per minute per milligram (dry weight) of bacteria. β-Galactosidase activity was measured by following the degradation of *o*-nitrophenyl-β-D-galactoside into *o*-nitrophenol that absorbs at 420 nm (37). Specific activity is expressed as nanomoles of products liberated per minute per milligram (dry weight) of bacteria.

Virulence tests. Potato tuber treatment and infection were performed as described by Lojkowska et al. (33). Usually three pipette tips were used per tuber; two contained suspensions of *crp* mutants (A2494 and A2507), and the third contained the wild-type strain. Five tubers were inoculated with each mutant and incubated at 30°C in a dew chamber at 100% relative humidity. Disease severity was determined 3 days after inoculation as described by Lojkowska et al. (33). For the determination of bacterial multiplication, rotted tissues from five samples were collected and homogenized in M63. CFU counts

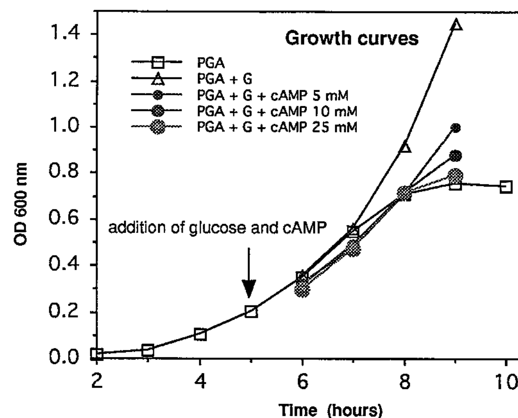
were determined by plating appropriate dilutions on agar plates. Chicory leaves, celery petioles, and saintpaulia infections were performed as described by Beaulieu et al. (7) and Expert and Toussaint (20). Pathogenicity on potted saintpaulia (24 plants) was assayed as described by Sauvage and Expert (52), with minor modifications: overnight bacterial cultures grown in M63 medium containing glucose as a carbon source were diluted to reach an optical density at 600 nm of 0.3. The inoculum was about 100 μl of the resulting suspension (approximately 3×10^7 bacteria). Progression of the symptoms was scored daily for 8 days. Concurrent enumeration of the bacterial population (CFU) present in extracted fluids from inoculated leaves was determined as previously described (36). Pectate lyase activity was determined in filter-sterilized supernatant fluids collected after centrifugation of diseased tissues at $20,000 \times g$.

Recombinant DNA techniques. Preparation of plasmid DNA, restriction digestion, ligation, DNA electrophoresis, DNA labelling, Southern blot hybridization, and transformation were carried out as described by Ausubel et al. (3). Nucleotide sequence analysis was performed by the chain termination method on double-stranded DNA templates. Two universal oligodeoxyribonucleotides (M13 primer and M13 reverse primer from Pharmacia) were used as primers, and [α-^{35}S]dATP (Amersham) was used to label the product. Extension of primers was achieved with T7 DNA polymerase. The resulting data were analyzed by using the MAC MOLLY TETRA program (SoftGene, Berlin).

Generation of a gene library from *E. chrysanthemi*. Chromosomal DNA was extracted as described by Ausubel et al. (3) and was subjected to partial digestion with the endonuclease *Sau*3A. Fragments of between 5 and 12 kb were separated electrophoretically, ligated into the unique *Bam*HI site of pBR322, and transformed into *E. coli* NM522. About 75% of the transformants carried a plasmid with an insert.

Primer extension analysis. Total RNA was extracted from *E. chrysanthemi* 3937 cells by the frozen-phenol method described by Maes and Messens (35). RNA concentration was estimated spectrophotometrically and after electrophoresis on a formaldehyde denaturing 1% agarose gel. For primer extension, 30 and 100 μg of total RNA was annealed in S1 hybridization solution with about 6×10^4 cpm of a ^{32}P end-labeled 28-mer oligonucleotide (5′CTGTTTGCGGT TTGCCGAGAACCATTCG3′) purchased from Eurogentec. This oligonucleotide hybridized to the coding strand between nucleotides +252 and +280 of the *crp* gene. Extension reactions were performed with 20 U of avian myeloblastosis reverse transcriptase (Promega) as described by Ausubel et al. (3), and products were fractionated on sequencing gels by using dideoxy sequencing reactions of double-stranded DNA from plasmid pWN2156 primed with the same oligonucleotide as size markers.

Nucleotide sequence accession number. The nucleotide sequence of the 2.5-kb *Sal*I-*Nsi*I fragment containing the putative *E. chrysanthemi crp* gene (Fig. 3) will appear in the EMBL gene bank under accession no. X89443.



Medium	PL activity (units/mg of BDW)	
	at 1 h	at 2 h
PGA	2.22	3.61
PGA + G	1.44	1.58
PGA + G + cAMP 5 mM	3.52	5.81
PGA + G + cAMP 10 mM	5.46	6.64
PGA + G + cAMP 25 mM	6.46	6.41

FIG. 1. Effect of an exogenous supply of cAMP on growth and on pectate lyase production in *E. chrysanthemi* 3937 during glucose repression. Log-phase cultures growing on polygalacturonate (PGA) minimal medium were supplemented with 5, 10, and 25 mM cAMP simultaneously with the addition of 0.2% glucose (G). Samples were removed for determination of pectate lyase specific (PL) activity 1 and 2 h later. Specific activity is expressed as micromoles of products liberated per minute per milligram (dry weight) of bacteria (BDW). OD, optical density.

RESULTS

Catabolite repression of pectate lyase synthesis in *E. chrysanthemi* 3937. To determine whether pectate lyase synthesis in *E. chrysanthemi* 3937 is subjected to catabolite repression as reported for other *Erwinia* strains, we analyzed pectate lyase production in cultures grown to log phase on M63 minimal medium supplemented with 0.4% polygalacturonate at 30°C. Under these conditions, *E. chrysanthemi* had a generation time of about 1.6 h. The addition of glucose to such cultures resulted in a twofold decrease in pectate lyase specific activity after 2 h (1.25 generations) (Fig. 1). When cAMP was added at 5, 10, and 25 mM to cells subjected to glucose repression, the pectate lyase specific activity increased above that observed in the nonrepressed control culture (Fig. 1). These results, which are similar to those obtained for *E. carotovora* by Hubbard et al. (24), confirm that pectate lyase synthesis is subjected to cAMP-controlled catabolite repression in *E. chrysanthemi* 3937. Moreover, the surplus of pectate lyase specific activity obtained in the presence of cAMP relative to the level obtained in the polygalacturonate control culture confirms that polygalacturonate exerts self-catabolite repression.

Isolation of the *E. chrysanthemi crp* gene. *E. coli crp* mutants are unable to catabolize several carbohydrates, such as maltose and lactose. The *E. chrysanthemi crp* gene was cloned by complementation of the maltose defect of an *E. coli crp* mutant. The *E. coli crp* mutant M182Δ*crp* was transformed with an *E. chrysanthemi* gene library constructed in pBR322, and maltose-fermenting transformants were then sought. Among 5,000 transformants, one clone able to catabolize maltose was obtained. The corresponding plasmid, pWN1989, contains a chromosomal DNA fragment of about 8.4 kb. The capacity of this clone to metabolize some other *crp*-dependent carbohydrates was tested. pWN1989 totally restored fermentation of maltose, lactose, arabinose, gluconate, glycerol, xylose, ribose, and galacturonate. Therefore, the insert encodes a common activator of these different catabolic pathways, which is probably the *E. chrysanthemi* CRP.

To further localize the *E. chrysanthemi crp* gene, the cloned 8.4-kb fragment was subjected to deletion analysis. The smallest fragment able to complement the *crp* mutation is the 0.9-kb *Ava*I-*Mam*I fragment (Fig. 2).

Nucleotide sequence of the *E. chrysanthemi crp* locus. The nucleotide sequence of the 2.5-kb *Sal*I-*Nsi*I fragment containing the putative *E. chrysanthemi crp* gene (Fig. 3) contains a 630-bp open reading frame (ORF) between nucleotides 1275 and 1904 which corresponds to the *E. chrysanthemi crp* gene. A putative ribosome binding site (AGAGGA) is located 9 bp upstream of the ATG translation start codon of the *crp* gene. Sequences matching the consensus of the *E. coli* CRP binding site (19) are found at two locations; the first one, from 1247 to 1262, overlaps the ribosome binding site, and the second one, from 1618 to 1633, is located in the *crp* ORF. The deduced *E. chrysanthemi* CRP protein is 210 amino acids long and has a calculated molecular mass of 23,600 Da. This protein shows 98% identity with the CRPs from *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella flexneri*, and *E. coli*. Weaker similarity was observed with other members of the CRP family

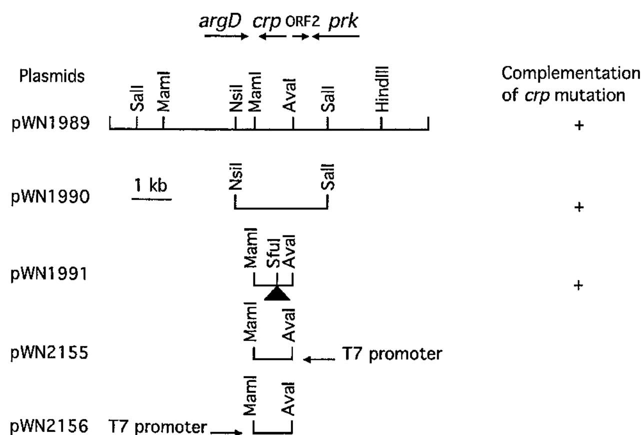


FIG. 2. Physical map of the 8.4-kb *Sau*3A-*Sau*3A DNA fragment containing the *E. chrysanthemi crp* gene. Localization of the *crp* gene on plasmid pWN1989 was achieved by complementation of the *E. coli crp* mutant M182Δ*crp*, using deletion derivatives such as pWN1990 and pWN1991. Transcriptional organization of *prk*, *argD*, and the *crp* divergent RNA was proposed, taking into account the nucleotide sequence of the *Sal*I-*Nsi*I fragment. The *Sfu*I site of insertion of the *uidA*-Km^r or Cm^r resistance cartridge is shown by an arrowhead.

(29), including the *Haemophilus influenzae* CRP protein (68%) and the *Xanthomonas campestris* catabolite activator-like protein C1P (45%).

The 0.9-kb *Ava*I-*Mam*I fragment containing the *crp* coding sequence was cloned into plasmid T7-6 (pWN2155). The proteins encoded by this fragment were specifically labelled after induction of the T7 polymerase. We detected one protein with a molecular mass of 24,000 Da, which is in agreement with the size predicted for CRP by sequence data (data not shown).

Another part of the sequenced region (nucleotides 919 to 1016) shows 58% identity with the DNA encoding the *E. coli crp* divergent RNA (44). The 2.5-kb *Sal*I-*Nsi*I fragment contains three additional ORFs: ORF1 (nucleotides 1 to 427), which has the same transcriptional direction as *crp*, and ORF2 (477 to 882) and ORF3 (1994 to 2491), which have transcriptional directions opposite that of *crp*. The truncated protein encoded by ORF1 shows 64% identity with the C-terminal sequence of the phosphoribulokinase from *E. coli*. ORF2 encodes a 135-amino-acid protein which shows 83% identity with the product of an ORF located at the same position in *E. coli*. This ORF overlaps the region encoding the *crp* divergent RNA (10). As observed in *E. coli*, the start of this ORF is located at position +135 relative to the transcription initiation site of the *crp* divergent RNA. ORF3 encodes a truncated protein exhibiting 59% identity with the C-terminal part of the acetylornithine aminotransferase from *E. coli*, encoded by *argD* (23).

The *E. chrysanthemi crp* regulatory region contains a 42-nucleotide (1103 to 1145) motif within the transcript (Fig. 3) that is highly conserved (82% identity) in the regulatory regions of the *E. chrysanthemi outC* operon (22) and in an *E. carotovora* protease (*prt*) gene (30). This motif is localized at nucleotides −207 and −300 relative to the translation start codons of the *prt* and *outC* genes, respectively, and overlaps the promoter region in the case of *prt*. The significance of this motif will be investigated by mutagenesis experiments.

Mapping of the transcriptional initiation site of *crp* and regulation of *crp* expression. RNA was isolated from strain

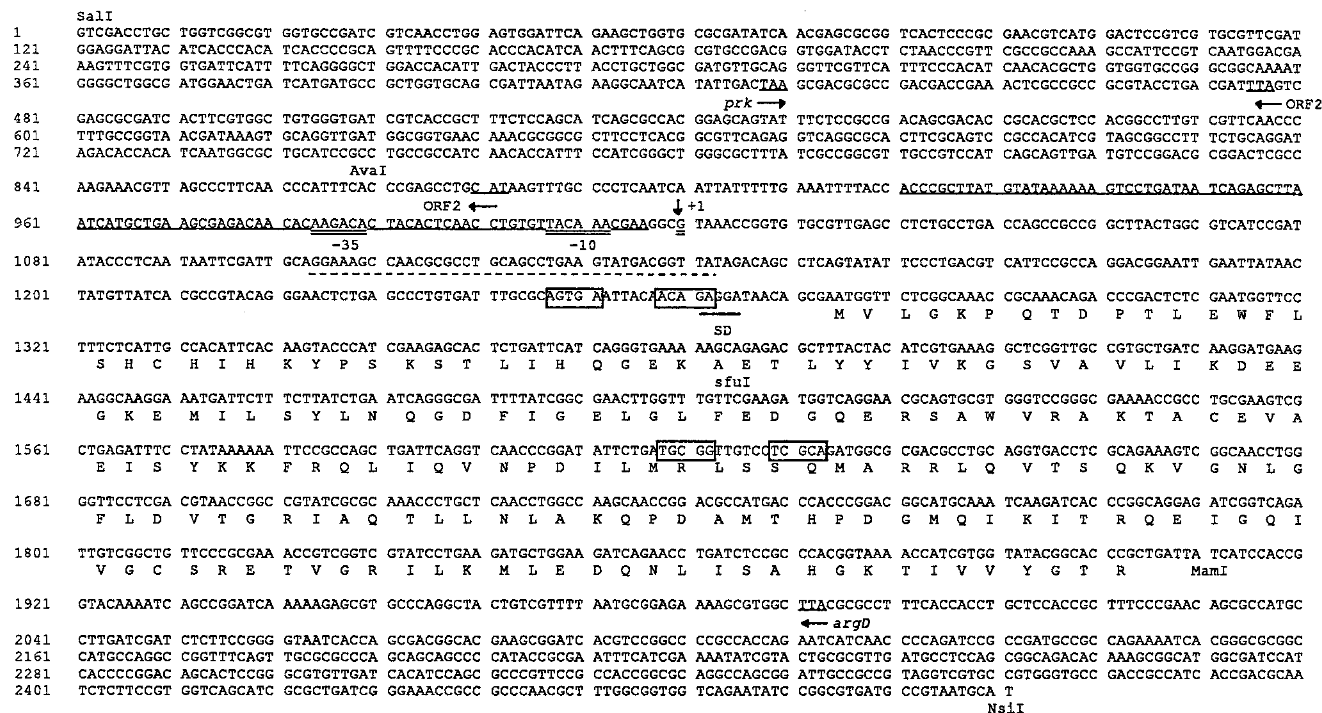


FIG. 3. Nucleotide sequence of the 2.5-kb *Nsi*I-*Sal*I fragment containing the *crp* gene. The putative promoter region (−35, −10) and the start of transcription are doubly underlined. The peptidic sequence corresponding to the *crp* ORF is shown under the nucleotide sequence. The putative *crp* ribosome binding site and sequence matching the *E. coli crp* divergent RNA are underlined. The putative CRP binding sites are boxed. The DNA sequence which shows high similarity with the regulatory regions of the *E. chrysanthemi outC* operon (22) and an *E. carotovora prt* gene (30) is indicated by a dashed line. The unique *Sfu*I restriction site used for a Cm^r cartridge or a *uidA*-Km^r cassette insertion is indicated. The transcriptional directions of *prk*, *argD*, and ORF2 are shown by arrows.

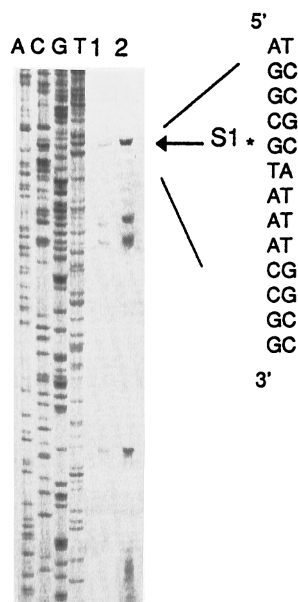


FIG. 4. Identification of the *E. chrysanthemi crp* transcription initiation. RNAs (30 μg [lane 1] and 100 μg [lane 2]) from *E. chrysanthemi* 3937 cells grown on LB medium were submitted to primer extension analysis using a *crp*-specific primer. DNA-sequencing ladders were generated from the pWN2156 recombinant plasmid with the same primer (lanes A, C, G, and T). The nucleotide sequences of both the coding and noncoding strands are shown on the right, with the specific transcription initiation site S1 indicated by an asterisk.

3937 cells and used in primer extension experiments. Extension terminated at the G base located 255 bp upstream from the translational start of *crp*. In addition to this major site, three other bands were detected at positions 217 (base C), 270 (base G), and 136 (base G) relative to the translation start position (Fig. 4). Since these three shorter elongation products could be due to artifacts such as a secondary structure formation in the *crp* mRNA or a partial degradation of this mRNA during the experiments, we retained the position 255 bp upstream the *crp* ATG as the transcription start site. Inspection of the DNA sequence of this *crp* region revealed the presence of a possible sigma 70 promoter with a −10 box (TACAAA) and a −35 box (AAGACA) spaced by 17 nucleotides. As reported for the *E. coli crp* gene, the *E. chrysanthemi crp* sigma 70 promoter is overlapped by the sequence of the putative divergent RNA (44). In *E. coli*, the expression of *crp* is negatively autoregulated and decreases in the presence of glucose in the growth medium (17). To determine whether the autoregulation is conserved in *E. chrysanthemi*, expression of the *crp*::*uidA* fusion was analyzed in the presence of the *E. chrysanthemi crp* gene on a multicopy plasmid (Table 2). Expression of this fusion was repressed twofold in the presence of multicopy of the *crp* gene. The strongest repression was observed when cells were grown in minimal medium supplemented with 5 to 10 mM cAMP as a CRP coactivator (Table 2). *crp* gene expression was also analyzed in the presence of polygalacturonate or plant extracts which induce *E. chrysanthemi* virulence factors. None of these compounds were found to significantly modify *crp* transcription. These findings suggested that the *crp* genes of the two bacteria *E. chrysanthemi* and *E. coli* are similarly regulated.

Construction and characterization of an *E. chrysanthemi crp*-deficient strain. To study the function of CRP in *E. chrysanthemi*, a *crp*-deficient mutant was constructed by reverse genetics. A Cm^r (chloramphenicol resistance) cartridge or a *uidA*-Km^r (kanamycin resistance) cassette was introduced into the unique *Sfu*I site located in *crp* (Fig. 2). These mutations were then recombined into the *E. chrysanthemi* chromosome by marker exchange, and the resulting mutants (A2507 and A2494) were confirmed by Southern blot hybridization (data not shown). The capacity of strains A2494 and A2507 to grow on minimal M63 medium supplemented with glycerol, glucose, gluconate, galacturonate, or polygalacturonate was analyzed. Only glucose could be used as a carbon source for growth by these mutants. The *E. chrysanthemi crp* mutants thus present a phenotype similar to that of the *E. coli crp* mutants. The *crp* locus was localized on the *E. chrysanthemi* chromosome by using the Km^r marker of the *crp*::*uidA*-Km^r fusion. Chromosomal mobilization mediated by plasmid pULB110 was used for conjugation with various polyauxotrophic recipient strains (27). The Km^r marker appeared closely linked to the mutations *met-555* and Sm^r (streptomycin resistance), with cotransfer frequencies of 68 and 72%, respectively. Using a three-point test, the Sm^r mutation was located between the *met-555* and *crp*::*uidA*-Km^r markers. Similarly, the *E. coli crp* gene is linked to the *rpsL* locus responsible for streptomycin resistance.

Since *E. chrysanthemi* is a phytopathogenic bacterium, we compared production of virulence-associated factors in the *crp* mutants and in the wild-type strain. Cellulase activity was not affected in a *crp* mutant (data not shown), in contrast to the pectate lyase activity, which was only barely detectable (Table 2).

TABLE 2. Complementation of the *crp* mutant and expression of the *crp*::*uidA* fusion[a]

Strain	Plasmid	Rotted tissue (g)	CFU/g of rotted tissue (10^{11})	Pectate lyase activity in glucose minimal medium supplemented with:				β-Glucuronidase activity[b] in glucose minimal medium supplemented with:			
				Nothing	cAMP	PGA[c]	PGA + cAMP	Nothing	cAMP	PGA	Plant extract
A2494 (*crp*::*uidA*)	None	0.3	1.75	0.04	0.06	0.10	0.14	1,987	2,079	2,081	2,111
	pWN1990[d]	3.1	2.4	0.45	0.51	3.65	5.12	1,282	896	1,403	1,801
A350 (wild type)	None	2.2	2.2	0.25	0.24	2.01	4.2				
	pWN1990	4.8	2.4	0.62	0.53	3.33	6.41				

[a] Enzyme assays were performed on cells grown to early stationary phase. Pectate lyase specific activity is expressed as micromoles of products liberated per minute per milligram (dry weight) of bacteria. β-Glucuronidase specific activity is expressed as nanomoles of products liberated per minute per milligram (dry weight) of bacteria. For the estimation of macerating capacity, potato tubers were inoculated with 10^8 bacteria and incubated at 30°C. Weight of rotted tissue was determined 3 days after inoculation. For the determination of bacterial multiplication during infection, 100 mg of rotted tissue was homogenized in 1 ml of M63. CFU counts were determined by plating appropriate dilutions on agar plates. The results reported are the averages of at least three independent experiments; the standard deviation was, in each case, less than 15%.

[b] Reflects expression of the *crp*::*uidA* fusion.

[c] PGA, polygalacturonate.

[d] A multicopy plasmid harboring the *E. chrysanthemi crp* gene.

TABLE 3. Effects of the *crp* mutation on the expression of pectinolysis genes[a]

Fusion	Enzyme activity (nmol of products liberated/min/mg [dry wt] of bacteria)							
	Wild type		*crp*		*kdgR*		*crp-kdgR*	
	G	G + PGA	G	G + PGA	G	G + PGA	G	G + PGA
pemA	41	356	40	66	118	350	43	72
pelA	6	19	6	7	14	29	170	186
pelB	20	296	11	12	97	299	22	31
pelC	19	234	8	13	57	252	9	13
pelD	12	2,558	6	16	905	2,969	5	13
pelE	140	1,324	13	17	677	1,304	11	15
ogl	18	44	21	23	45	58	19	20
kduI	11	712	13	40	2,140	2,608	134	196
kduD	30	2,109	26	152	2,329	2,484	1,517	2,452
kdgT	3	457	1	27	180	500	6	51
kdgK	42	569	37	40	415	614	403	542

[a] Each fusion was assayed after culture in glucose (G) minimal medium supplemented or not with polygalacturonate (PGA) as the inducer. Enzyme assays were performed on cells grown to early stationary phase. The results reported are the averages of at least three independent experiments; the standard deviation was, in each case, less than 15%.

Effect of *crp* mutation on the transcription of pectinolytic genes. To determine if CRP activates their transcription, the expression of genes involved in pectin catabolism was analyzed in a *crp* background by using the following chromosomal gene fusions: *ogl*::*lacZ*, *kdgT*::*lacZ*, *pemA*::*uidA*, *pelA*::*uidA*, *pelB*::*uidA*, *pelC*::*uidA*, *pelD*::*uidA*, *pelE*::*uidA*, *kduI*::*uidA*, *kduD*::*lacZ*, and *kdgK*::*lacZ*. Assay of enzymatic activities encoded by these reporter genes revealed that the expression of all of the pectinolysis genes mentioned above was reduced 2- to 160-fold in the *crp* mutant under inducing conditions (Table 3). These data do not indicate whether CRP acts by transcriptional activation of all pectinolysis genes or controls the expression of only some genes (oligogalacturonate lyase and pectinase genes) essential for formation of the inducers (DKI, DKII, and KDG). To elucidate this point, expression of the fusions in pectinolytic genes was analyzed in a *kdgR* background as well as in the double *kdgR-crp* mutant. Inactivation of KdgR, the main mediator of induction via pectic compounds, leads to a derepression of pectinolytic genes and enables us to observe the regulatory effect of CRP independently of induction. In comparison with the *kdgR* mutant, the expression of *pemA*, *pelB*, *pelC*, *pelD*, *pelE*, *ogl*, *kduI*, and *kdgT* genes decreased in a *kdgR-crp* background. Such results indicated that the expression of these genes is controlled by CRP either via a direct interaction with the regulatory region of these genes or by an indirect mechanism involving another regulator not yet identified. In contrast, expression of the *kduD* and *kdgK* genes is identical in the *crp-kdgR* and *kdgR* mutants. Therefore, the decrease of *kduD* and *kdgK* expression in a *crp* mutant in inducing conditions is due to the lack of inducer formation rather than to the absence of CRP. The *pelA* gene behaves quite differently from the other pectinolytic genes. Expression of the *pelA* gene in the double *crp-kdgR* mutant was 27- and 6-fold higher than that observed in *crp* and *kdgR* mutants, respectively. This result suggests that CRP could be involved in a negative regulation of *pelA* expression, detectable only in the absence of the KdgR repressor. This result was confirmed by an isoelectrofocusing analysis of the profiles of the different pectate lyase isoenzymes in the *crp*, *kdgR*, and *crp-kdgR* mutants (Fig. 5). In the double mutant, PelA is easily detectable while the other isoenzymes are barely detectable.

Plant tissue maceration and pathogenicity on saintpaulia plants. The tissue-macerating ability of the *crp* mutant (A2494) was compared with that of the parental strain, A350, by inoculation of potato tubers (Table 2), chicory leaves, and celery petioles. The macerating capacity of the *crp* mutant was strongly reduced in these different plant organs. In addition, the *E. chrysanthemi crp* mutant, carrying the *E. chrysanthemi crp* gene on a pBR322 derivative, macerates potato tubers more efficiently than the parental strain (Table 2). Such results suggest that the maceration capacity of *E. chrysanthemi* is directly correlated with pectinase production, which is strongly decreased in *crp* mutants but is increased when the *crp* gene is present on the multicopy plasmid (Table 2). Bacterial multiplication was monitored by estimation of the bacterial cell concentration in the rotted tissue. No significant difference between the *crp* mutant and the parental strain was observed (Table 2). The particular behavior of the *pelA* gene relative to CRP and KdgR regulation and its predominant role in *E. chrysanthemi* virulence led us to analyze the macerating capacity of the *kdgR*, *kdgR-crp*, and *kdgR-crp-pelA* mutants on celery petioles and chicory leaves. The *kdgR* mutant presented a slightly higher macerating capacity than the parental strain, whereas the *crp* mutant strain displayed no significant macerating capacity and the *crp-kdgR-pelA* mutant displayed a very weak macerating capacity (Fig. 6). In contrast, the double *crp-kdgR* mutant, which overproduces the PelA isoenzyme, showed a significant maceration capacity intermediate to those of the parental and *crp* mutant strains (Fig. 6). These results suggested that the lack of the maceration capacity of the *crp* mutant could be directly correlated to its poor production of pectinases.

The pathogenic behavior of the *crp* mutant was compared to that of the parental strain 3937 after inoculation of saintpaulia potted plants. In all plants, inoculation of strain 3937 resulted in maceration of the whole leaf after 3 days. The rotting symptom continued to spread throughout the petiole, and after 6 days, maceration expanding to a second petiole was detected in 2 of the 24 infected plants. In contrast, the *crp* mutant failed to induce any symptoms in seven plants. In the 17 other plants, the inoculation gave rise to a brown-colored water-soaked lesion after 1 to 2 days. The lesion remained confined to the

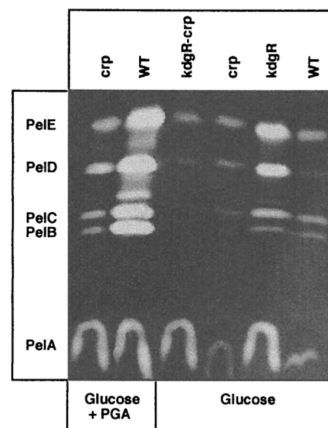


FIG. 5. Electrofocusing of pectate lyases produced by the *E. chrysanthemi* A350 (wild type [WT]), A2507 (*crp*), and A2518 (*kdgR-crp*). Pectate lyase activity was revealed by the sandwich technique (9). Each lane contained 20 μl of culture supernatant. Growth conditions are indicated at the bottom. PGA, polygalacturonate.

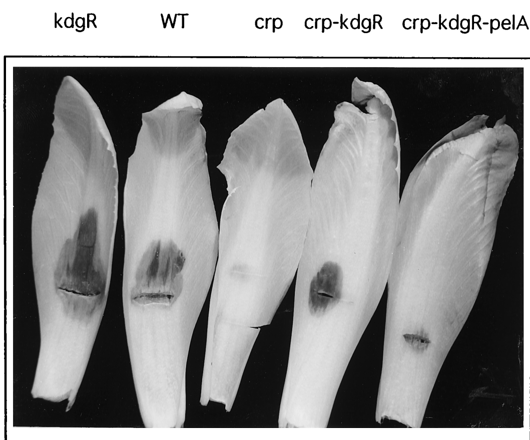


FIG. 6. Plant tissue maceration induced by *E. chrysanthemi* 3937 strains. About 10^7 cells were injected into chicory leaves at each inoculation site. Inoculated leaves were incubated in a moist chamber at 30°C for 24 h. From left to right: the *kdgR* mutant A837, the parental (wild-type [WT]) strain A350, the *crp* mutant A2507, the *crp-kdgR* mutant A2518, and the *crp-kdgR-pelA* mutant A2688.

inoculated area and generated a chlorotic border within a few days. However, the lesion did not dry out, as previously observed with iron uptake mutants. On average, a threefold increase in viable counts (CFU) relative to the initial inoculum was detectable after 1 day in leaves inoculated with the wild-type strain. Within the same period of time, the population of the *crp* mutant decreased threefold. No pectate lyase activity was recovered from 4-day-old lesions induced by the *crp* mutant, while the presence of PelA to PelE isoenzymes and of minor Pel activities was detected in macerated tissues induced by the wild-type strain (data not shown). This result indicates that factors other than the pectate lyases are responsible for the residual symptom induced by the *crp* mutant.

DISCUSSION

Although it has been known for a number of years that pectate lyase production in *Erwinia* species is subject to catabolite repression, direct involvement of CRP in this process has never been demonstrated. Indeed, since the physiological or molecular data suggesting involvement of CRP in the expression of some pectinolysis genes were obtained in various backgrounds and are sometimes contradictory, it is difficult to appreciate the role of this general regulator in the regulation of pectin catabolism. The complexity of the situation can be illustrated by the results obtained for the *E. carotovora* regulatory *aepA* locus by Liu et al. (32). Putative CRP binding sites were identified in front of the *aepA* gene. However, expression of this gene is not subject to catabolic repression, indicating that these CRP binding sites are not functional. The goal of our study was to clone the *crp* gene of *E. chrysanthemi* and to construct a *crp* mutant, in order to understand more precisely the role of CRP in the coordinate synthesis of pectate lyases and other enzymes of the intracellular part of pectin catabolism.

An *E. chrysanthemi* gene able to restore catabolism of several carbohydrates in an *E. coli crp* mutant was cloned. This gene encodes a protein showing 98% identity with *E. coli* CRP. By constructing an *E. chrysanthemi crp* mutant, we present a direct evidence that pectate lyase production in this bacterium is activated by CRP. The regulatory effect of *E. chrysanthemi* CRP, evaluated independently of induction by pectic compounds in a *kdgR* background, demonstrates that this protein activates the transcription of the major pectinase genes (*pemA*, *pelB*, *pelC*, *pelE*, and *pelD*), as well as the genes of some intracellular steps of pectin catabolism (*ogl*, *kduI*, and *kdgT*) responsible for the formation and transport of the inducers (DKI, DKII, and KDG). In contrast, the decreased expression of the *kduD* and *kdgK* genes observed in the simple *crp* mutant results from a lack of inducer formation that does not permit to abolish the repression exerted by the KdgR repressor. Therefore, CRP is required for the expression of all of the pectinolytic genes either by a transcriptional activation mechanism (*pemA*, *pelB*, *pelC*, *pelE*, *pelD*, *ogl*, *kduI*, and *kdgT*) or by an indirect effect due to the inhibition of inducer formation (*kduD* and *kdgK*). These results clearly establish that CRP plays a crucial role in pectin catabolism by *E. chrysanthemi.*

Regarding the effect of CRP, *pelA*, one of the *pel* genes most important for bacterial virulence in plants (7, 11), behaves quite differently from the other pectinolytic genes. Indeed, a negative regulation by CRP was observed for *pelA* expression only in the absence of KdgR, the main repressor of pectinolysis genes. The effect of CRP on the *pelA* gene could occur through a direct inhibition of transcription after binding of CRP to the *pelA* regulatory region, in the absence of the KdgR repressor. Such negative regulation due to a direct binding of CRP has been observed for some *E. coli* genes (1, 2, 40). Alternatively, the CRP effect could be explained by an indirect mechanism, requiring the intervention of another protein. Indeed, it is possible that CRP either activates the synthesis of a specific repressor of the *pelA* gene, which could act only in the absence of KdgR, or interacts with another regulator protein, and the resulting complex could then bind to the regulatory region of *pelA* in the absence of KdgR. The behavior of the *pelA* gene is surprising. PelA isoenzyme represents a small contribution to the total pectate lyase activity in vitro and is unable to elicit maceration when incubated alone on potato disks (6), but paradoxically, virulence of the *pelA* mutant is strongly reduced in a wide range of plants (7, 11). Moreover, for all *pel* genes except *pelA*, similar expression levels could be obtained in synthetic medium with polygalacturonate and potato tuber extract as during tuber infection (33). In contrast, *pelA* expression is barely detectable in synthetic medium, even in inducing conditions, but it is strongly stimulated when bacteria invade potato tubers (33). The *pelA* expression level in potato tubers is comparable to that obtained in the double *kdgR-crp* mutant, suggesting that a repression mechanism specific to the *pelA* gene is suppressed in these two conditions.

Analysis of the 5′ untranslated ends of the *E. chrysanthemi* CRP-regulated genes (*pemA*, *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *ogl*, *kdgT*, and *kduI*) revealed the presence of sequences matching the *E. coli* CRP binding consensus in all of these genes (Fig. 7).

		CRP-boxes			
		-------->		<-------	
Consensus	AAN	TGTGA	NNTANN	TCACA	NTT
pemA	TAC	CGTGA	TCAGGC	CGGCA	GCC
pelA	CAA	TGTTA	CATTTA	AAACA	TCG
pelB	GCA	AGTGA	CCGGTT	TCACA	GTT
pelC	AAA	AGTGA	CGCCTG	TCAAA	ATT
pelD	AAA	CGAGA	TTTTGA	TCACA	AAA
pelE	CAT	TGTCA	TCATCG	TGACA	AAG
ogl	AAA	TAAAA	CCACGA	TCACG	GAA
kduI	TTG	TGTGA	ACAAGG	TAACA	CAA
kdgT	TTG	TTTGC	AAGCGA	TCACT	TTT

FIG. 7. Multiple sequence alignment of the putative CRP boxes found in front of pectinolytic genes in *E. chrysanthemi.*

This result suggests a direct control by CRP of the *pemA*, *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *ogl*, *kdgT*, and *kduI* expression. In vitro analysis of the interaction between CRP and the regulatory region of the controlled genes will enable us to elucidate the mechanism by which this regulatory protein controls transcription of the pectinolysis genes in *E. chrysanthemi.*

Production of extracellular pectinases is central for pathogenicity (13). We have demonstrated that the CRP mutants not only are deficient in pectinase production but appear to be strongly reduced in maceration capacity and in pathogenicity. These data demonstrate that in addition to the control of general metabolic functions such as sugar catabolism, *E. chrysanthemi* CRP plays a central role in maceration and in pathogenicity. This physiological role is different from those recently reported for other pathogenic bacteria such as *Pseudomonas aeruginosa* (60) and *X. campestris* (18). In these two bacteria, a protein homologous to CRP plays a role in pathogenicity but has no involvement in sugar catabolism, as is the case in *E. coli*. In *E. chrysanthemi*, CRP appears to act as a global regulatory protein that coordinates various essential functions, including phytopathogenicity.

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