Characterization of a Novel Regulatory Gene *aepA* that Controls Extracellular Enzyme Production in the Phytopathogenic Bacterium *Erwinia carotovora* subsp. *carotovora*

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***Erwinia carotovora* subsp. *carotovora* strain Ecc71 produces an array of extracellular enzymes including pectate lyase (Pel), polygalacturonase, cellulase, and protease. In strain Ecc71, these enzymes are coregulated by *aepA*, which encodes an *a*ctivator of *e*xtracellular protein *p*roduction (H. Murata, J. L. McEvoy, A. Chatterjee, A. Collmer, and A. K. Chatterjee, Mol. Plant-Microbe Interact, 4:239-246, 1991). The nucleotide sequence of a 2.7-kb *aepA*$^+$ DNA segment revealed an open reading frame (ORF) of 1,395 bp which matches with the size of the *aepA* transcript determined by Northern blot analysis. *aepA* is predicted to encode a protein of 465 amino acid residues with a molecular mass of approximately 51 kDa and a pI of 6.52. The occurrence of a putative signal sequence and several hydrophobic domains suggest membrane localization of AepA. An *aepA-lacZ* operon fusion was constitutively expressed in *E. coli* (DH5α) but inducible by pectate and celery extract in *E. c.* subsp. *carotovora* (AC5006). These findings suggest that *aepA* expression may be negatively regulated in *E. c.* subsp. *carotovora*. By assaying for the transcript of *pel-1*, which specifies a major secreted Pel species in strain Ecc71, and by following the expression of a *pel1-lacZ* operon fusion we determined that AepA activates *pel-1* transcription. The characteristics of *aepA* including the lack of homology with other prokaryotic regulatory genes indicate that *aepA* encodes a novel regulatory protein required for extracellular protein production. Whereas homologs of Ecc71 *aepA* occur in *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica* strains, activation of exoenzyme production is markedly stimulated by *aepA* in *E. c.* subsp. *carotovora* than in *E. c.* subsp. *atroseptica*.**

Additional keywords: gene regulation, soft rot, transcriptional activator.

Erwinia carotovora subsp. *carotovora* causes soft-rot disease in a wide variety of plants (Perombelon 1987; Perombelon and Kelman 1980). The ability of this bacterium to elicit soft rot to a large extent depends on the production of extracellular enzymes, for example, pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt), that depolymerize plant cell and cell wall components (Chatterjee and Vidaver 1986; Collmer and Keen 1986; Kotoujansky 1987). Of these enzymes, pectinases such as Pel, Pnl (pectin lyase), and Peh have attracted the most attention because of their ability to macerate plant tissues (Collmer and Keen 1986; Lei *et al.* 1985; Mount *et al.* 1970; Roberts *et al.* 1986; Tanabe and Kobayashi 1986; Tsuyumu *et al.* 1991; Willis *et al.* 1987; Zink and Chatterjee 1985; Zink *et al.* 1985). However, the presence of Cel and Prt, in addition to Peh and Pel, in macerated tissues (Stephens and Wood 1975) and activation of the production of these enzymes in media supplemented with plant extracts (Chatterjee *et al.* 1991; Murata *et al.* 1991) raised the following possibilities: 1) a concerted action of Pel, Peh, Cel, and Prt is required for an efficient degradation of plant cell wall components, and 2) the production of these enzymes is coordinately regulated in *E. c.* subsp. *carotovora*.

The isolation of mutants pleiotropically deficient in extracellular enzymes provided additional evidence for their coregulation in *E. c.* subsp. *carotovora* strains (Beraha and Garber 1971; Murata *et al.* 1991; Pirhonen *et al.* 1991). Such enzyme-deficient mutants are severely attenuated in their pathogenicity. From gene libraries of wild-type strains plasmids that complemented the mutants were isolated; the genes responsible for the pleiotropic phenotype were designated as *aepA* (*a*ctivator of *e*xtracellular protein *p*roduction [Murata *et al.* 1991]) and *exp* (extracellular protein production [Pirhonen *et al.* 1991]). A plasmid carrying *aepA* also activated enzyme production in the wild-type strain Ecc71 (Murata *et al.* 1991). We have since characterized the regulatory system further by determining the structure, expression, and function of *aepA*. In this report, we 1) present the nucleotide sequence of *aepA* and the characteristics of the predicted product; 2) document that AepA activates transcription of *pel-1*, which specifies a pectate lyase isozyme of *E. c.* subsp. *carotovora* strain Ecc71; 3) show that *aepA* expression is induced by plant components; and 4) provide evidence for the presence of *aepA* homologs in the other *E. c.* subsp. *carotovora* strains as well as in the black leg pathogen, *E. c.* subsp. *atroseptica*.

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RESULTS

Sequence of *aepA*.

By sequencing the internal fragment of pAKC654, an open reading frame (ORF) was identified that corresponded to the limit of *aepA* and the direction of transcription as determined by analyzing Mu*d*I1734 insertions (Murata *et al.* 1991; Fig. 1). The 1,395-bp ORF is localized within the *Fsp*I-*Hin*dIII sites (Figs. 1 and 2). The transcript size inferred from the nucleotide sequence matches well with the size determined by a RNA blot analysis (Fig. 3). A potential ribosome binding site (RBS) (AAGGAA) is present 15 bp upstream of the putative ATG start codon. However, a GTG start codon present inframe just before the ATG codon brings the RBS close to the putative translational start site. The transcriptional start site is located at the adenine residue at 36 position relative to the presumed translational start site at the ATG codon (Fig. 4). The sequences exhibiting homologies to σ^{70} promoters were found at −40 bp and −17 bp (instead of the typical −35 and −10 sequences) upstream of the transcriptional start site in *aepA* (Fig. 2). Sequences matching the consensus KdgR binding site (Nasser *et al.* 1992; Reverchon *et al.* 1991) are found at two locations upstream of the transcriptional start site: the first one from −433 to −417, and the second one from −562 to −546. It remains to be determined if these binding sites located far upstream (>430 bp) of the transcriptional start site have any physiological significance. The sequence stretching from −30 to −15 relative to the transcriptional start site showed homologies with part of the cyclic AMP receptor protein (CRP)-binding site (De Crombrugghe *et al.* 1984). Whereas the highly conserved consensus CRP-binding sequences (TCTGA) match quite well with sequences in *aepA*, the less conserved sequences match rather poorly. This observation, the presence of the putative CRP-binding site within the σ^{70}-like promoter region, and the lack of evidence for catabolite repression (see below) raise doubts concerning the significance of these putative CRP-binding sequences in *aepA* expression.

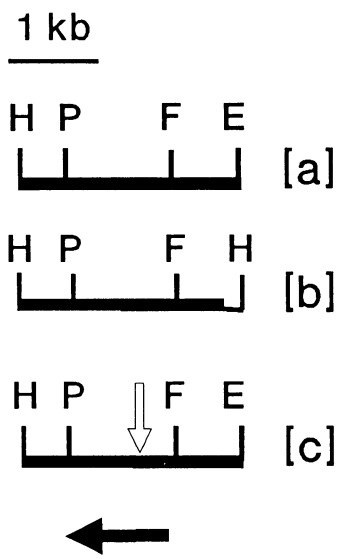

Fig. 1. Restriction endonuclease map of Ecc71 DNA containing *aepA*. The bold lines represent Ecc71 genomic DNA. The open arrow indicates the site of Mu*d*I1734 insertion. Predicted location and direction of transcription of *aepA* are indicated by the bold arrow at the bottom of the figure. Abbreviations: E; *Eco*RI, F; *Fsp*I, H; *Hin*dIII, P; *Pst*I. **A,** A portion of pAKC264 DNA containing *aepA*. **B,** A portion of pAKC602 DNA (= pRK404 + *aepA*$^+$; see Murata *et al.* 1991). The *Hin*dIII fragment was cloned into pCL1920, creating a plasmid, pAKC654. The *Hin*dIII DNA fragment contains, in addition to Ecc71 DNA, a part of the multiple cloning site of pRK404 as indicated by the thin line. **C,** A portion of pAKC612 DNA showing the site of Mu*d*I insertion within *aepA* (Table 1). pAKC612 was used to study *aepA* expression (see text).

The AepA protein is predicted to consist of 465 amino acid residues having a molecular mass of 51 kDa and an isoelectric point of 6.52. The characteristics of the 21 amino acid residues at the N-terminal segment of AepA are typical of prokaryotic signal sequences (Duffaud *et al.* 1985; Pugsley and Schwartz 1985). A potential signal peptide cleavage site is present between Ala-His residues at positions 21 and 22. The AepA polypeptide is also predicted to possess three additional hydrophobic domains (Fig. 5). The deduced amino acid sequence of AepA has not shown significant homology with protein sequences stored in SwissProt database.

Expression of *aepA-lacZ* operon fusion in *E. coli* and in derivatives of *E. c.* subsp. *carotovora* strain Ecc71.

To test the expression of *aepA*, pAKC612, the low copy plasmid carrying an *aepA-lacZ* operon fusion (Table 1), was introduced into *E. coli* strain DH5α and *E. c.* subsp. *carotovora* strain AC5006. DH5α(pAKC612) and AC5006(pAKC612) were grown in SYG medium at 28° C, and culture samples for β-galactosidase assay were collected at a Klett value of approximately 300. While the level of β-galactosidase activity in DH5α (pAKC612) was about 3,000 Miller units, about 500 Miller units were detected in AC5006(pAKC612). By gel electrophoretic analysis, we estimated that the copy number of pAKC612 in *E. coli* (DH5α) and *E. c.* subsp. *carotovora* (AC5006) was very similar (data not shown). AC5006(pAKC612), when grown in SYG supplemented with celery extract, produced 2,500–3,000 Miller units of β-galactosidase activity, which was five times higher than the level of activity detected in AC5006(pAKC612) grown in the SYG medium and similar to the level of β-galactosidase produced in DH5α grown in the SYG medium or in SYG medium containing celery extract. Thus, the expression of *aepA* appeared to be derepressed in DH5α but inducible in AC5006. The inducibility of *aepA-lacZ* in *E. c.* subsp. *carotovora* was further confirmed by constructing a strain, AC5031, carrying a chromosomal copy of *aepA-lacZ* (Table 1 and Materials and Methods) and assaying for β-galactosidase activity under various growth conditions. β-Galactosidase activity in AC5031 was induced about twofold with pectate and about fivefold in the presence of celery extract (SYG medium, 267 Miller units; SYG + pectate, 525 Miller units; SYG + CE, 1097 Miller units). There was no adverse effect of glucose on the expression of *aepA* in AC5031 (data not shown).

Activation of *pelI-lacZ* transcription by AepA.

To determine if AepA activates transcription, we used *pel-1*, which encodes one of the secreted Pels of *E. c.* subsp.

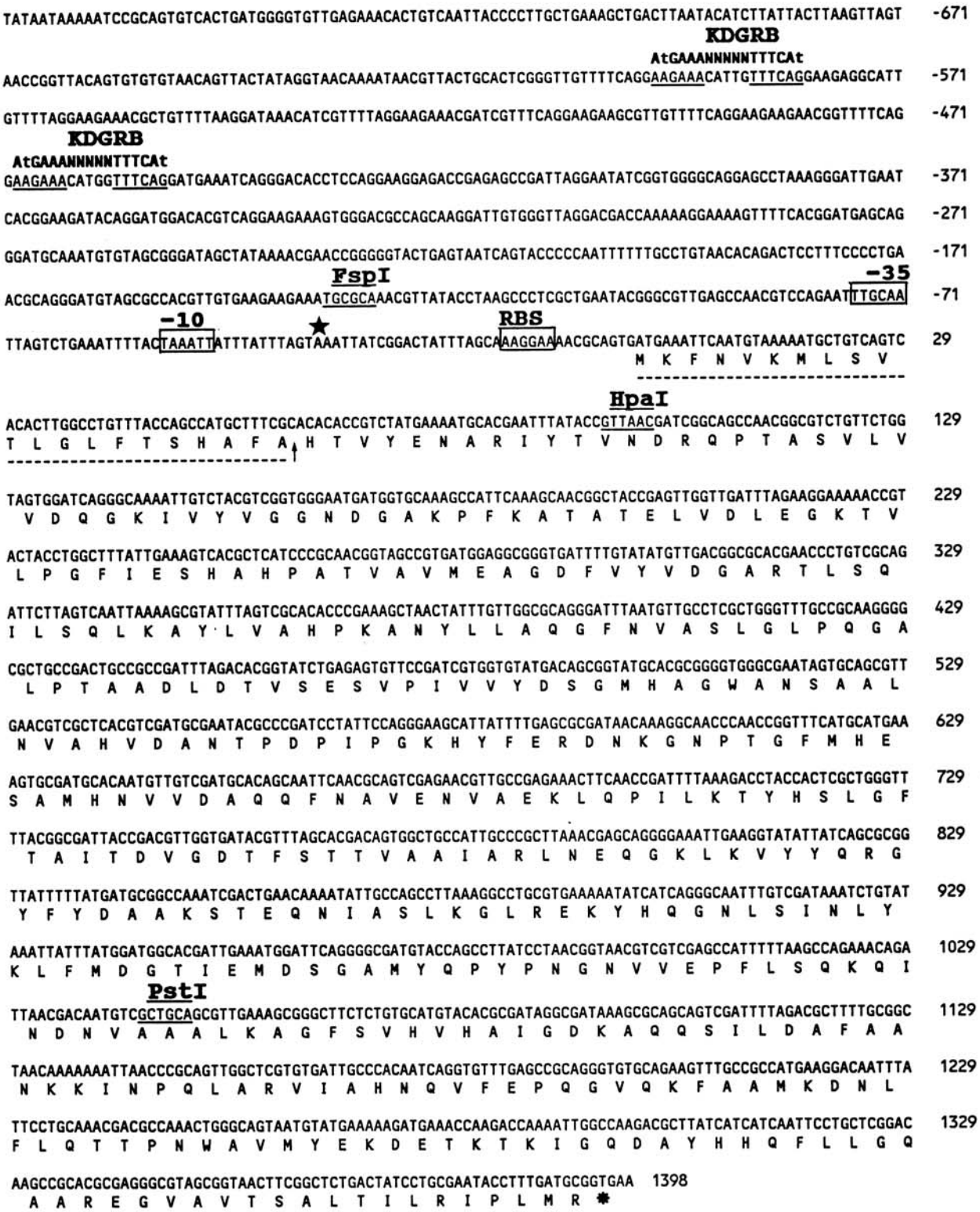

Fig. 2. Nucleotide sequence of the *aepA* structural gene and flanking DNA. The deduced amino acid sequence of *aepA* is also given. The putative ribosome binding site (RBS) and sequences similar to the −10 and −35 consensus sequences are boxed in. The transcriptional start site is indicated (★). The putative KdgR-binding site is shown at bases −469 to −453 and −598 to −582; uppercase letters indicate identity. The predicted signal sequence containing the first 21 amino acid residues is indicated by the broken line. The arrow between Ala and His residues at positions 21 and 22 indicates the putative signal peptide cleavage site. Sites of restriction endonucleases, *Fsp*I, *Hpa*I, and *Pst*I, are also indicated.

carotovora strain Ecc71, as the prototype of the genes specifying exoenzymes. Two types of experiments were performed. In one set of experiments, we examined the effect of gene dosage by transferring the *aepA*$^+$ plasmid, pAKC264 to the AepA$^+$, Pel1-LacZ strain, AC5030, and then assaying for the stimulation of β-galactosidase activity. Bacterial strains carrying the AepA$^+$ plasmid, pAKC264 or the cloning vector, pSF6, were grown in the SYG + CE medium at 28° C. When the cultures reached approximately 350 Klett units, samples were removed and assayed for β-galactosidase activity. AC5030(pSF6) produced about 1,060 Miller units of β-galactosidase compared to about 2,540 Miller units in AC5030(pAKC264). This 2.5-fold stimulation in *pel1-lacZ* expression was attributed to the dosage of the *aepA*$^+$ DNA.

In the second set of experiments we determined if transcription of *pel-1* was affected in the absence of AepA. For this we assayed the levels of the *pel-1* RNA in AepA$^+$ and AepA$^-$ bacterial constructs by conducting dot blot analysis (Fig. 6). *pel-1* RNA was barely detectable in a sample of the AepA$^-$ strain, AC503l(pSF6), whereas high levels of *pel-1* RNA were detected in samples of the AepA$^+$ bacteria.

Evidence for the occurrence of an *aepA*-like regulatory system in *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica*.

To determine if an *aepA*-like regulatory system is also operative in the other *E. c. subsp. carotovora* strains and in the related bacterium, *E. c.* subsp. *atroseptica*, we determined 1) the effect of the *aepA*$^+$ gene of the strain Ecc71 on exoenzyme production in the wild-type strains, and 2) the presence of *aepA* homologs in these bacteria.

1) Effect of *aepA* on the production of extracellular enzymes in wild-type strains.

As indicated in Table 2, *E. c.* subsp. *carotovora* strain Ecc193 carrying pAKC264 produced Pel, Peh, Cel, and Prt at five, two, eight, and eight times higher levels, respectively, than the levels in the strain carrying the cloning vector, pSF6. Similarly, the levels of Pel, Peh, Cel, and Prt in *E. c.* subsp. *carotovora* strain SCRI193 carrying pAKC264 were significantly higher compared to those in SCRI193 carrying pSF6: Pel was stimulated sevenfold, Peh threefold, Cel sixfold, and Prt threefold. In contrast, in *E. c.* subsp. *atroseptica* strains Eca12 and EC, stimulation of the levels of Cel and Peh activities by pAKG264 was less compared to the stimulation of the Pel and Prt levels (Table 2).

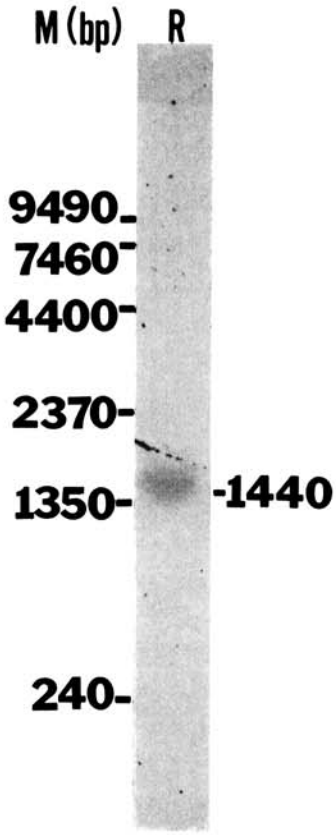

Fig. 3. Northern blot analysis of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 RNA probed with the antisense RNA of the 231-bp *Fsp*I-*Hpa*I fragment containing the 5′ portion of *aepA* (Fig. 2).

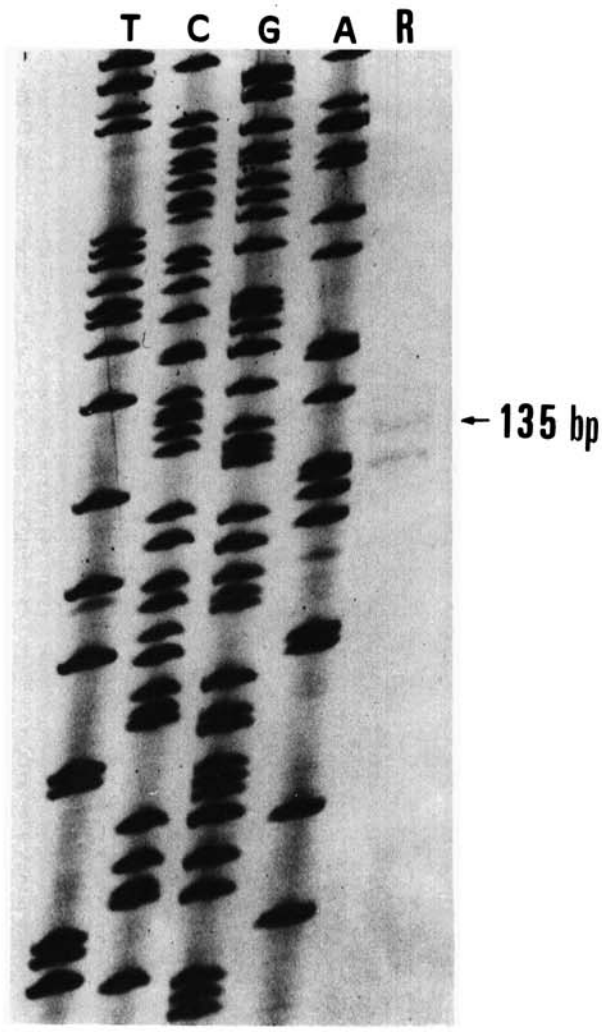

Fig. 4. RNase protection assay of the *aepA* transcript (described in the text). The sample containing RNA/RNA hybrids (lane R) was electrophoresed in parallel with a sequence ladder (TCGA). The portion of the sequence pertinent to the transcriptional start site is shown.

2) Presence of *aepA* homologs.

By Southern hybridization analysis homologs of *aepA* were searched in strains of *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica*. Genomic DNAs were digested by the endonuclease *Pst*I, transferred to Biotrans nylon membrane and probed with the *Eco*RI-*Pst*I DNA segment containing most of the coding region of *aepA* (Fig. 7). Following high-stringency washes, hybridization signals were detected in *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica* strains. Three different size fragments of *E. c.* subsp. *carotovora* strains hybridized with the *aepA* DNA. With EC153, a 3.8-kb fragment hybridized with *aepA*, whereas 6.2-kb fragments of Ecc71 and Ecc193 contained the *aepA* sequences. SCRI193 is unusual in that a much larger (i.e., an 18.0 kb) DNA segment hybridized with *aepA*. *E. c.* subsp. *atroseptica* strains, EC, Eca5, Eca12, and Eca31 contain *Pst*I fragments of about 19 kb that hybridized to the probe. The *Pst*I fragments of EC153 and SCRI193 hybridizing with *aepA* generated weaker signals compared to the signals produced by the corresponding fragments from the other bacterial strains (Fig. 7). This difference could be attributed to divergence in the *aepA* sequence of these *E. c.* subsp. *carotovora* strains.

DISCUSSION

Our discovery of the regulatory gene, *aepA*, that controls the production of Pel, Peh, Cel, and Prt in *E. c.* subsp. *carotovora* strain Ecc71 (Murata *et al.* 1991), had raised several significant issues, some of which have now been resolved. For example, we have provided evidence through the analysis of *pel-1* RNA and the expression of a *pel1-lacZ* operon fusion that AepA activates transcription. Although transcripts of the other target genes, i.e., *pel-2, pel-3, peh, cel,* or *prt* have not yet been investigated, extrapolating from the data for *pel-1* and taking into consideration the finding that an AepA function is required for the production of all of these enzymes, we suggest that AepA activates transcription of these genes as well. It should be noted that in the absence of a functional

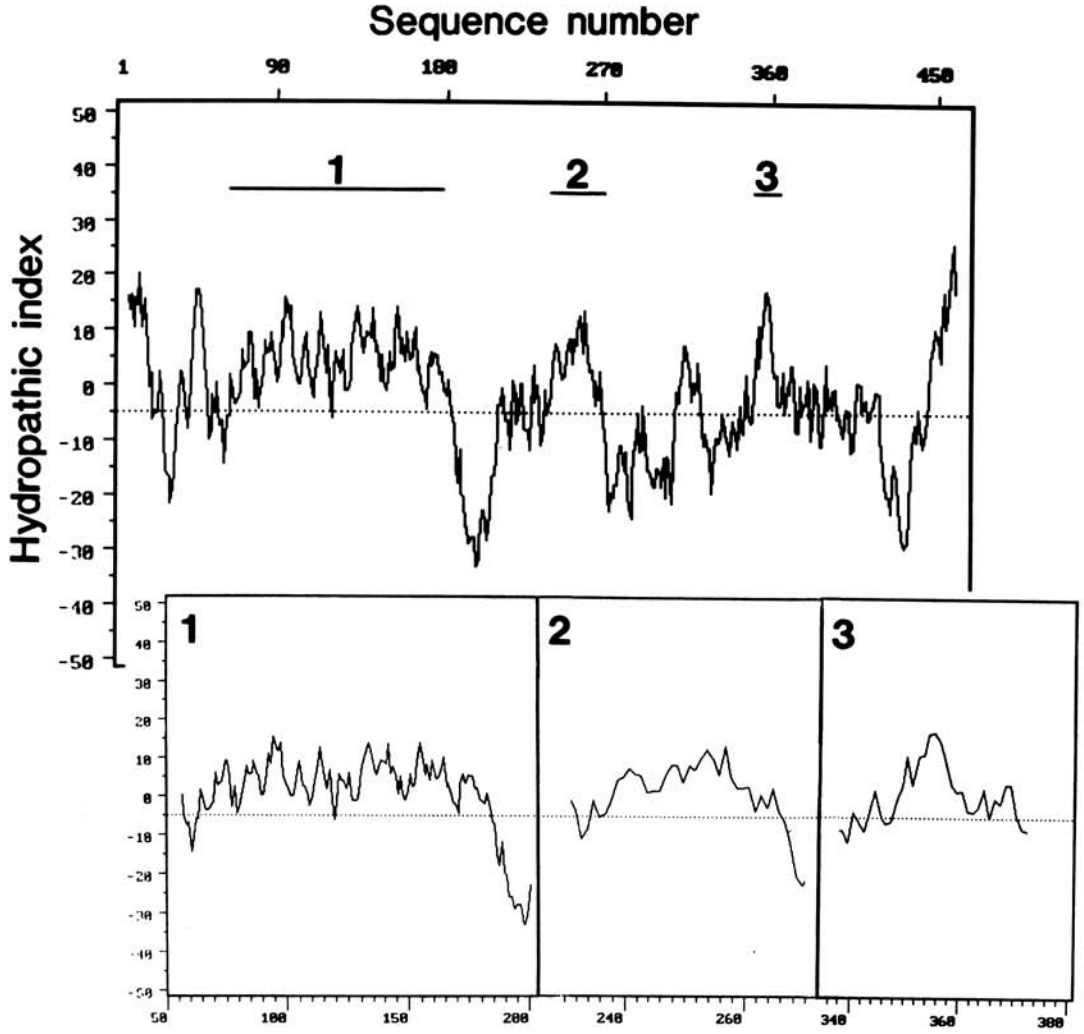

Fig. 5. Hydropathicity profile of *Erwinia carotovora* subsp. *carotovora* Ecc71 AepA determined according to the method of Kyte and Doolittle (1982) with a window of 15 amino acids. The portions of the protein sequence above the line indicate predicted hydrophobic regions and the portions below indicate predicted hydrophilic regions. The bars marked 1–3 are possible membrane-spanning domains shown in an expanded scale in the lower panel.

Table 1. Bacterial strains and plasmids used in this study

Bacteria and plasmids	Relevant characteristics	Reference or source
Strains		
Erwinia carotovora subsp. *carotovora*		
Ecc71	Wild type	Zink *et al.* 1984
EC153	Wild type	Chatterjee *et al.* 1979
Ecc193	Wild type	Zink *et al.* 1984
SCRI193	Wild type	Salmond *et al.* 1986
AC5006	Lac$^-$	Murata *et al.* 1990
AC5030	Lac$^-$, *pel1-lacZ*, Km^r	Derived from AC5006; this study
AC5031	*aepA-lacZ*, Km^r	Derived from AC5006; this study
E. carotovora subsp. *atroseptica*		
EC	Wild type	Lei *et al.* 1985
Eca5	Wild type	Zink *et al.* 1984
Eca12	Wild type	Zink *et al.* 1984
Eca31	Wild type	Zink et al. 1984
Escherichia coli		
DH5α	*ϕ80lacZ* Δ M15, Δ (*lacZYA-argF*), *U169, hsdR17, recA1, endA1, thi-1*	Bethesda Research Laboratories[a]
HB101	*proA2, lacY1, hsdS20* ($r^-_Bm^-_B$), *recA56, rpsL20*	Zink *et al.* 1984
M8820	Δ (*proAB-argF-lacPOZYA*), *recA*$^+$, Sm^r	Castilho *et al.* 1984
POI1734	Mu*dI*1734 *ara*::(Mu cts), Δ (*proAB-argF-lacIPOZYA*)	Castilho *et al.* 1984
Plasmids		
pBluescript SK$^+$	Ap^r	Stratagene[b]
pCL1920	Sp^r, Sm^r	Lerner and Inouye 1990
pRK2013	Mob$^+$, Tra$^+$, Km^r	Figurski and Helinski 1979
pSF6	Mob$^+$, Sp^r, Sm^r	Selvaraj *et al.* 1984
pRK404	Mob$^+$, Tc^r	Ditta *et al.* 1985
pAKC228	*pel-1*$^+$, Ap^r, Tc^r	The 2.2-kb *EcoRI* fragment of pAKC227 (Willis *et al.* 1987) cloned into pBR329; laboratory collection
pAKC264	*aepA*$^+$, Sp^r, Sm^r	Murata *et al.* 1991
pAKC602	*aepA*$^+$, Tc^r	The 7-kb *aepA*$^+$ fragment, obtained by *sau3A* partial digest of pAKC264, cloned into pRK404; Murata *et al.* 1991
pAKC612	*aepA-lacZ*, Km^r, Sp^r, Sm^r	pAKC264 containing an *aepA-lacZ* operon fusion; see Fig. 1C for the site of Mu*dI*1734
pAKC619	*pel1-lacZ*, Ap^r, Km^r, Tc^r	pAKC228 containing a *pel1-lacZ* operon fusion constructed by Mu*dI*1734 insertion; this study
pAKC647	Ap^r	The 231-base pair *Fsp*I-*Hpa*I fragment of pAKC264 cloned into pSK$^+$; Fig. 2
pAKC654	*aepA*$^+$, Sp^r, Sm^r	The 2.2-kb *Hin*dIII fragment of pAKC602 cloned into pCL1920; Fig. 1

[a]Gaithersburg, MD.
[b]La Jolla, CA.

aepA, the levels of enzymatic activities (Murata *et al.* 1991) and *pel-1* expression (Fig. 6) remained low under conditions that would normally induce the expression of the cognate genes. These data, therefore, indicate that while a low level of expression of *pel, peh, cel,* and *prt* may occur in the absence of AepA, the full expression of these genes in the presence of inducing signals requires AepA. How such signals affect AepA action is not yet known. However, the data presented here show that *aepA* is itself regulated. Those substances that stimulate extracellular enzyme production in *E. c.* subsp. *carotovora* also activate the expression of an *aepA-lacZ* operon fusion (see above). These observations taken along with the influence of the dosage of the *aepA* DNA in *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica* (Table 2) raise the possibility that the pool size of AepA is limiting in these bacterial cells. When bacteria are grown under inducing conditions, i.e., in the presence of celery extract, *aepA* expression is activated, thereby increasing the pool size of the regulatory

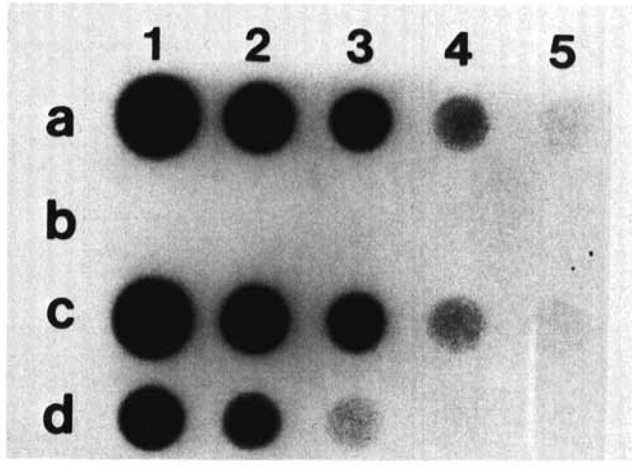

Fig. 6. Dot blot analysis showing the effect of *aepA* on the *pel-1* transcription in derivatives of *Erwinia carotovora* subsp. *carotovora* Ecc71. For the details of experimental conditions see Materials and Methods. The relevant genotypes of bacterial constructs are shown within the parentheses. Line a, AC5031(pAKC264) (*aepA*/*aepA*$^+$); line b, AC503l(pSF6) (*aepA*); line c, AC5006(pAKC264) (*aepA*$^+$/*aepA*$^+$); line d, AC5006(pSF6) (*aepA*$^+$). Lane 1, undiluted; lane 2, 1/3 dilution; lane 3, 1/9 dilution; lane 4, 1/27 dilution; lane 5, 1/81 dilution.

Table 2. The effect of *aepA*$^+$ plasmid on the levels of extracellular enzymatic activities in *E. carotovora* subsp. *carotovora* (strains Ecc193 and SCRI193) and *E. c.* subsp. *atroseptica* (strains EC and Eca12)[a]

Bacterial strain	Plasmid	Specific activity (units/mg protein) of Pel	Peh	Cel	Prt
Ecc193	pSF6	2	7	3	1
Ecc193	pAKC264	10	18	24	8
SCRI193	pSF6	27	164	20	19
SCRI193	pAKC264	183	413	110	56
EC	pSF6	45	135	130	11
EC	pAKC264	108	155	205	27
Eca12	pSF6	26	143	14	5
Eca12	pAKC264	55	202	16	9

[a]Cultures were grown in the SYG + CE medium to Klett value of about 350. Culture supernatants were assayed for the enzymatic activities.

protein, which then stimulates the expression of the target genes.

The following evidence implicates a negative regulation of *aepA*. The expression of an *aepA-lacZ* operon fusion is derepressed in *E. coli* but remains inducible in *E. c.* subsp. *carotovora* strain AC5006. Moreover, the basal level of expression in *E. coli* and the induced level of expression in an AC5006 derivative are comparable. This differential expression of *aepA* is presumed to be due to a repressor that is present in *E. c.* subsp. *carotovora* strain AC5006 but absent in *E. coli* DH5α. Whether the two putative KdgR-binding sites upstream of the translational start site of *aepA* (Fig. 2) are relevant in *aepA* expression remains to be determined.

The nucleotide sequence (Fig. 2) predicts that AepA possesses hydrophobic domains and a signal sequence, suggesting a membrane localization of the AepA polypeptide. The lack of significant homology of AepA with other regulatory proteins and the absence of a helix-turn-helix motif in AepA are apparently inconsistent with its purported role as a transcriptional activator. We should, however, note that the product of *hlyT*, which is the transcriptional activator of haemolysin (*hly*) genes, also has no similarity to established transcriptional modulators or DNA-binding proteins (Bailey *et al.* 1992). It is possible

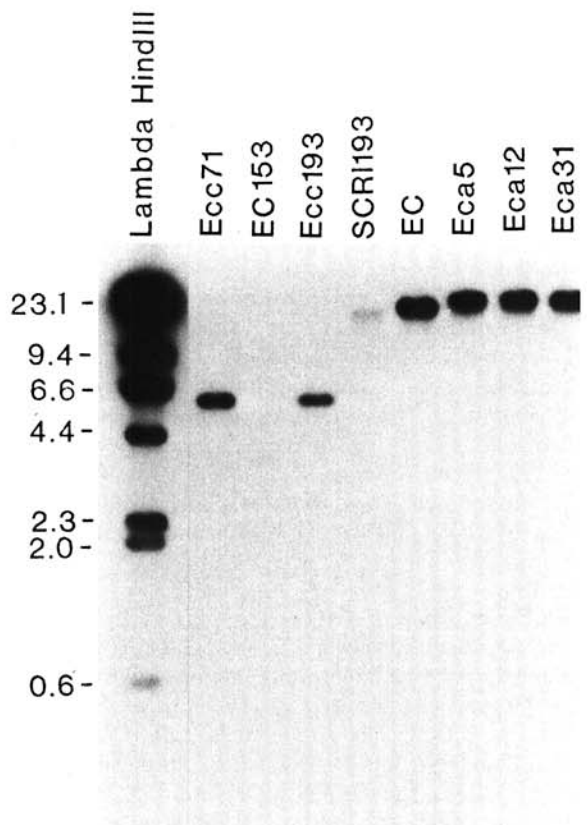

Fig. 7. Southern hybridization of *Pst*I-digested chromosomal DNAs of *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica* wild-type strains (Table 1). The 2.0-kb *Eco*RI-*Pst*I fragment of pAKC264 labeled with [^{32}P]dCTP was used as a probe. The lambda size markers are indicated at the left side of the figure.

that AepA interacts with another regulator protein and the resulting complex then binds to regulatory sequences activating transcription of the cognate genes. Alternatively, AepA may act as a sensor of environmental signals, transmitting them to other regulatory components. Noteworthy in these contexts is our finding of another gene, *aepB*, which in conjunction with *aepA* activates Pel, Peh, Cel, and Prt production. Molecular characterization of *aepB* in progress should provide useful insights into the mechanisms underlying activation of *pel, peh, cel,* and *prt* transcription by the *aep* product. It also would be instructive to ascertain structural and functional relationships between the *exp* gene, which controls exoenzyme production in *E. c.* subsp. *carotovora* strain SCC3193 (Pirhonen *et al.* 1991), the *pehR* gene, which specifically activates Peh production in SCC3193 (D. Flego, M. Pirhonen, and E. T. Palva; abstract presented at 6th International Symposium on Molecular Plant-Microbe Interactions, Seattle, WA; Saarilahti *et al.* 1992) and the *aep* genes of *E. c.* subsp. *carotovora* strain Ecc71.

It is likely that AepA and HlyT are components of "transcriptional" machineries that specifically control expression of the genes whose products are secreted to the milieu. The burgeoning issues are the mechanisms by which the Aep system and the HlyT system activate transcription of the exoprotein genes and how these processes are coupled with the protein export systems. In contrast to the Aep system of *E. carotovora* and the HlyT system of *E. coli*, the conventional two-component regulatory systems control the production of certain exoenzymes in other bacterial species such as *Bacillus subtilis* (Dahl *et al.* 1991; Kunst *et al.* 1988; Tanaka *et al.* 1991), *Staphylococcus aureus* (Janzon and Arvidson 1990; Peng *et al.* 1988), *Pseudomonas syringae* pv. *syringae* (Hrabak and Willis 1992), and *Xanthomonas campestris* pv. *campestris* (Tang *et al.* 1991). In *P. solanacearum*, Schell and associates (M. A. Schell, T. P. Denny, J. Huang, S. Clough, B. Carney, and S. Brumbley, abstract presented at 6th International Symposium on Molecular Plant-Microbe Interactions, Seattle, WA) have detected coregulation of extracellular proteins, virulence factors and EPS production by a LysR-like protein. Why these bacteria employ several different systems to regulate exoprotein production remains an open issue.

The uncertainty about the mechanism of AepA action notwithstanding, the data presented here suggest that as in *E. c.* subsp. *carotovora* strains, an AepA-like system may also occur in *E. c.* subsp. *atroseptica.* This is evident from the stimulatory effect of *aepA* on exoenzyme production (Table 2) as well as the presence of *aepA* homologs (Fig. 7). The stimulatory effect of *aepA* was generally greater in *E. c.* subsp. *carotovora* than in *E. c.* subsp. *atroseptica.* Also, in *E. c.* subsp. *atroseptica* a better stimulation was noted with Pel and Prt activities than with Peh and Cel. These differences could be due to a less efficient action of *aepA* of *E. c.* subsp. *carotovora* in heterologous systems. It would be of interest to ascertain if the *aepA*-like gene of *E. c.* subsp. *atroseptica* will also function in *E. c.* subsp. *carotovora*, as would be predicted from the data presented here.

In conclusion, taking into account all the available data,

we propose that *aepA* encodes a key regulatory protein which activates transcription of exoenzyme regulons in *E. c.* subsp. *carotovora*. The coregulated exoenzymes cause depolymerization of plant cell wall components; the degradation of one such component, i.e., pectin, is the primary cause of plant tissue maceration. The findings presented here and the observation that AepA$^-$ mutants of *E. c.* subsp. *carotovora* are severely attenuated in pathogenicity (Murata *et al.* 1991) establish a critical role of *aepA* in the adaptation of these bacteria as plant pathogens.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids are described in Table 1. The strains carrying drug markers were maintained on L-agar medium supplemented with the appropriate antibiotics. The wild-type strains were maintained on yeast extract-glucose-calcium carbonate (YGC) agar (Chatterjee 1980).

Media.

Cellulase detection agar, L medium, minimal medium, nutrient gelatin agar, polygalacturonate-yeast extract (PY) agar, salts-yeast extract-glycerol medium (SYG), and YGC agar were described elsewhere (Barras *et al.* 1986; Chatterjee 1980; Chatterjee *et al.* 1985; Murata *et al.* 1990; Murata *et al.* 1991). Preparation of celery extract (CE) and reconstitution of the SYG + CE medium were previously described (Murata *et al.* 1991). For isolation of RNA, 50 ml of celery extract was used to reconstitute 100 ml of the SYG + CE medium, instead of 95 ml of celery extract, which we routinely use (Murata *et al.* 1991). This modification was necessary since in preliminary trials we found that components of the SYG + 95% (v/w) CE precipitated upon storage and interfered with the recovery of RNA. Antibiotics were supplemented, if required, as follows (μg/ml): ampicillin (Ap), 50; kanamycin (Km), 50; spectinomycin (Sp), 50; streptomycin (Sm), 100; and tetracycline (Tc), 10. For the growth of *E. coli* DH5α, SYG medium was supplemented with 50 μg/ml of thiamine. Media were solidified by the addition of Difco agar (1.5%) (Difco, Detroit, MI).

Preparation of samples for enzyme assays and assay conditions.

Bacteria were grown at 28° C to a Klett value of 300 in SYG and to a Klett value of 350 in SYG + CE. The preparation of culture supernatants has been described (Murata *et al.* 1990; Murata *et al.* 1991). Cellulase, polygalacturonase, pectate lyase, and protease activities were quantitatively assayed and expressed as detailed in our previous publications (Chatterjee *et al.* 1985; Murata *et al.* 1990; Thurn and Chatterjee 1985). β-Galactosidase activity was assayed according to Miller (1972).

Construction of strains carrying a chromosomal copy of *aepA-lacZ* fusion.

aepA-lacZ transcriptional fusions were derived from an AepA$^+$ plasmid, pAKC264, with Mu*d*I1734 as reported in our previous publication (Table 1; Murata *et al.* 1991). An *aepA-lacZ* fusion plasmid, pAKC612 (Table 1; Fig. 1), was mated into AC5006, a Lac$^-$ derivative of *E. c.* subsp. *carotovora* strain Ecc71. The *aepA-lacZ* DNA on the plasmid was replaced for a chromosomal copy of *aepA* by marker exchange recombination according to Zink *et al.* (1985). The fidelity of marker exchange was confirmed by Southern hybridization. As expected, the AepA-LacZ strain, AC5031 was defective in the production of Pel, Peh, Cel, and Prt.

Construction of a strain carrying a chromosomal copy of *pel1-lacZ* fusion.

A *pel1-lacZ* operon fusion was constructed by mutagenizing a *pel-1* plasmid, pAKC228 (Table 1) with Mu*d*I1734 (Castilho *et al.* 1984). Following induction of Mu in *Escherichia coli* (P0I1734) carrying pAKC228, Mu lysates containing mini-Mu plasmid cointegrates were used to infect the *E. coli* strain, M8820. The resulting Ap^r, Km^r, and Tc^r colonies were scored on pectate-yeast extract agar for a deficiency in pectolytic activity resulting from insertions of mini-Mu-*lacZ* within the *pel-1* DNA. A plasmid, pAKC619 (Table 1), which carries *pel1-lacZ* was transformed into AC5006 and further characterized by testing for β-galactosidase production on agar media containing X-Gal. The location and orientation of the mini-Mu-*lacZ* insertion in pAKC619 were determined by restriction analysis.

The *pel1-lacZ* DNA on pAKC619 was replaced for the chromosomal copy of *pel-1* in AC5006 by maker exchange recombination as described above. The fidelity of marker exchange in the resulting strain, AC5030, was confirmed by Southern hybridization (data not shown) and by the absence of Pel-1 as determined by isoelectric focusing-activity overlay staining technique (Zink and Chatterjee 1985).

Recombinant DNA techniques.

Standard published procedures were used for the isolation of plasmid and chromosomal DNAs, transformation of *E. coli* and *E. c.* subsp. *carotovora*, restriction digest, gel electrophoresis, electroelution of DNA fragments, DNA ligation, and Southern hybridizations (Crouse *et al.* 1983; Maniatis *et al.* 1982; McEvoy *et al.* 1990; Murata *et al.* 1990; Whitford and DiCioccio 1988). Restriction and modifying enzymes were obtained from Promega Biotech (Madison, WI) and U.S. Biochemicals (Cleveland, OH).

Nucleotide sequence of *aepA*.

aepA was localized within a 2.5-kb *Eco*RI-*Hin*dIII DNA fragment by mutagenizing pAKC264 and pAKC602 with Mu*d*I1734 and constructing various subclones (Fig. 1A; Murata *et al.* 1991; our unpublished data). When *aepA* with flanking DNA was cloned into moderate to high copy number plasmids, such as pBR322, pUC18, pSPORT, and pBluescript, deletions and rearrangements occurred. Therefore, nucleotide sequence of *aepA* was determined by using a low copy number vector, pCL1920 (Lerner and Inouye 1990). The 2.2-kb *Hin*dIII fragment of pAKC602 (Table 1; Fig. 1B), which contains the entire *aepA*, was cloned into the *Hin*dIII site of pCL1920, creating a plasmid,

pAKC654. Starting with the known sequence of the multiple cloning site in pCL1920, successive oligonucleotide primers were synthesized to sequence both strands of *aepA* in pAKC654. The sequencing reactions were conducted by using the Sequenase System Version II of U.S. Biochemicals (Cleveland, OH). The data were analyzed by the PC/Gene program (Intelligenetics Inc., Mountain View, CA). The sequence of the 2.3-kb fragment containing *aepA* has been assigned the GenBank accession number L13457.

Determination of *aepA* transcript size.

RNA was isolated from *E. c.* subsp. *carotovora* strain Ecc71 grown in SYG + CE in a 28° C shaker. When cultures reached a Klett value of about 240, bacterial cells were collected and RNA was extracted according to the procedure of Aiba *et al.* (1981).

RNA samples (20 μg) and a size marker RNA ladder (GIBCO BRL, Gaithersburg, MD) were electrophoresed in a formaldehyde-agarose gel, transferred to a Biotrans (ICN, Irvine, CA) nylon membrane, and probed with the antisense *aepA* RNA labeled with [^{32}P]dUTP. The antisense RNA was obtained by *in vitro* transcription of the insert DNA of pAKC647 according to Sambrook *et al.* (1989). The blots were washed under stringent conditions as described for Southern blots (Maniatis *et al.* 1982; Murata *et al.* 1990).

Dot blot analysis of the *pel-1* mRNA in AepA$^-$ and AepA$^+$ strains.

Total RNA samples, obtained from AC5006(pSF6), AC5006(pAKC264), AC5031(pSF6), and AC5031 (pAKC264) grown in the SYG + CE, were treated according to Sambrook *et al.* (1989). Ten micrograms of RNA in 200 μl of treatment buffer represented the undiluted sample; this sample was then diluted to 1/3, 1/9, 1/27, and 1/81 of the original concentration and transferred to Biotrans (ICN, Irvin, CA) membrane by using a filtration manifold (Sambrook *et al.* 1989). The 2.2-kb internal fragment of pAKC228 was digested by restriction endonucleases, *Xho*II and *Kpn*I to obtain the 0.9-kb internal *pel-1* DNA (A. Chatterjee *et al.*, unpublished). The *Xho*II-*Kpn*I fragment was eluted, labeled with [^{32}P]dCTP, and used in hybridization.

Determination of the transcriptional start site of *aepA*.

The RNase protection assay was used to determine the transcriptional start site in *aepA* (Sambrook *et al.* 1989). By using T3 RNA polymerase, antisense RNA was produced from pAKC647 (Table 1) in the presence of [^{32}P]dUTP and other standard reaction constituents (Sambrook *et al.* 1989). The radio-labeled antisense RNA was hybridized to the RNA sample previously used for the determination of the *aepA* transcript size (see above). The reaction mixture was treated with RNase A and RNase T1 to remove single stranded RNAs and the sample of RNA:RNA hybrids was denatured and run in an 8% acrylamide-urea sequencing gel in parallel with a DNA sequencing ladder.

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