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 activator of extracellular protein production (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 pl 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-I*, which specifies a major secreted Pel species in strain Ecc71, and by following the expression of a *pell-lacZ* operon fusion we determined that AepA activates *pel-I* 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.

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*Erwinia carotovora* subsp. *carotovora* causes soft-rot disease in a wide variety of plants (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* (activator of extracellular protein production [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-I*, 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*.
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. The direction of transcription as determined by analyzing ModII1734 insertions (Murata et al. 1991; Fig. 1). The 1,395-bp ORF is localized within the FspI-HindIII 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 in frame 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 position 36 relative to the presumed translational start site at the ATG codon (Fig. 4). The sequences exhibiting homologies to σ70 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 (TCTGTA) 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 σ70-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.

The AepA protein is predicted to consist of 465 amino acid residues having a molecular mass of 51 kDa and an isolectric 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 peptate and about fivefold in the presence of celery extract (SYG medium, 267 Miller units; SYG + peptate, 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 pel-lacZ transcription by AepA.

To determine if AepA activates transcription, we used pel-l, 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, FspI, HpaI, and PstI, 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+, Pell-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 pell-lacZ expression was attributed to the dosage of the aepA+ DNA.

In the second set of experiments we determined if transcription of pel-l was affected in the absence of AepA. For this we assayed the levels of the pel-l RNA in AepA+ and AepA− bacterial constructs by conducting dot blot analysis (Fig. 6). pel-l RNA was barely detectable in a sample of the AepA− strain, AC5033(pSF6), whereas high levels of pel-l 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. atroseptica strain SCHR193 carrying pAKC264 were significantly higher compared to those in SCHR193 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 FspI-HpaI fragment containing the 5' portion of aepA (Fig. 2).](image)

![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.](image)
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 PstI, transferred to Biotrans nylon membrane and probed with the EcoRI-PstI 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 PstI fragments of about 19 kb that hybridized to the probe. The PstI 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-l RNA and the expression of a pel-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-l 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

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**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

<table>
<thead>
<tr>
<th>Bacteria and plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Strains</td>
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<tr>
<td><em>Erwinia carotovora</em></td>
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<tr>
<td>Ec71</td>
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<td>Zink et al. 1984</td>
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<td>EC153</td>
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<td>SCR193</td>
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<td>Murata et al. 1980</td>
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<td><em>aepA</em>-lacZ, Km’</td>
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<td>Wild type</td>
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</tr>
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<td>Eca5</td>
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<td>Zink et al. 1984</td>
</tr>
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<td>Zink et al. 1984</td>
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<td>Wild type</td>
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<td><em>Escherichia coli</em></td>
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<td>DH5α</td>
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<td>Bethesda Research Laboratories*</td>
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<tr>
<td>HB101</td>
<td>Δ (lacZYA-argF), U609, hsdR17, recA1, endA1, thi-1 proA2, lacY1, hsdS20 (r6* m*b), recA56, rpsL20</td>
<td>Zink et al. 1984</td>
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<td>M8820</td>
<td>Δ (proAB-argF-lacZPOZYA), recA’, Sm’</td>
<td>Castilho et al. 1984</td>
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<td>PO1734</td>
<td>MutI734 arcd: (Mu cts), Δ (proAB-argF-lacZPOZYA)</td>
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<td>Plasmids</td>
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<tr>
<td>pBluescript SK*</td>
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<td>Strageneb</td>
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<tr>
<td>pCL1920</td>
<td>Sp’, Sm’</td>
<td>Lerner and Inouye</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Mob’, Tra’, Km’</td>
<td>Figurski and Helsinski</td>
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<td>pRK404</td>
<td>Mob’, Te’</td>
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<td>Murata et al. 1991</td>
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<td>pAKC612</td>
<td>aepA-lacZ, Km’, Sp’, Sm’</td>
<td>pAKC264 containing an aepA-lacZ operon fusion; see Fig. IC for the site of MutI734</td>
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<td>pAKC619</td>
<td><em>pel</em>-lacZ, Ap’, Km’, Te’</td>
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<td>pAKC647</td>
<td>Ap’</td>
<td>The 231-base pair FspI-HpaI fragment of pAKC264 cloned into pSK'; Fig. 2</td>
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<tr>
<td>pAKC654</td>
<td>aepA’, Sp’, Sm’</td>
<td>The 2.2-kb HindIII fragment of pAKC602 cloned into pCL1920; Fig. 1</td>
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</tbody>
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*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. auroseptica* (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* Ec71. 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, AC5031(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 Ec93 and SCR193) and *E. c. subsp. auroseptica* (strains EC and Eca12)

<table>
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<th>Bacterial strain</th>
<th>Plasmid</th>
<th>Specific activity (units/mg protein) of</th>
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<td></td>
<td></td>
<td>Pel</td>
<td>Peh</td>
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<tr>
<td>Eca12</td>
<td>pAKC264</td>
<td>55</td>
<td>202</td>
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</table>

*Culture* 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 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; Saariluhtu 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 (Janzen 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 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 coleoptil 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 mutant strains 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 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 MudI734 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 Eec71. 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, Cfl, and Pfl.

Construction of a strain carrying a chromosomal copy of pell-lacZ fusion.

A pell-lacZ operon fusion was constructed by mutagenizing a pel-l plasmid, pAKC228 (Table 1) with MudI734 (Castillo et al. 1984). Following induction of Mu in Escherichia coli (P01734) carrying pAKC228, Mu lysates containing mini-Mu plasmid cointegrates were used to infect the E. coli strain, M8820. The resulting Ap', Km', and Tc' colonies were scored on pectate-yeast extract agar for a deficiency in pectolytic activity resulting from insertions of mini-Mu-lacZ within the pel-l DNA. A plasmid, pAKC619 (Table 1), which carries pell-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 pell-lacZ DNA on pAKC619 was replaced for the chromosomal copy of pel-l in AC5006 by marker 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-l 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, electroleution 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 EcoRI-HindIII DNA fragment by mutagenizing pAKC264 and pAKC602 with MudI734 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, pC1920 (Lerner and Inouye 1990). The 2.2-kb HindIII fragment of pAKC602 (Table 1; Fig. 1B), which contains the entire aepA, was cloned into the HindIII site of pC1920, 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. coli subsp. carotovora strain Eec71 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 [32P]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-I 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, XhoII and KpnI, to obtain the 0.9-kb internal pel-I DNA (A. Chatterjee et al., unpublished). The XhoIIKpnI fragment was eluted, labeled with [32P]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 [32P]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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**LITERATURE CITED**


