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Molecular Cloning of an *aepA* Gene that Activates Production of Extracellular Pectolytic, Cellulolytic, and Proteolytic Enzymes in *Erwinia carotovora* subsp. *carotovora*

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Strain 71 of *Erwinia carotovora* subsp. *carotovora* produces extracellular enzymes such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt). The levels of extracellular Pel, Cel, and Prt were higher in a medium containing crude celery extract than in a medium containing pectate. Using transposons (Tn*5*, Tn*phoA*, and Tn*10-lacZ*), we isolated pleiotropic mutants that were deficient in extracellular levels of these enzymes and attenuated in their ability to macerate plant tissues. The mutants, however, were similar to the parent in their ability to utilize various sugars and to produce periplasmic enzymes. In an *E. c.* subsp. *carotovora* 71 gene library, we detected a cosmid, pAKC264, that restored extracellular enzyme production and tissue maceration in all the mutants. The cosmid appears not to carry *pel*, *peh*, *cel*, or *prt* genes. In *E. c.* subsp. *carotovora* 71, pAKC264 stimulated the production of Pel, Peh, Cel, and Prt, but it did not affect the levels of the periplasmic enzymes, cyclic phosphodiesterase, or β-lactamase. pAKC602, a subclone of pAKC264, stimulated enzyme production in *E. c.* subsp. *carotovora* 71 and did not complement mutations in *cya*, the gene specifying adenylate cyclase, or *crp*, the gene specifying cyclic AMP receptor protein, in *Escherichia coli*. The *E. c.* subsp. *carotovora* 71 gene that *a*ctivates *e*xtracellular protein *p*roduction was designated as *aepA*. Sixteen mini-Mu-*lacZ* (Mu*d*1734) insertions inactivating *aepA* spanned a DNA region of about 0.8 kilobases and allowed determination of the direction of *aepA* transcription by screening for β-galactosidase production. By further subcloning and localizing the sites of mini-Mu-*lacZ* insertions not inactivating *aepA*, the gene was localized within a 1.1-kilobase DNA segment.

Erwinia carotovora subsp. *carotovora* (Jones) Bergey *et al.* causes tissue-macerating (= soft rot) diseases in a wide variety of plant hosts. Pathogenicity of these bacteria is correlated with their ability to produce extracellular enzymes, specifically pectinases such as pectate lyase (Pel) or endopolygalacturonase (endo-Peh) (Lei *et al.* 1985; Mount *et al.* 1970; Roberts *et al.* 1986; Willis *et al.* 1987). They also produce extracellular protease (Prt), cellulase (Cel), and phospholipase, which are known not to macerate plant tissues. These enzymes may nevertheless contribute to virulence by augmenting the action of pectinases, by inflicting stress onto plant cells previously damaged by pectolytic activities, and by providing the bacterium with readily utilizable sources of carbon and nitrogen through the degradation of polymeric substances.

These ecological considerations and a lack of understanding of the processes affecting extracellular protein production in gram-negative bacteria have generated considerable interest in the *Erwinia* enzymes (for reviews see Chatterjee and Vidaver 1986; Collmer and Keen 1986; Kotoujansky 1987). Aside from structural and functional aspects of the genes specifying extracellular enzymes (see, for example, Hinton *et al.* 1989, 1990; Lei *et al.* 1988; Reverchon *et al.* 1989; Saarilahti *et al.* 1990; Tamaki *et al.* 1988; Van Gijsegem 1989), the issues currently attracting the most attention are 1) biochemical mechanisms by which proteins are translocated across the tripartite cell envelope (He *et al.*, in press; Ji *et al.* 1989; Murata *et al.* 1990) and 2) molecular aspects of regulation of the expression of genes specifying secreted proteins (Beaulieu and Van Gijsegem 1990; Chatterjee *et al.*, in press; Kelemu and Collmer 1990; Reverchon *et al.* 1989; Yang *et al.* 1989).

Investigations with *Bacillus subtilis* (Ehrenberg) Cohn (Henner *et al.* 1988; Wang and Doi 1990), *Staphylococcus aureus* Rosenbach (Peng *et al.* 1988; Recsei *et al.* 1985), and *Streptomyces griseus* (Krainsky) Waksman and Henrici (Daza *et al.* 1990) have disclosed global regulation of secreted proteins. That Pel, Peh, Cel, Prt, and other extracellular proteins may also be similarly regulated in *E. c.* subsp. *carotovora* was suggested by the phenotypes of mutants isolated by Beraha and Garber (1971). The nitrosoguanidine-induced mutants were pleiotropically defective in the production of these extracellular enzymes as well as phospholipase. They also isolated a one-step revertant that regained the ability to produce all of those enzymes. These findings could have resulted from a defect in a "positive regulator (= activator) gene" required for the production of extracellular proteins. However, since the mutants and the revertant were not subjected to further genetic analyses, the basis for the pleiotropic effect remained unresolved.

In the course of isolating mutants defective in the secretion (Out) pathway (Murata *et al.* 1990), we came across a few mutants wherein the production of Peh, Pel, Cel, and Prt was affected. Such mutants, phenotypically similar to those reported by Beraha and Garber (1971), were different from the bona fide Out$^-$ mutants (Murata *et al.*

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1990) in 1) the detrimental effect of mutations on the production of extracellular Prt in addition to Peh, Pel, and Cel, 2) the absence of high levels of cell-bound Peh, Pel, and Cel, and 3) the lack of restoration of the mutational phenotype by the *out*$^+$ DNA. We describe here the characteristics of the mutants and a wild-type allele designated as *aepA*$^+$ for the *a*ctivation of *e*xtracellular protein *p*roduction. In addition, our finding that the *aepA*$^+$ DNA restores plant tissue macerating ability to the mutants suggests a key role of the *aepA* gene product in pathogenesis of *E. c.* subsp. *carotovora*. (Preliminary accounts of parts of this work will be or have been published [Chatterjee *et al.*, in press; Murata and Chatterjee 1990a, 1990b].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. Bacterial strains, plasmids, and the bacteriophage that were used in this study are described in Table 1. The strains carrying drug markers were maintained on Luria-Bertani agar supplemented with the appropriate antibiotics. The wild-type strains of *Erwinia* were maintained on yeast extract-glucose-calcium carbonate (YGC) agar. Lambda lysates were kept in SM buffer (Maniatis *et al.* 1982).

Media. Cel detection agar, Luria-Bertani medium, minimal medium, nutrient gelatin agar, polygalacturonate-yeast extract-citrus pectin (PYCP) agar, and YGC agar were described previously (Barras *et al.* 1986; Chatterjee 1980; Chatterjee *et al.* 1985; Murata *et al.* 1990; Thurn and Chatterjee 1985). Salts-yeast extract-glycerol (SYG) medium consisted of 0.4 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 55 mM potassium phosphate (pH 7.0), 0.025% yeast extract, and 0.2% glycerol. SYG medium was supplemented either with pectate (0.2%) or with plant extracts prepared as follows. Carrot roots, celery petioles, or potato tubers obtained from a local supermarket were cut into pieces; 100 g of the cut material was suspended in 100 ml of distilled water and then autoclaved at 121° C for 20 min. After autoclaving, the liquid material was decanted and centrifuged at 12,000 × *g* at 4° C for 30 min to remove debris. To 95 ml of

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

Designation	Relevant characteristics[a]	References or sources
Erwinia carotovora subsp. *carotovora*		
Strain 71	Wild type	Zink *et al.* 1984
AC5006	Lac$^-$	Derivative of *E. c.* subsp. *carotovora* 71; Murata *et al.* 1990
AC5012	Tn*5* insertion mutant, AepA$^-$, Km^r	Derivative of *E. c.* subsp. *carotovora* 71; this study
AC5026	Tn*10-lacZ* insertion mutant, AepA$^-$, Tc^r	Derivative of AC5006; this study
AC5027	Tn*phoA* insertion mutant, AepA$^-$, Km^r	Derivative of *E. c.* subsp. *carotovora* 71; this study
Escherichia coli		
AC8001	*arg*, *his*, Δ*lac*, Sm^r, Km^r, Gm^r (pJB4JI)	Chatterjee *et al.* 1983
CGSC6027	Δ*cya-854*, *relA1*, *spoT1*, *thi-1*, λ$^-$	*E. coli* Genetic Stock Center[b] (B. J. Bachmann); Brickman *et al.* 1973
CGSC7043	Δ*crp-45*, *relA1*, *rpsL136*, *spoT1*, *thi-1*, λ$^-$	*E. coli* Genetic Stock Center (B. J. Bachmann); Brickman *et al.* 1973
DH5α	φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*), *U169*, *recA1*, *thi-1*	Bethesda Research Laboratories[c]
DW75	*lamB*$^+$, Ap^r Cm^r, Tc^r	Salmond *et al.* 1986
M8820	Δ(*proAB-argF-lac-POZYA*), *recA*$^+$, Sm^r	Castilho *et al.* 1984
MM294A	*pro-82*, *thi-1*, *endA1*, *hsdR17*, *supE44*	Long *et al.* 1988
POI1734	Mu*dI*1734 *ara*::(Mu *cts*), Δ(*proAB-argF-lacIPOZYA*), Km^r, Sm^r	Castilho *et al.* 1984
HB101	*pro*, *leu*, *thi*, *lacY*, *recA*, *hsdR*, *hsdM*, Sm^r	Zink *et al.* 1984
Bacteriophage		
λ1045	λ-Tn*10-lacZ*, Tc^r	Way *et al.* 1984
Plasmids		
pHCP2	*lamB*$^+$, Mob$^+$, Ap^r	Clement *et al.* 1982
pJB4JI	pPHIJI::Mu::Tn*5*, Mob$^+$, Tra$^+$, Gm^r, Km^r	Hirsch and Beringer 1984
pRK404	Mob$^+$, Tc^r	Ditta *et al.* 1985
pRK609	pRK600::Tn*phoA*, Km^r, Cm^r	Long *et al.* 1988
pRK2013	Mob$^+$, Tra$^+$, Km^r	Figurski and Helsinki 1979
pSF6	Mob$^+$, Sp^r, Sm^r	Selvaraj *et al.* 1984
pAKC264	AepA$^+$, Sp^r, Sm^r	pSF6 containing *E. c.* subsp. *carotovora* 71 genomic DNA; this study
pAKC602	AepA$^+$, Tc^r	Subcloning of *aepA*$^+$ DNA into pRK404; this study
pAKC607	AepA$^+$, Km^r, Tc^r	Mu*dI*1734 insertion in pAKC602; this study
pAKC608	AepA$^+$, Tc^r	2.2-kilobase (kb) *Hin*dIII *aepA*$^+$ subclone of pAKC607 containing 1.2 kb of *E. c.* subsp. *carotovora* 71 DNA and 1.0 kb of Mu DNA in pRK404; this study
pAKC609	AepA$^+$, Tc^r	Same as pAKC608 except the insert DNA is cloned in the opposite orientation; this study

[a] Km = kanamycin, Tc = tetracycline, Sm = streptomycin, Gm = gentamycin, Ap = ampicillin, Cm = chloramphenicol, Sp = spectinomycin, and ^{r} = resistant.
[b] Yale University, School of Medicine, New Haven, CT.
[c] Gaithersburg, MD.

this solution, these stock solutions were added as follows: 1 ml of 40 mM $MgSO_4$, 1 ml of 760 mM $(NH_4)_2SO_4$, 1 ml of 20% glycerol, 2 ml of 2.75 M potassium phosphate (pH 7.0), and 0.25 ml of 10% yeast extract. When required, antibiotics were added as follows: ampicillin, 50 μg/ml; chloramphenicol (Cm), 10 μg/ml; gentamycin, 10 μg/ml; kanamycin (Km), 50 μg/ml; spectinomycin, 50 μg/ml; streptomycin, 100 μg/ml; and tetracycline (Tc), 10 μg/ml. The minimal medium was supplemented with 50 μg of amino acid per milliliter as necessary. Media were solidified by the addition of Difco agar (Difco Laboratories, Detroit, MI) (1.5%). Soft (top) agar contained 0.6% Difco agar.

Transposon mutagenesis. Tn*5* and Tn*10-lacZ* insertion mutants were obtained by using the plasmid pJB4JI and λ-Tn*10-lacZ*, respectively (Murata *et al.* 1990). Tn*phoA* mutations were generated in *E. c.* subsp. *carotovora* 71 by mating with *Escherichia coli* MM294A carrying the plasmid pRK609 (Long *et al.* 1988). Antibiotic-resistant colonies were scored for defects in pectolytic, proteolytic, and cellulolytic activities on PYCP agar, nutrient gelatin agar, and Cel detection agar, respectively. *aepA-lacZ* transcriptional fusions were derived from pAKC264 and pAKC602 (Table 1 and Fig. 1) with the mini-Mu-*lacZ* element, Mu*d*I1734, as described previously (Castilho *et al.* 1984; McEvoy *et al.* 1990). Individual plasmids containing Mu*d*I1734 insertions were transferred to the AepA$^-$ mutant AC5012 by triparental matings. The transconjugants that remained deficient in pectolytic, proteolytic, and cellulolytic activities, as determined by agar plate assays, were presumed to result from inactivation of the *aepA*$^+$ gene. Plasmids containing *aepA-lacZ* were further characterized by screening the transconjugants for β-galactosidase production on agar media containing X-gal. *Hin*dIII, which cleaves pAKC602 twice (within the polylinker and the internal fragment; Fig. 1) and Mu*d*I1734 twice (0.1 kb from the 5′ end of the *lacZ* transcriptional unit and 1.0 kb from the 3′ end, McEvoy *et al.* 1990), and *Sst*I, which cleaves once within the insert DNA (Fig. 1) and once within Mu*d*I1734 (Metcalf *et al.* 1990), were used in determining the location and orientation of the insertions. *Eco*RI, *Eco*RV, and *Pst*I digests were used to confirm mapping of the insertions.

Preparation and assay of samples for enzymatic activities. Bacteria were grown in desired media to about 250 Klett units (Klett-Summerson colorimeter, No. 66 filter; Klett Manufacturing Co., Inc., New York), and cells and supernatants were separated by centrifugation (12,100 × *g*) at 4° C. The protocols for preparing periplasmic fractions, cellular extracts, and dialyzed samples; the procedures for the quantitative assays of Cel, cyclic phosphodiesterase, β-lactamase, Pel, Peh, and Prt activities; and the definitions of the enzymatic units have been described previously

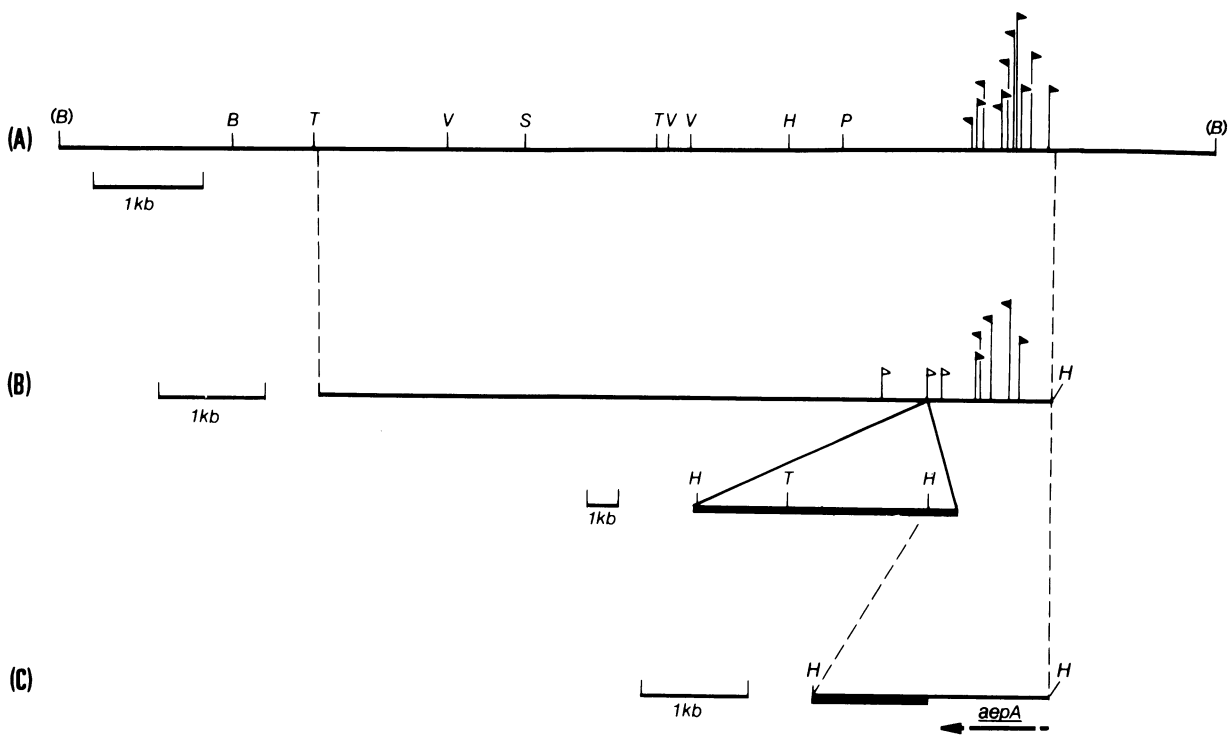

Fig. 1. Cloned portion of the *Erwinia carotovora* subsp. *carotovora* 71 genome harboring the *aepA* gene. **A**, Restriction map of the 10.5-kilobase (kb) region of *E. c.* subsp. *carotovora* 71 DNA contained in the cosmid pAKC264. The solid flags denote the sites and orientations of Mu*d*I1734 insertions inactivating *aepA*; flags pointing to the left indicate insertions that produced high levels of β-galactosidase. **B**, A 6.5-kb *Sau*3A subclone of *aepA* DNA contained in pAKC602. The solid flags indicate the sites and orientations of Mu*d*I1734 insertions inactivating *aepA*; the open flags indicate insertions not inactivating *aepA*. Solid flags pointing to the left indicate the insertions from which high levels of β-galactosidase were produced. Also shown in detail is the Mu*d*I1734 insertion (bold line) found in pAKC607. **C**, A *Hin*dIII fragment from pAKC607 that contains about 1.0 kb of Mu*d*I1734 DNA (bold line) and 1.2 kb of *E. c.* subsp. *carotovora* 71 DNA including the *aepA* gene. This fragment was cloned in both orientations in pRK404 resulting in the *aepA*$^+$ plasmids pAKC608 and pAKC609. The direction of transcription and extent of *aepA* are shown at the bottom of the figure. Abbreviations are as follows: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; T, *Sst*I; and V, *Eco*RV.

(Barras *et al.* 1986; Murata *et al.* 1990; Thurn and Chatterjee 1985). Protein content was determined by using the BCA reagent (Pierce Chemical Co., Rockford, IL).

Isolation of the AepA$^+$ cosmid. The procedures used for the screening of an *E. c.* subsp. *carotovora* 71 gene bank have been described in a previous publication (Murata *et al.* 1990). An AepA$^-$ mutant, AC5012, was used as the recipient. The transconjugants resulting from triparental matings were scored for the production of pectolytic, cellulolytic, and proteolytic activities using agar plate assays.

Recombinant DNA techniques. Standard published procedures were used for the isolation of plasmid and chromosomal DNAs, transformation of *E. coli*, restriction digests, gel electrophoresis, and electroelution (Crouse *et al.* 1983; Maniatis *et al.* 1982). DNA-modifying enzymes were obtained from Promega (Madison, WI).

Plant tissue maceration. The potato tuber assay was described previously (Murata *et al.* 1990). The celery petiole assay was done in the same way as the potato tuber assay except that celery petioles were soaked in 0.5% sodium hypochlorite for 5 instead of 10 min, and inoculation sites were not sealed with Vaseline. Surfaces of celery petioles were punctured, and bacterial suspensions, which were prepared as described previously (Murata *et al.* 1990), were inoculated through the wounds into the vascular tissues.

RESULTS

Effects of crude plant extracts on enzyme production. To develop a medium that could sustain the production of high levels of Pel, Peh, Cel, and Prt in *E. c.* subsp. *carotovora* 71, we tested the effects of crude extracts from various plant tissues. In preliminary trials, we found higher levels of extracellular Pel in SYG medium containing celery extract than in SYG medium supplemented with pectate, carrot extract, or potato extract. Subsequently, we compared the levels of various enzymes in SYG and SYG supplemented with pectate or celery extract. The data (Table 2) show that *E. c.* subsp. *carotovora* 71 produced higher levels of extracellular Pel as well as Cel and Prt in SYG plus celery extract than in SYG or SYG plus pectate. However, extracellular Peh activity was similar in media supplemented with pectate or celery extract. A particularly striking feature was that 94% of Pel, 92% of Cel, and >99% of Prt activities in SYG plus celery extract were present in the culture supernatant. Because of this stimulatory effect of celery extract on the production of extracellular Pel, Cel, and Prt, we routinely used SYG plus celery extract in our subsequent work.

Isolation of transposon insertion mutants defective in extracellular enzyme production. As was done in our previous studies (Murata *et al.* 1990; Zink *et al.* 1984), we introduced Tn*5* into *E. c.* subsp. *carotovora* 71 using pJB4JI and Tn*10-lacZ* into AC5006 using λ::Tn*10*. For mutagenesis with Tn*phoA*, the Tra$^+$ plasmid pRK609 (Long *et al.* 1988) was used. This plasmid is unstable in *E. c.* subsp. *carotovora* 71 because it contains a ColE1 origin of replication. Therefore, in the absence of Cm selection, a high proportion (>99%) of Km^r Cm^s transconjugants were produced. In gel electrophoretic analysis of plasmid preparations, repre-

Table 2. Effect of pectate and celery extract on extracellular enzyme production in *Erwinia carotovora* subsp. *carotovora* 71

	Activity[b] (units/mg protein)									
	Pel		Peh		Cel		Prt		Bla	Cpd
Media[a]	S	C	S	C	S	C	S	C	C[c]	C
SYG	0.3	2.3	<0.1[d]	5.6	<0.1	<0.1	<0.1	<0.1	8.5	2.4
SYG + pectate	16.7	13.3	4.1	8.1	5.6	10.4	<0.1	<0.1	5.5	2.4
SYG + celery extract	29.6	1.9	3.5	1.3	34.3	3.0	8.0	<0.1	6.6	1.0

[a] Bacteria were grown in minimal salts-yeast extract-glycerol (SYG) medium or in this medium supplemented with pectate (0.2%) or celery extract. For additional details see the text.
[b] Supernatants (S) and cell extracts (C) of cultures grown to about 250 Klett units in a shaker at 28°C were assayed. Pel = pectate lyase, Peh = polygalacturonase, Cel = cellulase, Prt = protease, Bla = β-lactamase, and Cpd = cyclic phosphodiesterase.
[c] Bla and Cpd activities were only detected in cell extracts.
[d] <0.1 units for Peh, Cel, or Prt represent the limits of detection of activities under the assay conditions used.

Table 3. Effect of *aepA*$^+$ DNA on the production of extracellular and periplasmic enzymes in *Erwinia carotovora* subsp. *carotovora* 71 and a Tn*5* insertion mutant, AC5012[a]

	Activity[c] (units/mg protein)							
	Strain 71/pSF6[d]		Strain 71/pAKC264[e]		AC5012/pSF6		AC5012/pAKC264	
Enzyme[b]	S	C	S	C	S	C	S	C
Pel	13.7	0.7	72.5	2.0	2.0	2.3	70.5	3.0
Peh	1.9	0.9	13.1	2.1	<0.1[f]	1.3	10.6	2.0
Cel	15.6	<0.1	83.6	7.3	<0.1	6.5	149.1	15.3
Prt	3.6	<0.1	15.2	<0.1	<0.1	<0.1	12.2	<0.1
Bla	<0.02[g]	2.4	<0.02	2.5	<0.02	3.3	<0.02	3.0
Cpd	<0.02	1.1	<0.02	0.9	<0.02	2.3	<0.02	2.0

[a] Bacteria were grown in minimal salts-yeast extract-glycerol (SYG) medium supplemented with celery extract. For additional details see the text.
[b] Pel = pectate lyase, Peh = polygalacturonase, Cel = cellulase, Prt = protease, Bla = β-lactamase, and Cpd = cyclic phosphodiesterase.
[c] Supernatants (S) and cell extracts (C) of cultures grown to about 250 Klett units in a shaker at 28°C were assayed for enzymatic activities.
[d] pSF6 = the cloning vector (Table 1).
[e] pAKC264 = pSF6 + *aepA*$^+$ DNA (Table 1 and Fig. 1).
[f] <0.1 units of activity for Peh, Cel, or Prt represent the limit of detection under the assay conditions used.
[g] <0.02 units of activity for Bla and Cpd represent the limit of detection under the assay conditions used.

sentative Km^r Cm^s derivatives were found not to contain pRK609 (data not shown). These data collectively indicate that the Km^r and Cm^s colonies resulted from the loss of the plasmid and the presence of Tn*phoA* in the *E. c.* subsp. *carotovora* 71 genome.

Agar plate screening and subsequent quantitative assays for the production of extracellular enzymes in strains carrying transposon insertions revealed the occurrence of two classes. Class I was represented by Out$^-$ mutants previously described by Murata *et al.* (1990). Class II is represented by three independently isolated AepA$^-$ mutants (Table 1); quantitative data for one mutant (AC5012) is given in Table 3. While the levels of cell-bound Pel, Peh, and Cel as well as Prt in this mutant were generally low and comparable to those in the parent, extracellular activities were much reduced. The absence of high levels of Cel, Pel, and Peh in cells and the deficiency in Prt production (Table 3) clearly distinguish the AepA$^-$ mutants from the bona fide Out$^-$ mutants of *E. c.* subsp. *carotovora* (Hinton and Salmond 1987; Murata *et al.* 1990) or *E. chrysanthemi* Burkholder *et al.* (Andro *et al.* 1984; Thurn and Chatterjee 1985). In addition, the plasmids carrying the *E. c.* subsp. *carotovora* 71 *out* cluster (Murata *et al.* 1990) did not restore enzyme production in any of the AepA$^-$ mutants (data not shown). However, these mutants, like the Out$^-$ mutants (Murata *et al.* 1990), were severely attenuated in their ability to macerate celery petioles or potato tuber tissue (Fig. 2). The AepA$^-$ mutants, like their parents, utilized the following compounds as carbon sources: glycerol, arabinose, ribose, xylose, glucose, galactose, galacturonate, mannitol, sucrose, raffinose, melibiose, and polygalacturonate.

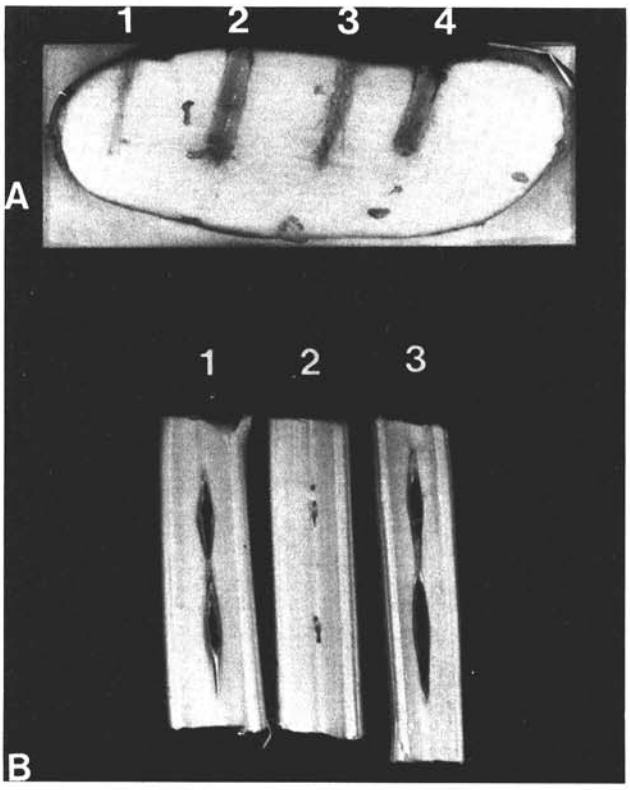

Fig. 2. Tissue maceration by *Erwinia carotovora* subsp. *carotovora* 71, an AepA$^-$ strain AC5012, and AC5012 carrying an *aepA*$^+$ plasmid. Whole potato tubers and celery petioles were inoculated and incubated as described in the text. After 48 hr of incubation, pathogenic reactions were recorded. **A**, Potato tuber: section 1, buffer control; section 2, AC5012(pAKC602); section 3, AC5012(pRK404); and section 4, *E. c.* subsp. *carotovora* 71(pRK404). **B**, Celery petioles: segment 1, *E. c.* subsp. *carotovora* 71(pRK404); segment 2, AC5012(pRK404); and segment 3, AC5012(pAKC602).

Molecular cloning of the *aepA*$^+$ DNA. By mobilizing an *E. c.* subsp. *carotovora* 71 library of about 1,000 cosmids into AC5012, we obtained one cosmid, pAKC264, that restored extracellular enzyme production. This cosmid restored extracellular enzyme production in all of the AepA$^-$ mutants but not in Out$^-$ mutants. Along with extracellular enzyme production, the AepA$^-$ mutants carrying pAKC264 regained the ability to macerate plant tissue like the parent strain (Fig. 2). pAKC264 appears not to carry the structural genes for the extracellular enzymes Pel, Cel, Peh, or Prt, because these activities were not detected in HB101 carrying the cosmid (data not shown).

We subcloned the *aepA*$^+$ segment of pAKC264 by preparing a *Sau*3A partial digest and ligating the fragments to *Bam*HI-cut pRK404 treated with phosphatase. The Tc^r DH5α transformants that remained white in the presence of X-gal were picked, and their plasmids were mobilized into AC5012. Of the several plasmids that restored enzyme production in AC5012, we selected pAKC602 for our subsequent analysis, because it carried the smallest insert DNA of about 6.5 kb. pAKC602, like its parent pAKC264, restored enzyme production in AC5012 and AC5027. To localize the *aepA*$^+$ DNA, we mutagenized the insert DNAs of pAKC264 and pAKC602 with Mu*d*I1734 (mini-Mu-*lacZ*; Castilho *et al.* 1984) and tested Km^r derivatives for their ability to restore enzyme production. The results of such analyses, taken along with the sites of mini-Mu-*lacZ* insertions (indicated in Fig. 1), allowed localization of *aepA* within a 1.1-kb DNA stretch. Indeed, 16 plasmids containing Mu*d*I1734 insertions that mapped within about 800 bp of DNA (Fig. 1) failed to restore extracellular enzyme production in the AepA$^-$ mutants. To confirm the limits of *aepA*, we subcloned into pRK404 a 2.2-kb fragment spanning from the *Hin*dIII site of the vector polylinker to the *Hin*dIII site within the Mu DNA of the Mu*d*I1734 insertion in pAKC607 (Fig. 1). This fragment, containing about 1.2 kb of *E. c.* subsp. *carotovora* 71 DNA and 1.0 kb of Mu DNA, was cloned in both orientations in pRK404 and produced plasmids pAKC608 and pAKC609. Both of these plasmids restored enzyme production in AC5012 and AC5027. Thus, the 1.2-kb *E. c.* subsp. *carotovora* 71 DNA appears to contain sequences encoding a functional *aepA* gene as well as the *aepA* promoter.

Stimulation of extracellular enzyme production by the *aepA*$^+$ DNA. The data in Table 3 show that in the SYG medium supplemented with celery extract, AC5012 carrying pAKC264 produced higher levels of enzymes than AC5012 carrying the vector pSF6; the levels of extracellular Pel, Peh, Cel, and Prt were 35-fold, >100-fold, >150-fold, and >10-fold higher, respectively. We should point out that the levels of these enzymes within the cells were not markedly affected by the presence of pAKC264. The data also show that cyclic phosphodiesterase and β-lactamase activities in the mutant were comparable to those produced by wild-type strain 71 of *E. c.* subsp. *carotovora* and that

pAKC264 did not affect the levels of these periplasmic enzymes.

pAKC264 also stimulated the production of extracellular enzymes in wild-type strain 71 of *E. c.* subsp. *carotovora*. In SYG plus celery extract, the levels of Pel, Peh, Cel, and Prt were higher in extracellular fluid of *E. c.* subsp. *carotovora* 71(pAKC264) than in *E. c.* subsp. *carotovora* 71(pSF6); Pel was stimulated fivefold; Cel, fivefold; Prt, fourfold; and Peh, sevenfold (Table 3). Although the levels of Pel and Peh in cells were not significantly affected by pAKC264, Cel activity was somewhat higher in cells carrying the AepA$^+$ plasmid than in the cells carrying the vector.

Previous studies with *E. c.* subsp. *carotovora* (Mount *et al.* 1979) have shown a requirement for a functional cAMP-CRP system for Pel production. Consistent with this requirement is the occurrence of cAMP receptor protein (CRP)-binding sites upstream of the coding regions of the various *Erwinia pel* and *peh* genes (Hinton *et al.* 1989; Reverchon *et al.* 1989; Saarilahti *et al.* 1990; Tamaki *et al.* 1988). To rule out the possibility that the stimulatory effect of the cloned DNA was due to the presence of *cya* or *crp* DNAs, pAKC602 was transformed into an *E. coli* Cya$^-$ mutant (CGSC6027) and a Crp$^-$ mutant (CGSC7043), and the antibiotic-resistant transformants were tested for the utilization of various sugars. The transformants carrying pAKC602, like those carrying the vector pRK404, failed to utilize arabinose, maltose, mannitol, and sorbitol. In light of a previous finding with an *E. chrysanthemi cya*$^+$ DNA (Hedegaard and Danchin 1985), complementation of *E. coli* mutations should have occurred if the *E. c.* subsp. *carotovora* 71 *cya* and *crp* genes were present on pAKC602.

DISCUSSION

The findings of this study show that function of a gene locus, designated as *aepA*, is required for the production of extracellular enzymes in *E. c.* subsp. *carotovora* 71. Several lines of evidence (see above) established that the pleiotropic mutants were genetically and physiologically distinct from the bona fide Out$^-$ mutants of *E. chrysanthemi* and *E. c.* subsp. *carotovora* (Andro *et al.* 1984; Hinton and Salmond 1987; Murata *et al.* 1990; Thurn and Chatterjee 1985). The cloned DNA restored production of the extracellular enzymes in all of the transposon-generated mutants. Restoration of the AepA phenotype is most likely due to allelic complementation. However, at this juncture we cannot eliminate the possibility of an extragenic suppression of the mutational phenotype by the cloned DNA.

The AepA$^+$ plasmid stimulated enzyme production in the wild-type strain of *E. c.* subsp. *carotovora*. This stimulation, apparently due to a gene dosage effect, appeared to be specific for extracellular proteins, because the levels of periplasmic enzymes were not altered by the presence of the *E. c.* subsp. *carotovora* 71 *aepA*$^+$ DNA (Table 3). Thus, the specificity of the *aepA* function appears to be similar to the *B. subtilis senS* gene function (Wang and Doi 1990) in regulating extracellular enzyme production.

The pleiotropic phenotype in the AepA$^-$ mutants did not result from a defect in the cAMP-CRP system as indicated by the utilization of various sugars whose catabolism requires *cya* and *crp* functions (Mount *et al.* 1979; Pastan and Adhya 1976) and by the inability of the cloned *aepA*$^+$ DNA to complement *cya* and *crp* mutations in *E. coli*. This is in contrast with the findings of De Crecy-Lagard *et al.* (1990) with a *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson gene, *clp*, that apparently regulates various traits, including pathogenicity and extracellular enzyme production. The *clp* gene product (a *CAP-like protein*; CAP, catabolic activator protein) also partially restored the carbohydrate fermentation pattern of a *cya crp* strain of *E. coli*, and the *clp* gene shared some homology with the *E. coli crp* DNA. Whether the *E. c.* subsp. *carotovora* 71 *aepA*$^+$ DNA, which specifically affects genes for extracellular proteins, has sequence homology with *clp*, *cya*, *crp*, or other genes mediating global regulation is currently under investigation.

We do not yet know how the *aepA* gene product stimulates extracellular enzyme production. However, several possibilities can be considered in explaining the *trans*-dominant effect of *aepA*. It is conceivable that the *aepA* gene product is a component of a cotranslational export machinery required in the production of extracellular proteins, including enzymes such as Pel, Cel, Prt, and Peh. In its normal state, AepA by itself or as a component of secretion machinery may stabilize the mRNA-polysome complex, thereby increasing the half-life of the message and stimulating translation of mRNA. We should note that precedence exists for translational control of exported proteins in *E. coli* (Ferro-Novick *et al.* 1984; Hall *et al.* 1983; Hengge-Aronis and Boos 1986; Watanabe *et al.* 1988). Alternatively, the *aepA* gene product may stimulate gene expression by functioning as a transcriptional activator or as a component of transcriptional machinery such as a sigma factor or a sensor protein. In light of an apparently similar effect of the *Erwinia aepA* gene and the *B. subtilis senS* gene on the regulation of extracellular enzyme production, we can entertain the possibility that the products of these genes act in a similar fashion. Noteworthy in this context is the finding of Wang and Doi (1990) that the *senS* gene product showed partial homology with sigma factors and possessed a helix-turn-helix motif found in DNA-binding proteins. Much additional work is clearly needed to clarify the role of the *aepA* gene product, to determine the relationship between the expression of *aepA* and activation of extracellular enzyme production, and to identify the components of the signal transduction pathway.

Our demonstration that a gene locus controls the production of multiple extracellular enzymes and pathogenicity of *E. c.* subsp. *carotovora* 71 has an additional ramification. *E. c.* subsp. *carotovora* is often considered to be an opportunistic pathogen that causes soft rots in susceptible plants as an inevitable consequence of its secretion of massive quantities of pectic enzymes. The finding of the *aepA* gene, while supporting this concept, also provokes the idea that the bacterium has developed highly sophisticated mechanisms to modulate the production of extracellular enzymes during its interaction with host plants.

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