Mobilization, Cloning, and Sequence Determination of a Plasmid-Encoded Polygalacturonase from a Phytopathogenic *Burkholderia* (*Pseudomonas*) *cepacia*

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Phytopathogenic strains of *Burkholderia cepacia* (synonym *Pseudomonas cepacia*) produce endopolygalacturonase, whereas strains of clinical and soil origin do not. Growth of a phytopathogenic strain (ATCC25416) at elevated temperatures resulted in nonpectolytic derivatives that were either cured of a resident plasmid or contained a plasmid of reduced mass. The resident 200-kb plasmid (pPEC320) in strain ATCC25416 was tagged with Tn5-Mob. The pPEC320::Tn5-Mob (pPEC321) plasmid was mobilized in *B. cepacia* strains of soil and clinical origin. Transconjugants containing pPEC321 expressed the endopolygalacturonase and showed differential activity on plant tissue. No evidence for self-transfer of pPEC320 or the tagged derivative was observed. A 2.85-kb cloned fragment from pPEC320 containing the plasmid-borne *pehA* gene was sequenced and compared to the *pehA* gene from *Erwinia carotovora* subsp. *carotovora* and *Ralstonia solanacearum* and the polygalacturonase sequence from *Lycopersicon esculentum.*

Additional keywords: Pseudomonas solanacearum, virulence factor.

Burkholderia cepacia (synonym *Pseudomonas cepacia*; Yabauuchi et al. 1992) is a highly versatile bacterium that has adapted to a number of different environments. It is the causal agent of decay of onions (Burkholder 1950), is an opportunistic pathogen of man (Conly et al. 1986; McKevitt and Woods 1984; Tablan et al. 1987), and is commonly found in soil environments (Ballard et al. 1970; McArthur et al. 1988). In a previous study by Gonzalez and Vidaver (1979), strains of *B. cepacia* of plant (BCP) and clinical (BCC) origins were compared. In Gonzalez and Vidaver's (1979) study, conventional biochemical tests and antibiotic sensitivity patterns supported synonymy between strains of *B. cepacia*, *P. kingii*, and *P. multivorans*. However, bacteriocin production patterns, onion maceration tests, and hydrolysis of low-pH pectate agar clearly differentiated BCC and BCP strains into two distinct

groups. The same study (Gonzalez and Vidaver 1979) found plasmid DNA in 15 of 16 strains examined. However, the role of the resident plasmids in the ecological fitness of the diverse *B. cepacia* was not determined. Lennon and DeCicco (1991) conducted a plasmid screen of 37 *B. cepacia* isolates from clinical, pharmaceutical-industrial, and environmental origins and found that 84% of the strains examined contained plasmid DNA. However, no phenotypic attributes were assigned to the plasmids.

The unusual catabolic potential and wide distribution of *B. cepacia* in the environment suggest the possibility of novel mechanisms for the adaptability of this microorganism. Genetic studies have determined that one of the most important factors underlying the extraordinary adaptability and catabolic potential of *B. cepacia* may be the prevalence of insertion elements in its genome and associated plasmids (Lessie et al. 1990; Wood et al. 1990). A number of insertion sequences have been identified in *B. cepacia* based on their ability to promote genomic rearrangement (Beckman et al. 1982; Gaffney and Lessie 1987), to recruit foreign genes by replicon fusion (Barsomian and Lessie 1986), and to cause insertional activation of gene(s) (Scordilis et al. 1987). This genomic rearrangement presumably contributes to the adaptability of *B. cepacia*.

Phytopathogenic strains of *B. cepacia* have been implicated in both field and postharvest disease problems (Cother and Dowling 1985; Kawamoto and Lorbeer 1972, 1974). Plant-pathogenic *B. cepacia* strains characteristically produce a polygalacturonase (Peh) that is responsible for the maceration of both bulb scale and leaf tissue and is implicated in disease development (Ulrich 1975). In the current study, we demonstrate the conjugal mobilization of a Peh-encoding plasmid, and the cloning and sequence analysis of the *pehA* (pectic enzyme hydrolase) gene from *B. cepacia*.

RESULTS

Selection for Peh⁻ derivatives.

Phenotypically polygalacturonase negative (Peh⁻) derivatives of ATCC25416 (Table 1) were obtained by growing the strain at elevated temperatures. The Peh⁻ derivatives were detected on Hildebrand's medium A (HA) (Hildebrand 1971) and were readily distinguishable from parental Peh⁺ colonies that produced pitting. The frequency of Peh⁻ derivatives ranged

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from 1 to 2% of colonies tested (2,000) in four independent experiments. The plasmid content of the parental strain and the Peh⁻ derivatives was examined to determine whether the Peh⁺ phenotype was plasmid associated. The parental strain harbored a single plasmid of approximately 200 kb (Fig. 1, lane A). A plasmid survey of 20 Peh⁻ derivatives indicated that two classes of Peh⁻ derivatives were obtained from the heat treatment. Strain BCP251 (Fig. 1, lane B) is representative of derivatives that are Peh⁻ and contains a deletion of the resident plasmid, designated pPEC322. A second class of Peh⁻ derivatives that shows no evidence of the resident plasmid is represented by strain BCP252 (Fig. 1, lane C). Of the Peh⁻ derivatives examined, half harbored a plasmid of reduced mass, and half were cured of the resident plasmid. Restriction enzyme analysis of the parental plasmid (pPEC320) and the Peh⁻ derivative, pPEC322, confirmed a deletion of 35 kb (data not shown). Derivatives of ATCC25416 that harbored pPEC322 or no longer contained the Peh-encoding plasmid were unable to macerate plant tissue but were able to cause restricted necrosis of plant tissue at the site of inoculation.

Tagging and mobilization of the Peh-encoding plasmid.

Plasmid pPEC320 from strain ATCC25416 was tagged with the selective marker kanamycin (Km), using the suicide plasmid pSUP5011, which contains Tn5-Mob. Tagging experiments yielded Km-resistant (Km^r) transconjugants at a frequency of 2×10^{-6} per recipient. Conjugal mobilization of pPEC320::Tn5-Mob(pPEC321) was accomplished with plasmid pRK2013 in triparental matings. Strain BCP253 (Peh⁺, catabolizes myo-inositol [ino⁺], does not catabolize itaconic acid [ita⁻]), a representative transposon-tagged derivative containing pPEC321, was used as the donor strain in a series of triparental crosses with Peh⁻, ino⁻, ita⁺, and trimethoprim-resistant (Tp^r) recipient *B. cepacia* BCS or BCC strains (Table 1). Initial mobilization of pPEC321 into BCS or BCC strains of *B. cepacia* was detected at a frequency of 1×10^{-6} to 6.2×10^{-6} per recipient (Table 2). The BCS and BCC transconjugants expressed a Peh⁺, ino⁻, ita⁺, and Tp^r phenotype and harbored plasmid pPEC321. Physical evidence for the presence of pPEC321 in the transconjugants was confirmed by agarose gel electrophoresis of survey lysates (data not shown) and Southern blot

Table 1. Bacterial strains and plasmids used in this study[a]

Strain	Relevant characteristics	Source or Reference
Escherichia coli		
DH5αMCR	F⁻ *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1*rec*A1 *deo*R *thi*-1 *sup*E44λ⁻*gyr*A96 *rel*A1	Life Technologies, Gaithersburg, MD
C2110	Nal^r, *pol*A	Stachel et al. 1985
HB101	F⁻, *rec*A13	Boyer and Roulland-Dussoix 1969
Sm17-1	Mobilizing strain	Simon et al. 1983
Burkholderia cepacia		
ATCC25416	Plant pathogen, contains plasmid pPEC320, 200 kb, Peh⁺, ino⁺, ita⁻	American Type Culture Collection, Rockville, MD
BCP251	Harbors pPEC322, a 165-kb derivative of pPEC320, Peh⁻, ino⁺, ita⁻	This study
BCP2511	Nal^r, BCP251	This study
BCP252	Plasmid-free derivative of ATCC25416, Peh⁻, ino⁺, ita⁻	This study
BCP2521	Nal^r, BCP252	This study
BCP253	ATCC25416,contains pPEC320::Tn5-Mob, designated pPEC321	This study
TL249-2	Soil isolate, plasmid-free, *lys*-2, Peh⁻, ino⁻, ita⁺	Gaffney and Lessie 1987
BCS10	Tp^r, TL249-2	This study
DBO1	Soil isolate, Peh⁻, ino⁻, ita⁺	Zylstra et al. 1989
BSC20	Tp^r, DBO1	This study
B4648	Clinical isolate, Peh⁻, ino⁻, ita⁺	Gonzalez and Vidaver 1979
BCC10	Tp^r, B4648	This study
B5912	Clinical isolate, Peh⁻, ino⁻, ita⁺	Gonzalez and Vidaver 1979
BCC11	Tp^r, B5912	This study
Pseudomonas aeruginosa		
PAO25	*arg*F, *leu*-10, Peh⁻	R. H. Olsen, University of Michigan, Ann Arbor
Plasmids		
pSUP5011	Tn5-Mob vector, Km^r	Simon 1984
pSUP2017	Tn7 suicide vector, Tp^r	Simon et al. 1983
pRK2013	Km^r, Tra⁺, Mob⁺, ColE1 replicon	Figurski and Helinski 1979
pUFRO34	Inc W, Km^r, Mob⁺, *mob*(P) *lac*Zα⁺, Par⁺, cosmid	DeFeyter et al. 1990
pURFO47	Inc W, Amp^r, Gm^r, Mob⁺, *lac*Zα⁺, Par⁺, derivative of pURFO43	DeFeyter and Gabriel 1991
pPEC323	5.3-kb *Sst*I fragment from pPEC320 cloned in pUFRO34, Peh⁺	This study
pPEC324	5.3-kb *Sst*I fragment from pPEC320 cloned in pUFRO47, Peh⁺	This study
pPEC325	3.75-kb *Sst*I-*Bam*HI subclone of pPEC323 in pUFRO47, Peh⁺	This study
pPEC326	2.85-kb *Eco*RI-*Bam*HI subclone of pPEC323 in pUFRO47, Peh⁺	This study
pHoKmGus	Km^r, Ap^r, tnpA⁻; promoterless β-glucuronidase gene	Bonas et al. 1989
pSShe	Cm^r, *tnp*A	Stachel et al. 1985
pRZ102	ColE1::Tn5	Jorgensen et al. 1979

[a] Strains of plant, clinical, and soil origin have been designated with the prefix BCP, BCC, and BCS, respectively. Nal^r, Tp^r, Ap^r, Gm^r, and Km^r = resistance to nalidixic acid, trimethoprim, ampicillin, gentamicin, and kanamycin, respectively. Peh = polygalacturonase phenotype. Ino and ita = ability to catabolize myo-inositol and itaconic acid. + and – = positive and negative for phenotype, respectively.

analysis of the same gel with a radiolabeled Tn*5* probe (Fig. 2). Strain BCP253 (Fig. 2, lane B) and transconjugants containing plasmid pPEC321 showed hybridization to Tn*5* (Fig. 2, lanes H, J, and L), whereas ATCC25416 (Fig. 2, lane A) and the respective recipient strains showed no hybridization to the probe (Fig 2, lanes G, I, and K). Transconjugants BCS101 and BCS201 (Table 2) obtained in the initial mobilization experiments subsequently were used as donors in triparental matings. The frequency of mobilization observed in this series of matings ranged from 1×10^{-5} to 1×10^{-4} transconjugants per recipient (Table 2). The transconjugants expressed the Peh$^+$ phenotype and harbored pPEC321. The absence of pPEC321 in the recipients and its presence in the transconjugants was confirmed further by lack of homology to Tn*5* by the former (Fig. 2, lanes C and E) and homology by the respective transconjugants (Fig. 2, lanes D and F). No evidence for conjugal transfer of pPEC321 was observed in the absence of the mobilizing plasmid.

Supernatants from the parental recipient strains grown in M9GP medium (M9 medium supplemented with glycerol, polygalacturonic acid, and amino acids as required [Sambrook et al. 1989]) showed no Peh activity (Fig. 3, B1 to F1), whereas supernatants of their respective transconjugants harboring pPEC321, grown under the same conditions, showed Peh activity (Fig. 3, B2 to F2). Strain ATCC25416 produced a single polygalacturonase with a pI of 8.1 (Fig. 4, lane A). Isoelectric focusing (IEF) of culture supernatants from BCP253 and transconjugants harboring pPEC321 showed that they also produced a Peh with the same pI as that produced by ATCC25416 (Fig. 4, lanes B, C, and D). Supernatants from BCP251, BCP252, and the parental recipient strains, BCS10 and BCS20, grown
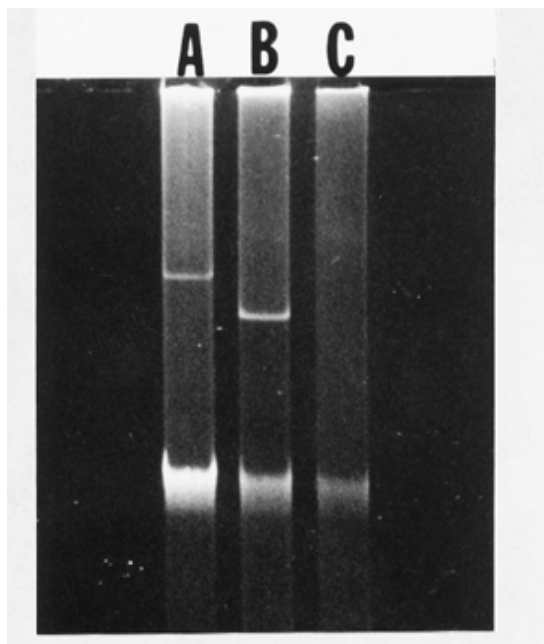
Fig. 1. Agarose gel electrophoresis of survey lysates from *Burkholderia cepacia* ATCC25416 and derivative strains. Lane A, ATCC25416; lane B, BCP251; and lane C, BCP252. Electrophoresis of DNA was in 0.7% agarose at 40 V for 20 h. The upper bands in lanes A and B represent plasmid DNA. The lower bands in lanes A and B represent chromosomal DNA and trapped linear plasmid DNA. The band in lane C represents chromosomal DNA.

under the same conditions, showed no Peh activity (data not shown).

Plant tissue inoculations.

None of the unwounded bulb scales inoculated with the strains tested (Table 3) showed any symptom development during a 72-h period. The inoculum droplet remained on the surface of the epidermal layer, and the pathogen was unable to penetrate the layer. Wounded bulb scales inoculated with ATCC25416 showed a large zone of macerated tissue (Table 3). The plasmid-cured derivative (BCP252) produced no maceration but did produce necrosis that expanded during the test period. The clinical strain (BCC10) produced necrosis of plant tissue only at the higher inoculum concentration (Table 3). However, BCC101 did cause maceration of plant tissue at both levels of inoculation (Table 3). The symptoms elaborated by BCC101 were shot-hole in appearance, whereas those produced by ATCC25416 were a general maceration of tissue. Plant tissue inoculated with the soil strain, BCS20 or the derivative harboring the Peh-encoding plasmid (BCS201), exhibited no necrosis or maceration symptoms (Table 3).

Enzyme activity.

Examination of hydrolytic products by thin-layer chromatography (TLC) demonstrated the ability of the Peh from *B. cepacia* to release dimers ($R_f = 0.67$), trimers ($R_f = 0.47$), and larger oligomers ($R_f = 0.30$) of galacturonic acid. The control exo-poly-α-D-galacturonidase from *Erwinia chrysanthemi* generated dimers ($R_f = 0.60$), whereas the endo-Peh from *Agrobacterium vitis* generated dimers ($R_f = 0.63$), trimers ($R_f = 0.43$), and larger oligomers of galacturonic acid ($R_f = 0.27$). Viscometry assays with equivalent units of each enzyme showed the ability of the Peh from *B. cepacia* and the endo-Peh from *A. vitis* to rapidly reduce viscosity compared to the slower acting exo-cleaving enzyme from *E. chrysanthemi*. Cell extracts of ATCC25416 grown in M9 medium containing polygalacturonate exhibited a 7.5-fold increase in Peh activity compared to those grown in M-9 glucose. Extracellular Peh activity was fivefold higher in cells grown in the presence of polygalacturonate compared to glucose-grown cells.

Cloning and expression of the *peh*A-encoding gene.

An *Sst*I library of plasmid pPEC320 was constructed in cosmid pUFRO34 and transfected into *Escherichia coli* DH5αMCR (Table 1). The cosmid clones were mobilized into *B. cepacia* strain DBO1, a Peh$^-$ BCS strain, and transcon-

Table 2. Conjugal mobilization of plasmid pPEC321 in *Burkholderia cepacia* strains

Donor strain	Recipient strain	Transfer frequency/ recipient[a]	Transconjugant designation[b]
BCP253	BCS10	2.0×10^{-6}	BCS101
BCP253	BCS20	1.0×10^{-6}	BCS201
BCP253	BCC10	6.2×10^{-6}	BCC101
BCP253	BCC11	1.5×10^{-6}	BCC111
BCS101	BCP2511	1.0×10^{-4}	BCP25111
BCS101	BCP2521	4.2×10^{-5}	BCP25211
BCS101	BCC10	7.5×10^{-5}	BCC102
BCS201	BCP2521	1.0×10^{-5}	BCP25212

[a] Frequency is the average of three independent triparental matings, using pRK2013 as the mobilizing plasmid.
[b] All transconjugants contained pPEC321 and expressed a Peh$^+$ phenotype.

jugants were assayed for Peh production on HA medium. Polygalacturonase activity was detected in an isolate harboring a cosmid (designated pPEC323) containing a 5.3-kb DNA fragment. The 5.3-kb fragment was subcloned as 3.75-kb *Sst*I-*Bam*HI and 2.85-kb *Eco*RI-*Bam*HI (Fig. 5) fragments into plasmid pUFR047, designated pPEC325 and pPEC326, respectively. Transconjugants of BCS20 and BCP252 that contained the subclones exhibited Peh activity on HA medium and produced Peh in M9GP medium. IEF of supernatant concentrates revealed a single activity band with a pI of 8.1 (data not shown). Cosmid pPEC324 (5.3-kb fragment in cosmid pUFRO47) also was mobilized in *P. aeruginosa* (Table 1) to determine expression of *pehA* in a heterologous background. Strain PAO25(pPEC324) grew on HA medium supplemented with leucine and arginine but did not pit the medium. Culture supernatants of PAO25(pPEC324) grown in supplemented M9GP medium exhibited no Peh activity, as determined by the pectate gel assay method. However, the osmotic shock fluid of PAO25(pPEC324) cells grown in M9GP medium exhibited Peh activity when assayed by the same method. Supernatants of PAO25, BCP251, and BCP252 grown and analyzed under the same conditions showed no Peh activity.

Sequence of the *pehA* gene from *B. cepacia*.

The nucleotide sequence of the 2.85-kb *Eco*RI-*Bam*HI fragment contained in pPEC325 that expressed Peh activity was determined by sequencing both strands. The portion of the sequence shown (Fig. 6) includes restriction sites that correspond to those designated in Figure 5. A consensus sequence (ATAGGTTA) for a *Pseudomonas* promoter (Mermod et al. 1984) was observed 63 bases upstream of the open reading frame (ORF) of the *pehA* sequence, suggesting a monocistronic operon. A possible ribosome-binding site was identified 4 bases upstream of the ATG translation initiation codon. The 459-amino acid ORF coded for a protein with a molecular mass of 46,260 Daltons. The translation stop codon TGA at position 1468 was followed by a putative transcription termination loop centered at position 1593. The loop was not followed by a polyT chain, suggesting the termination of transcription of *pehA* may be *rho* dependent. A putative signal sequence of 26 amino acids also was observed, beginning with the Met (position 1) and extending to Ala. The sequence contained a positively charged N-terminal region, a central hydrophobic region (12 hydrophobic residues), and a polar carboxyl-terminal region that may define a cleavage site. An individual comparison of the amino acid sequences of the predicted *pehA* product from *B. cepacia* to that of the *pehA* gene products from *E. carotovora* subsp. *carotovora* and *Ralstonia solanacearum* (previously *P. solanacearum*, reclassified as *B. solanacearum*, and recently reclassified as *R. solanacearum*; Yabauuchi et al. 1992, 1995) and the polygalacturonase amino acid sequence from *Lycopersicon esculentum* (PG-2a) was done by the BESTFIT program (University of Wisconsin Genetics Computer Group; Devereux et al. 1984). The amino acid sequence from *B. cepacia* showed 30.3, 25.4, and 27.6% identity to the sequences from *E. carotovora* subsp. *carotovora*, *R. solanacearum*, and *L. esculentum*, respectively. Figure 7 shows an alignment of the four amino acid sequences by the PILEUP program (University of Wisconsin Genetics Computer Group). Several regions of high similarity or identity were observed when sequences were aligned. A 9-residue sequence, GXG(T/A)I(D/N)GXG, at positions 178 to 185 and a 10-
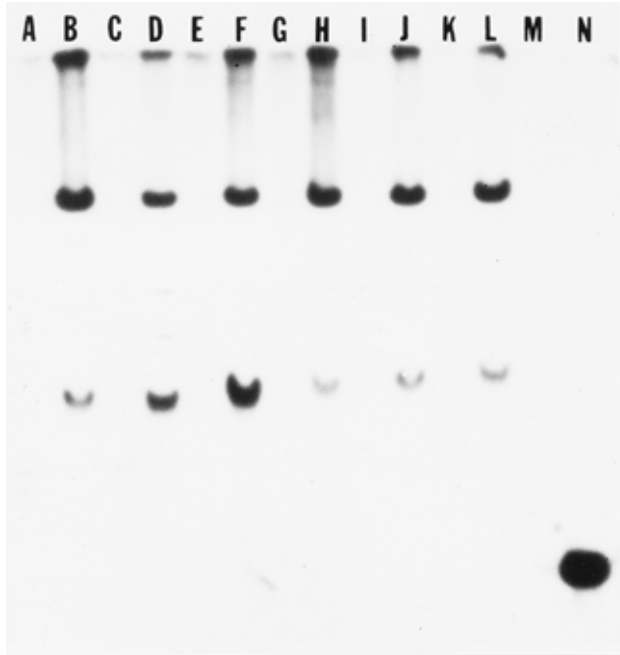
Fig. 2. Southern hybridization analysis of DNA from survey lysates of *Burkholderia cepacia*, donor, recipients, and transconjugant strains probed with Tn*5*. Lane A, ATCC25416; lane B, BCP253; lane C, BCP2511; lane D, BCP25111; lane E, BCP2521; lane F, BCP25212; lane G, BCS20; lane H, BCS201; lane I, BCS10; lane J, BCS101; lane K, BCC10; lane L, BCC101; lane M, blank; and lane N, probe plasmid pRZ102(Tn*5*). The upper bands represent plasmid DNA. The lower bands represent linear plasmid DNA trapped in chromosomal DNA of survey lysates.
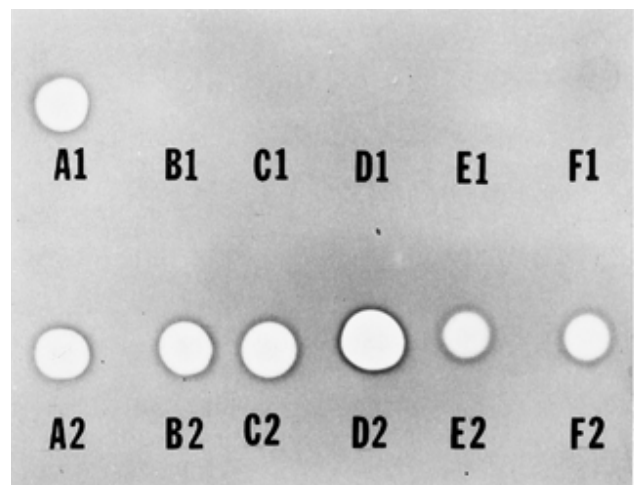
Fig. 3. Detection of polygalacturonase (Peh) activity in culture filtrates of *Burkholderia cepacia* ATCC25416 and donor, recipient, and transconjugant strains by the pectate-agar gel method: A1, ATCC25416; B1, BCP2511; C1, BCP2521; D1, BCS10; E1, BCS20; and F1, BCC10. B1 to F1 represent culture filtrates of Peh⁻ recipient strains. A2 represents activity from BCP253 filtrates. B2 to F2 represent cultural filtrate activities of transconjugants of strains B1 to F1, which harbor plasmid pPEC321, respectively. Gel was stained with ruthenium red to detect activity. A clear zone indicates activity.

residue sequence, (I/L)XTGDDX(V/I)(A/S)(I/V), at positions 322 to 331 appeared to be highly conserved. The sequences GHGXSIGS, GXRIK(S/T), and NTD located at positions 353 to 360, 393 to 398, and 301 to 303, respectively, also showed a high degree of identical or conserved residues.

Site-directed mutagenesis of the Peh-encoding plasmid.

Mutagenesis of the 3.75-kb *Sst*I-*Bam*HI fragment contained in pPEC325, which included the sequenced 2.85-kb *Eco*RI-*Bam*HI, was performed with the pHoKmGus transposon-reporter gene construct. Analysis of the mutants showed the same direction of transcription of *pehA* as that established by sequencing. The BCS20 transconjugants harboring the interrupted *pehA* gene between the *Dra*I and the *Bgl*I sites (Fig. 5) did not produce Peh in HA or M9GP medium (data not shown). Insertions 160, 155, and 82 exhibited normal pitting of HA medium (Fig. 5), whereas insert 50 showed reduced activity (Fig. 5).

It was of further interest to determine whether the Peh-encoding plasmid was transfer proficient. To examine this, three pHoKmGus (Fig. 5, number 23, 28, and 150) insertions of pPEC325 that exhibited a Peh⁻ phenotype were marker-exchanged into plasmid pPEC320 harbored by strain ATCC25416 and confirmed by survey lysates and Southern blot analysis of plasmid DNA (data not shown). The derivative plasmids were designated pPEC320, -23, -28, and -150, respectively. The Km^r, gentamicin-sensitive (Gm^s) mutants showed no expression of Peh activity on HA medium and did not produce Peh

in liquid M9GP medium or macerate plant tissue. However, plant tissue inoculated with the mutants did exhibit the same symptoms of limited necrosis observed with BCP251 and BCP252. Because plasmid pPEC321 had shown no evidence of self-transfer, the possibility existed that the Tn5-Mob had inserted in a region of the plasmid that altered transfer functions. Strains containing the site-directed mutation of pPEC320 (pPEC320, 23, -28, or -150) in the *pehA* gene allowed testing of self-transfer without concern for the interruption of transfer functions, if present. No evidence for self-transfer was detected for plasmids pPEC320, -23, -28, or -150 in conjugal matings with both isogenic and nonisogenic strains of *B. cepacia*.

DISCUSSION

The role of pectic enzymes in the pathogenesis of plant pathogens is well established (Collmer and Keen 1986; Collmer et al. 1990). Some *Erwinia* and *Pseudomonas* species are able to incite soft rot or tissue maceration in a variety of plants (Collmer et al. 1990). In bacterial systems, the pectic enzymes of the soft-rotting *Erwinia* spp. are the most well characterized (Collmer and Keen 1986; Collmer et al. 1990). *Erwinia* spp. produce several classes of pectate enzymes, including pectate lyase, exo-poly-galacturonate lyase, polygalacturonase, and exo-poly-α-D-galacturonosidase (Collmer and Keen 1986; Collmer et al. 1990). The nonfluorescent phytopathogenic pseudomonads *B. cepacia*, *B. caryophylli*, *B. gladioli*, and *R. solanacearum* all produce polygalacturonase (Gross and Cody 1985). Ulrich (1975) purified and characterized the pectolytic enzymes from *B. cepacia* and found that polygalacturonase was the principle enzyme produced during infection and that it had a pH optimum between 4.4 and 4.6. Infection of onion tissue resulted in tissue degradation and a lowering of the pH from 5.5 (healthy tissue) to near 4.0, which is conducive to polygalacturonase activity.

Evidence presented in this study demonstrates that the structural gene encoding for the production of polygalacturonase by ATCC25416, a phytopathogenic strain of *B. cepacia,* is plasmid determined. Growth of ATCC25416 under inducing and noninducing conditions indicates that production of Peh is regulated. Our current studies have not elucidated whether a hydrolytic product, presumably made by the basal level of Peh, is responsible for induction of Peh, as has been observed in other systems (Barras et al. 1994). Tagging of the Peh-encoding plasmid with Tn5-Mob allowed mobilization of plasmid pPEC321 into non–Peh-producing strains of *B. cepacia* of
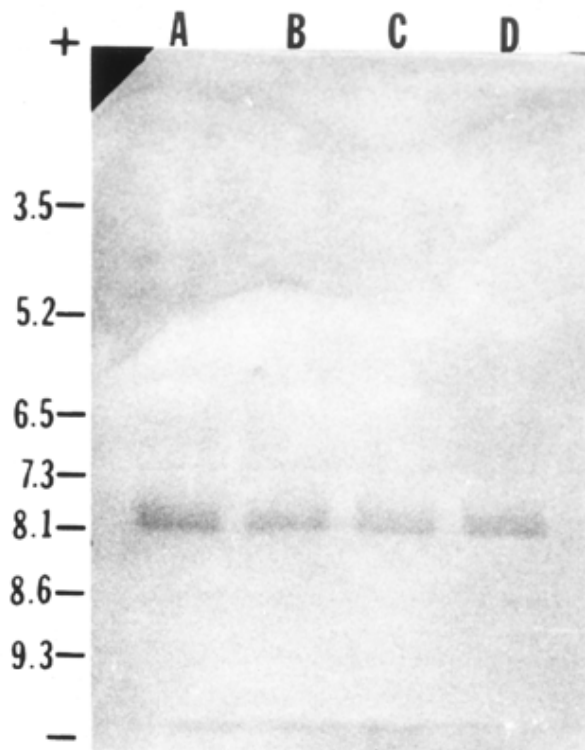
Fig. 4. Isoelectric focusing gel (pH 3 to 10) of extracellular proteins from *Burkholderia cepacia* ATCC25416 and transconjugant strains containing the polygalacturonase-encoding plasmid pPEC321. Lane A, ATCC25416; lane B, BCP253; lane C, BCS101; and lane D, BCS201. pI markers and anode and cathode are indicated to the left of the gel. The pectate-agarose overlay was stained with hexadecyltrimethylammonium to detect activity.

Table 3. Onion tissue maceration and/or necrosis by *Burkholderia cepacia* strains

Strain	Inoculum (CFU/bulb scale)	
	10^3	10^5
ATCC25416	26 ± 3.2[a,b]	29 ± 4[a,b]
BCP252	8.3 ± 1[c]	10.3 ± 2[c]
BCC10	0[a,c]	7.7 ± 1.1[c]
BCC101	9.5 ± 3[a,b]	16 ± 5[a,b]
BCS20	0[a,c]	0[a,c]
BCS201	0[a,c]	0[a,c]

[a] Number indicates the average macerating diameter (in centimeters) plus/minus the standard deviation at 48 h for four replicates.

[b] No necrosis was measurable due to masking by tissue maceration.

[c] Number indicates the average necrosis diameter (in centimeters) plus/minus the standard deviation at 48 h for four replicates.

plant, soil, and clinical origin. The transconjugants containing plasmid pPEC321 expressed a Peh⁺ phenotype (Fig. 3), produced a single polygalacturonase with a pI of 8.1 (Fig. 4), and acted as donors in subsequent mobilization experiments (Table 2). The gene encoding the enzyme (*pehA*) was isolated from a cosmid library of plasmid pPEC320 and expressed and secreted in both isogenic and nonisogenic strains of *B. cepacia*. Derivatives of ATCC25416 that were cured of the Peh-encoding plasmid (pPEC320), harbored the deleted plasmid pPEC322, or had an inactive *pehA* gene obtained by transposon mutagenesis were unable to macerate plant tissue but caused limited necrosis of plant tissue surrounding the point of inoculation. The plasmid-borne *pehA* gene, therefore, encoded for an extracellular enzyme that enhanced the aggressiveness of the pathogen in wounded tissue, because in the absence of Peh production the pathogen appeared to be restricted in its ability to cause extensive tissue damage (Table 3). We observed no disease development or necrosis of bulb scales in the absence of tissue wounding with any of the strains tested, confirming that *B. cepacia* is a wound pathogen, as had been observed by Kawamoto and Lorbeer (1974). The presence of the Peh-encoding plasmid enhanced the aggressiveness of ATCC25416 and clinical strain BCC101, which showed limited tissue necrosis without the plasmid. Introduction of the Peh-encoding plasmid into the BCS strain resulted in in vitro production of the enzyme but did not impart the ability to cause symptoms (Table 3), suggesting that the Peh enhanced virulence and the BCS strain lacked the primary determinant(s) required for pathogenesis. The role of the necrosis factor in pathogenicity awaits construction of mutants lacking this function.

Studies to examine expression of the *B. cepacia pehA* gene in heterologous backgrounds, such as *P. aeruginosa*, showed that the *B. cepacia pehA* gene was expressed. Osmotic shock experiments showed that the shock fluid had Peh activity, indicating a periplasmic location of the enzyme. Huang and Schell (1990) suggested that export of the polygalacturonase from *R. solanacearum* may be a two-step process, in which the Peh polypeptide first enters the periplasm before it crosses the outer membrane. The ability of the *P. aeruginosa* transconjugants to secrete the polygalacturonase into the periplasmic space suggests that the proposed 26-amino acid signal pep-

tide of the *B. cepacia* polygalacturonase is recognized by *P. aeruginosa*. The positively charged N-terminal, central hydrophobic, and C-terminal regions are indicative of a signal sequence (von Heijne 1985). In addition, the predicted cleavage site for the signal sequence adheres to the "(–3, –1)-rule" as defined by von Heijne (1986). However, because the enzyme is not exported through the outer membrane, it is possible that *P. aeruginosa* lacks the enzyme export trait found in many soft-rotting *Erwinia* and *Pseudomonas* spp. (Murata et al. 1990; Salmond 1994). Enzyme export is tightly linked to the ability of various plant pathogens to cause disease, and in *Erwinia* spp., the *Out* gene cluster is essential for secretion of the major pectate lyases, polygalacturonases, and cellulases (Barras et al. 1994).

The *pehA* gene product expressed by *B. cepacia* appeared to have different biophysical properties from that reported for *E. carotovora* subsp. *carotovora* and *R. solanacearum*. The former has a pI of 10.19 and a pH optimum of 5.5, whereas the latter may produce at least two extracellular Peh enzymes, with the major one having a pI of 9.0 and a pH optimum of 5.7 and the minor one a pI of 8.0 (Allen et al. 1991; Schell et al. 1988). The *pehA* gene is located chromosomally in both *E. carotovora* subsp. *carotovora* (Willis et al. 1987) and *R. solanacearum* (Allen et al. 1991; Huang and Schell 1990). In *Erwinia* spp., the endo- and exo-PG are ancillary to the multiple pectate lyases produced (Collmer and Keen 1986; Collmer et al. 1990). In the case of *R. solanacearum*, the *pehA* gene product is important but not required for pathogenesis (Allen et al. 1991; Schell et al. 1988). The Peh produced by *B. cepacia* has a pI of 8.1, has a pH optimum of 4.5, is an endo-cleaving polygalacturonase (based on examination of hydrolytic products by TLC and viscometry assays), and appears to be a virulence factor. Although there are some biophysical differences that are apparent among the *pehA* products of *E. carotovora* subsp. *carotovora*, *R. solanacearum*, and *B. cepacia*, an amino acid sequence alignment (Fig. 7) showed several highly conserved regions in all three bacterial Peh's and PG-2a from tomato. Analysis of known polygalacturonases from several sources suggested that conserved sequences in the enzyme are required for activity. The histidine residue in the GHGXSIGS sequence (residues 353 to 360), the three aspartic acid resi-
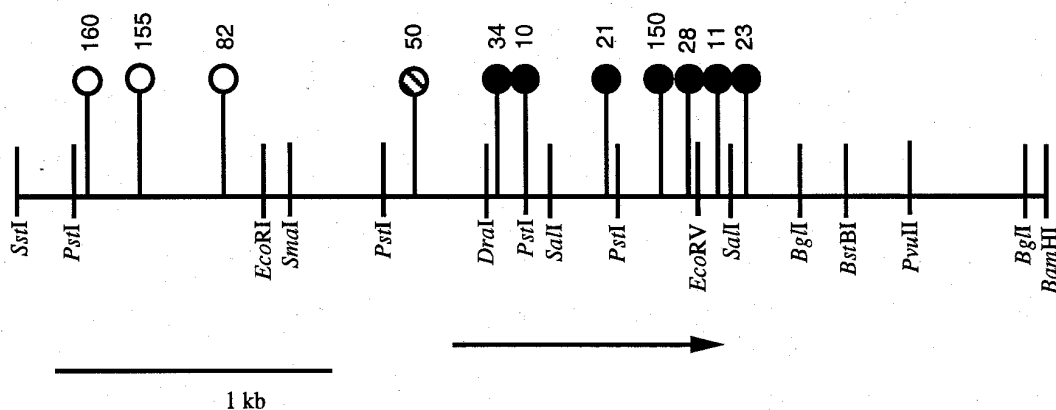
Fig. 5. Physical map of the 3.75-kb *Sst*I-*Bam*HI fragment containing the *pehA* gene cloned from plasmid pPEC320. Solid and cross-hatched circles represent pHoKmGus insertions of plasmid pPEC325 that resulted in polygalacturonase negative (Peh⁻) mutants or mutants with reduced activity, respectively, as determined in *Burkholderia cepacia* strain BCS20. The open circles represent insertions that are Peh⁺. The arrow indicates the transcriptional direction of the *pehA* gene. Restriction sites are indicated.

dues in NTD (residues 303 to 305) and TGDD (residues 324 to 327), the RIK sequence (residues 395 to 397), and the tyrosine residue at position 431 all have been implicated in the activity of Peh enzyme from different sources (Bussink et al. 1991; Cooke et al. 1976; Stratilova et al. 1996). The recently published amino acid sequence of the *pehA* gene from *Agrobacterium vitis* (Herlache et al. 1997) also shows these same conserved sequences, which have been implicated in the activity of the Peh enzyme from diverse backgrounds.

Transposon mutagenesis of cosmid pPEC325 aided in the

```
GTCCGGCGGCGAATTGCGACTCATAGGTTACCCCCCCCACTGCTTGCCGGCTGCACCTAT 60
                     DraI
TTCGCTCTCTTATTTTAAATACAAGGAAAACCAATGAAAGGCAAAAGCAGCACGCGCCTC 120
                                  M  K  G  K  S  S  T  R  L    9
GTCCTAAGGCTCTCCACCTTGGCCGCGCTCGCCGTGCAAGCGTCCGCGCAAGCGGCCACC 180
V  L  R  L  S  T  L  A  A  L  A  V  Q  A  S  A  Q  A  A  T    29
                                                    PstI
TGCACGCCGCAGTGGAGCAGTTCGGCCAGCACCAACACCACGAATCTGCAGAACGCCATC 240
C  T  P  Q  W  S  S  S  A  S  T  N  T  T  N  L  Q  N  A  I    49
                                        SalI
CAACAGTGCGCTGCCAGCGGCACCAGCAGCAGTCCGGGCCTGGTCGACCTGGCCAGCAAC 300
Q  Q  C  A  A  S  G  T  S  S  S  P  G  L  V  D  L  A  S  N    69
AACGGCATCTCGACGGCGGTCATCACCAGCGTGAATCTCGCCAACAATATCGTGCTGAAG 360
N  G  I  S  T  A  V  I  T  S  V  N  L  A  N  N  I  V  L  K    89
TTGGAGAAGGGCTTCACGCTGAAGGGCTCGCCCGCGCAGCCCTCGAGCGGCGCGATGCTG 420
L  E  K  G  F  T  L  K  G  S  P  A  Q  P  S  S  G  A  M  L    109
ACCGGCAGCAATCTGAGCAACCTGACCATTACCGGCACCGGCGCGATCGACGGCGACGGA 480
T  G  S  N  L  S  N  L  T  I  T  G  T  G  A  I  D  G  D  G    129
CAGGACTACTGGCCTGCCGCCAGTCGGGCAGAACAATACGGCCCGGCCCAAACTGATCGCC 540
Q  D  Y  W  P  A  A  V  G  Q  N  N  T  A  R  P  K  L  I  A    149
               PstI
ATCACCGGCTCAAACCTGCAGATTGGCTCGAACTTCACCGATGCCGGCCAAATCGCAGTCG 600
I  T  G  S  N  L  Q  I  G  S  N  F  T  D  A  G  K  S  Q  S    169
ATCGTGGCCTTCCCGAGTTCGTCCAATGCTACCGGCAGCGCACTGATCATCCGCAACTCG 660
I  V  A  F  P  S  S  S  N  A  T  G  S  A  L  I  I  R  N  S    189
CCGAAGGAGCAACTGGTGATTGAATCGGGCTCGAAGAACGTCACGATCGACGGCGTGTGG 720
P  K  E  Q  L  V  I  E  S  G  S  K  N  V  T  I  D  G  V  W    209
ATCTACGCGAATCCGAACCGCAATGCCAGCGGTGACGATCTCGCGCCGAACACCGACGCG 780
I  Y  A  N  P  N  R  N  A  S  G  D  D  L  A  P  N  T  D  A    229
ATCGACATCATCGGCACCCAGACGGCGACCATCAAGAACTGTCTGCTCGACACCGGCGAC 840
I  D  I  I  G  T  Q  T  A  T  I  K  N  C  L  L  D  T  G  D    249
        EcoRV
GACGATATCGCCATCAAGTCAAACGCGGGCGGGGCGGCCACCTCGAGCGTCAACGTCAGC 900
D  D  I  A  I  K  S  N  A  G  G  A  A  T  S  S  V  N  V  S    269
CATTGCGTGGTGGGCGGTGGTCATGGCATCTCGATTGGCGGCCAGGAGGCGGCCGGCACC 960
H  C  V  V  G  G  H  G  I  S  I  G  G  Q  E  A  A  G  T    289
                SalI
ACGCTCGCCAAGCCCGGCGTCTCGCAGGTGACGGTCGACACGATGCAGTTCAGCGGCACC 1020
T  L  A  K  P  G  V  S  Q  V  T  V  D  T  M  Q  F  S  G  T    309
GACTACGGCTACCGGATCAAGACCGACCAGACCGCCAAGGACAGCGGTGCCACCACCGGC 1080
D  Y  G  Y  R  I  K  T  D  Q  T  A  K  D  S  G  A  T  T  G    329
GTGACGTACCGGAACACCTGCATGCGCAACGTCCAGCAGCCCTTCCTGTTCACCTACGCC 1140
V  T  Y  R  N  T  C  M  R  N  V  Q  Q  P  F  L  F  T  Y  A    349
TACGCCTCGGGCACCGGCGGCGCCCTGCCGATCATCGCCAACGTCACCATCGACAATGTG 1200
Y  A  S  G  T  G  G  A  L  P  I  I  A  N  V  T  I  D  N  V    369
                                            BglII
ATCGCCACCGCCACCAAGCAGCAAGGCGCCATAATCGGGCTCTCGAACAGCCTGATGGGC 1260
I  A  T  A  T  K  Q  Q  G  A  I  I  G  L  S  N  S  L  M  G    389

GTGCCAAAATCGGGCGACACGGGAATCAGCATCACCAATAGCCAGATCAGTGGCGGTAAG 1320
V  P  K  S  G  D  T  G  I  S  I  T  N  S  Q  I  S  G  G  K    409
GCCTTCTCCGTGACCGACGGCGAATTGCAACTGGGCAGCCACAGCAGCGCGACGACCTCG 1380
A  F  S  V  T  D  G  E  L  Q  L  G  S  H  S  S  A  T  T  S    429
     BstBI
ACCGGTTCGAACGGACAAGTAGTGGGGATTCCGGATACCGGCGCGACGCTGTCCTGCCCG 1440
T  G  S  N  G  Q  V  V  G  I  P  D  T  G  A  T  L  S  C  P    449
AGCAGCATCACGATCCCTGCGCAGATTTGAGCGACATGGGGACACGTCTTCGGTAGGGGC 1500
S  S  I  T  I  P  A  Q  I  *    458
CAAATTAGGATCAAAACGCCTGATGCGTAAGCGCGAAATCCTCCGCACCTAGCCGGCACT 1560
GCTGAATCGATCCAGGATTGCGTCCGCCACTCACACTTGCGGACGCAACCACGATGACAG 1620
     ->-->-->-->-->        <--<--<--<--<--
                              PvuII
CTGACACGATTGACCTCGTTTACCTGCCCCTGAAGGTGA                      1659
```

Fig. 6. DNA sequence of the *pehA* gene from *Burkholderia cepacia* and the predicted amino acid sequence. Underlined are the putative promoter sequence, the Shine-Delgarno sequence, and the translation start codon. The translation termination codon is indicated by an asterisk. A potential transcription termination loop is underlined and marked with arrows. The restriction sites are indicated.

further characterization of the *pehA* gene and resulted in insertions useful for site-directed mutagenesis of plasmid pPEC320. Interruptions within the established *pehA* sequence resulted in loss of activity (Fig. 5). Insertion 50 (Fig. 5) in the pPEC325 clone was 210 base pairs upstream of the putative promoter for *pehA* and resulted in reduction in pitting activity. Because there is no significant ORF in the region, the observed reduction in activity is difficult to explain. Cosmid pPEC326 contains the 2.85-kb fragment from plasmid pPEC320 that encodes for the production of Peh. Homology to the 2.85-kb Peh-encoding fragment was observed in plasmids from eight wild-type *B. cepacia* strains expressing Peh activity by Southern blot analysis (C. F. Gonzalez, *unpublished data*). This homology indicates that the plasmid-borne *pehA* gene may be widely distributed in phytopathogenic *B. cepacia*. Our study, using Tn5-Mob–tagged pPEC320 plasmid (pPEC321) and the transposon-tagged Peh− mutants (pPEC320, -23, -28, and -150), suggests that pPEC320 is not transfer proficient. However, it is possible that other plasmids containing a homologous *pehA* sequence may be transfer proficient.

In summary, we have identified a 200-kb plasmid that encodes for Peh production in *B. cepacia*. Mobilization of the tagged plasmid or the cloned 2.85-kb fragment containing the *pehA* gene from pPEC320 imparts the ability to produce an endo-Peh to naturally occurring Peh− *B. cepacia* of clinical or soil origin and restores the phenotype to Peh− derivatives of a phytopathogenic strain. Peh activity, a virulence factor, is the only phenotypic characteristic that currently has been associated with the plasmid. To our knowledge, this is the first report of a plasmid-encoded polygalacturonase.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1. *B. cepacia* strains of plant, clinical, and soil origin are designated with the prefix BCP, BCC, and BCS, respectively.

Media and growth conditions.

Complex TN medium (Hansen and Olsen 1978) was used for routine maintenance of cultures. Minimal Vogel-Bonner glucose (VBG) medium (Vogel and Bonner 1965) was used in mating experiments. All media were supplemented with amino acids (0.50 mM) as required. Antibiotics were added to media at the following concentrations: Tp at 50 μg/ml, Km at 600 μg/ml, Gm at 100 μg/ml, and nalidixic acid (Nal) at 500 μg/ml for *B. cepacia*; Gm at 100 μg/ml for *P. aeruginosa*; and ampicillin at 40 μg/ml, Gm at 5 μg/ml, and Km 25 at μg/ml for *E. coli*. All antibiotics were purchased from Sigma Chemical Co., St. Louis. M9 medium (Sambrook et al. 1989), adjusted to pH 5.0 and supplemented with 0.05% glycerol, 0.20% polygalacturonic acid, and amino acids as required (medium M9GP) was used to test for production of Peh in liquid medium. To determine carbon source utilization, the medium of Bochner and Savageau (1977) was used with either itaconic (BSI medium) or myo-inositol (BSM medium) added as the carbon source at a final concentration of 0.01%. Phytopathogenic *B. cepacia* were routinely incubated at 28°C unless otherwise stated. Soil and clinical strains of *B. cepacia*, *P. aeruginosa*, and *E. coli* were incubated at 37°C.

```
         1                                                    50
Ecc    ...........  ......MEYQ  SGKRVLSLSL  GLIGLFSASA  WASDSRTVSE
Rs     MNHRYTLLAL  AAAALSAGAH  ATGTSVTAPW  GEVAEPSLPA  DSAVCKTLSA
Bc     ...........  ...........  ..MKGKSSTR  LVLRLSTLAA  LAVQASAQAA
PG-2a  .......MVI  QRNSILLLII  IFASSISTCR  SNVIDDNLFK  QVYD.NILEQ

         51                                                   100
Ecc    PKTP.....S  SCTTLKADSS  TATSTIQKAL  NNCDQ.....  GKAVRLSAGS
Rs     SITPIKGSVD  SVDGNPANSQ  PDASRIQSAI  DNCPA.....  GQAVKLVKGS
Bc     TCTPQWSSSA  S.........  TNTTNLQNAI  QQCAASGTSS  SPGLVDLASN
PG-2a  EFAHDFQAYL  SYLSKNIESN  NNIDKVDKNG  IKVINVLSFG  AKGDGKTYDN

         101                                                  150
Ecc    T..SVFLSGP  LSLPSGVSLL  IDKG......  .VTLRAVNNA  KSFENAPSSC
Rs     AGESGFLSGS  LKLKSGVTLW  IDTG......  .VTLFASRNP  ADYDNGLGTC
Bc     NGISTAVITS  VNLANNIVLK  LEKG......  .FTLKGS...  ..........
PG-2a  IAFEQAWNEA  CSSRTPVQFV  VPKNKNYLLK  QITFSGPCRS  SISVKIFGSL

         151                                                  200
Ecc    G.VVDKNG.K  GCDAFITAVS  TTNSGIYGPG  TIDGQGGVKL  ....QDKKVS
Rs     GTATTSND.K  SCNALIVARD  TAGSGIVGAG  AIDGRGGSLV  TSGPNANRLT
Bc     .....PAQ.P  SSGAMLTGSN  LSNLTITGTG  AIDGDGQDYW  PAAVGQNNTA
PG-2a  EASSKISDYK  DRRLWIAFDS  VQNLVVGGGG  TINGNGQVWW  PSSCKINKSL

         201                                                  250
Ecc    WWELAADAKV  K.......KL  KQNTPRLIQI  NKSKNFTLYN  VSLINSPNFH
Rs     WWDIAYLNKT  K.......GL  NQQNPRLIQT  YNGSAFTLYG  VTVQNSPNFH
Bc     RPKLIAITGS  NLQIGSNFTD  AGKSQSIVAF  PSSSNATGSA  LIIRNSPKEQ
PG-2a  ..........  ........P  CRDAPTALTF  WNCKNLKVNN  LKSKNAQQIH

         251                                                  300
Ecc    VVF.SDGDGF  TAWKTTIKTP  S.........  ..........  .........T
Rs     IVT.TGTSGV  TAWGIKIVTP  SLAYAVAGYK  CPSGSTPDKV  TPATCFTPET
Bc     LVIESGSKNV  TIDGVWIYAN  PNRNASGD..  ..........  ........DL
PG-2a  IKFESCT.NV  VASNLMINAS  A.........  ..........  .........K

         301                                                  350
Ecc    ARNTDGIDPM  SSKNITIAYS  NIATGDDNVA  IKAYKGRAET  RNISILHNDF
Rs     VKNTDGFDPG  QSTNVVLAYS  YINTGDDHVA  VKASSGP..T  RNLLFAHNHF
Bc     APNTDAIDII  GTQTATIKNC  LLDTGDDDIA  IKSNAGGAAT  SSVNVSHCVV
PG-2a  SPNTDGVHVS  NTQYIQISDT  IIGTGDDCIS  IVSGSQNVQA  TNITC.....

         351                                                  400
Ecc    GTGHGMSIGS  E.......T.  MGVYNVTVDD  LKMNG....T  TNGLRIKSDK
Rs     YYGHGLSIGS  E.......TN  TGVSNMLVTD  LTMDGNDSSA  GNGLRIKSDA
Bc     GGGHGISIGG  QEAAGTTLAK  PGVSQVTVDT  MQFSG....T  DYGYRIKTDQ
PG-2a  GPGHGISIGS  ...LGSGNSE  AYVSNVTVNE  AKIIG....A  ENGVRIKTWQ

         401                                                  450
Ecc    SA..AGVVNG  VRYSNVVMKN  VAKPIVIDTV  YEKKEGSNVP  DWSDITFK..
Rs     SR..GGKVTN  IVYDGICMRN  VKEPLVFDPF  YSSVKGSLYP  NFTNIVVKNF
Bc     TAKDSGATTG  VTYRNTCMRN  VQQPFLFTYA  YASGTGGALP  IIANVTIDN.
PG-2a  GG..SGQASN  IKFLNVEMQD  VKYPIIIDQN  YCDRVEPCIQ  QFSAVQVKNV

         451                                                  500
Ecc    .DVTSE....  .TKGVVVLNG  ENAKKPIEVT  MKNVKLTSD.  STWQIKNVNV
Rs     HDLGSAKSIK  RTMTFLGYKA  NKQKNPLTIT  LDNVVFDGTL  PAFEGSHYGG
Bc     ..........  .....VIATA  TKQQGAIIGL  SNSLMGVPKS  GDTGISITNS
PG-2a  V.YENIKGTS  ATKVAIKFDC  STNFPCEGII  MENINLVGES  GKPSEATCKN

         501                                                  550
Ecc    KK........  ..........  ..........  ..........  ..........
Rs     PASPNGVHFT  FGGTGPVSFA  DAIVTSSTTD  VTVTGTPGTA  AAVDCSKAFV
Bc     QISGGKAFSV  TDGELQLGSH  SSATTSTGSN  GQVVGIPDTG  ATLSCPSSIT
PG-2a  VHFNNAEHVT  PHCTSLEISE  DEALLYNY..  ..........  ..........

         551      561
Ecc    ..........  .
Rs     PLKSVAPTSP  I
Bc     IPAQI.....  .
PG-2a  ..........  .
```

Fig. 7. Alignment of the predicted amino acid sequence of the *pehA* gene from *Erwinia carotovora* subsp. *carotovora* (Ecc) (Saarilahti et al. 1990), *Ralstonia solanacearum* (Rs) (Huang and Schell 1990), *Burkholderia cepacia* (Bc), and polygalacturonase from *Lycopersicon esculentum* (PG-2a) (Grierson et al. 1986; Bird et al. 1988), using the algorithm PILEUP program (University of Wisconsin Genetics Computer Group; Devereux et al. 1984). Identical amino acids are indicated in bold.

Plasmid elimination.

The culture was grown for 18 h at 28°C in TN broth with shaking (200 rpm; model 4710, Queue Systems, Parkersburg, WV). The cells were subcultured into prewarmed TN broth (42°C) to a final concentration of 10^4 CFU/ml and grown with shaking (200 rpm; model G76, New Brunswick Scientific Co., Inc., Edison, NJ) in a water bath at 42°C for 18 h. Temperature-treated cultures were diluted and plated to TN agar. Individual colonies from the treatment were transferred to TN agar (stock culture) and stab-inoculated on HA medium (Hildebrand 1971) to test for pectolytic activity (pitting). Isolated colonies expressing a polygalacturonase negative (Peh⁻) phenotype were purified by streaking on TN agar, and the individual colonies were retested on HA medium. Individual colonies reconfirmed as Peh⁻ were subjected to further analysis.

Transposon labeling and matings.

Donor strain S17-1(pSUP5011) was used for tagging plasmid pPEC320, as described by Simon (1984). The filters were incubated at 37°C for 18 h, washed in phosphate buffer (0.01 M, pH 7.1), and plated to VBG medium containing Km (600 μg/ml). Km^r transconjugants were used as donors in triparental matings to determine mobilization of pPEC320::Tn5-Mob with *E. coli* HB101 containing helper plasmid pRK2013. Transconjugants were selected on VBG medium amended with Tp (50 μg/ml) and Km (600 μg/ml). Tp^r recipient strains were constructed by mobilization of plasmid pSUP2017 into the strain and selection on VBG medium amended with Tp (50 μg/ml). In experiments that involved the mobilization of plasmid pPEC321 into derivatives of ATCC25416 as recipients, a Nal^r recipient was used.

Characterization of transconjugants.

Parental strains and transconjugants were evaluated for Peh production on HA medium and M9GP broth and examined for plasmid content. Transconjugants were purified by serial colony isolation on medium containing selective antibiotic(s). Additionally, in matings that involved the transfer of pPEC321 from BCP strains to BCS or BCC recipients, the transconjugants were transferred to BSI and BSM media. Phytopathogenic *B. cepacia* strains used in this study were able to catabolize myo-inositol (ino⁺) and not itaconic acid (ita⁻), whereas the opposite was true for BCS and BCC strains. Colonies capable of catabolizing the substrate reduced the 2,3,5-triphenyltetrazolium chloride and produced a deep red formazan, whereas negative colonies remained colorless. The catabolic differences between the BCP and the BCS and BCC strains allowed confirmation of transconjugants that were of BCS or BCC recipient origin. HA medium plates incubated at 28 and 37°C were evaluated for pitting. Supernatants of 18-h cultures were assayed as described below. To confirm the physical presence of the mobilized plasmid, agarose gel electrophoresis of the lysates of parental and recipient strains and transconjugants were conducted as previously described (Gonzalez and Vidaver 1979). Southern blots of the same gels were probed individually with a Tn5 probe (Jorgensen et al. 1979).

Plant tissue maceration assay.

Yellow onion cv. Texas 1015Y was used to determine the potential of strains to cause tissue maceration or necrosis. Onions were rinsed, and the outer bulb scales were removed in a manner that would not disturb the integrity of the bulb scale. Using a flame-sterilized knife, a transverse cut was made across the top and bottom of the bulb. The bulb was sectioned into four parts, and the outer bulb scale was discarded. The average bulb scale portion used was 3 cm wide and 7.5 cm long. The bulb scale portions were placed with the abaxial (inner) part of the scale up in a sterile glass petri dish lined with a Whatman (Maidstone, England) No. 1 filter paper that was kept moist with sterile deionized water. The plates were placed in a sealed plastic container to maintain moisture. The bulb scales were pierced at the center to just break the abaxial epidermal tissue with a blunted 18-gauge sterile needle. The bulb scales, replicates of four, were inoculated with 25 μl of aqueous solution at a final concentration of 10^3 or 10^5 CFU per bulb scale portion. Sterile deionized water was used as a negative control. Additionally, unwounded bulb scales treated with the inoculum were included as controls. The bulb scales were incubated at 37°C and evaluated for necrosis and maceration at 24 and 48 h after inoculation. Cultures to be assayed were grown at 37°C on TN agar containing the appropriate antibiotic as required.

IEF and enzyme detection.

Samples of supernatant concentrated 15- to 20-fold from cultures grown in M9GP medium were dialyzed against 1% glycine. The supernatant samples (5 μl) and pI markers (pH 3 to 10, Pharmacia LKB Biotechnologies, Piscataway, NJ) were applied to IsoGel agarose IEF plates (pH 3 to 10; FMC Corp., Rockland, MD) and electrofocused at 25 W, with a 1,500-V maximum, for 45 to 60 min. Focusing was obtained when the visible pI markers were sharply resolved and the current stopped decreasing significantly (less than 1 mA in 10 min). IEF was performed at 5°C, using an Isobox model HE 950 (Hoefer Scientific Instruments, San Francisco). Pectolytic activity in IEF gels was determined by the method of Reid and Collmer (1985), using an ultrathin pectate-agarose overlay. The pI markers were stained with Coomassie, and agarose-pectate gels were stained with a 1% (wt/vol) solution of hexadecyltrimethylammonium bromide (Sigma).

Enzyme activity.

The endo or exo activity of the Peh from strain ATCC25416 on polygalacturonic acid was determined by analysis of the reaction products and examination of the rate of viscosity reduction. As controls, exo-poly-α-D-galacturonidase and endo-polygalacturonase were obtained by osmotic shock of strains DH5α(pPEH10) (He and Collmer 1990) and DH10B(pCPP2068) (Herlache et al. 1997), respectively, grown for 18 h in Luria-Bertani (LB) broth at 37°C. The osmotic shock fluids were obtained as described below. Supernatant concentrates of ATCC25416 were obtained as for IEF experiments, except they were dialyzed against 50 mM sodium acetate (pH 5) and 10 mM EDTA. Conditions for the production of reducing groups incorporating the enzyme preparations were as described by Collmer et al. (1988). Negative controls consisted of enzyme preparations boiled for 10 min. Samples (200 μl) were removed from reactions (4 ml) at 0 and 30 min and 1, 2, 4, 8, and 16 h and analyzed for the presence of reducing groups and hydrolytic products. Reducing groups were determined by the method of McFeeters (1980) and normalized to galacturonic acid equivalents. One unit of Peh activity was defined as

the amount of enzyme that produces one mole of galacturonic acid equivalent per minute at 30°C. Analysis of degradation products was determined by TLC, using thin-layer cellulose sheets (Baker-Flex, J. T. Baker Inc., Phillipsburg, NJ) that were developed for 7 h and allowed to air-dry and developed for an additional 7 h to obtain maximal separation in the solvent system described by Willis et al. (1987). Chromatography controls included D-galacturonic, digalacturonic, trigalacturonic, and polygalacturonic acids (Sigma). The products were visualized by the silver nitrate staining method as described by Hough and Jones (1962). R_f values were expressed as the ratio of cleavage product migration to the distance migrated by the D-galacturonic acid standard on each of the TLC plates. Changes in viscosity were determined at 30°C, as described by Willis et al. (1987).

To determine whether the production of Peh was regulated in ATCC25416, cells were grown under noninducing conditions (M9 medium supplemented with 0.5% glucose), washed, and transferred to M9 medium supplemented with glucose or 0.5% polygalacturonic acid. Cell cultures (5 ml) were harvested ($7,000 \times g$ at 5°C for 10 min) and resuspended in 1 ml of assay buffer. Cell extracts were prepared by sonication with a Heat Systems (Farmington, NY) sonicator (model XL2005) at 50% full power, using three 30-s pulses. The sonicate was centrifuged ($7,000 \times g$ at 5°C for 10 min) to remove cell debris. Supernatants and cell sonicates were assayed for activity by the method of Collmer et al. (1988). Reducing groups were determined by the method of McFeeters (1980) and normalized to galacturonic acid equivalents. Specific activity was expressed as micromoles of galacturonic acid equivalent per minute at 30°C per milligram of protein. Protein concentration was determined by the method of Koch and Putnam (1971).

DNA analysis.

Survey lysis of cells and agarose gel electrophoresis (Gonzalez and Vidaver 1979) were performed to determine plasmid content of temperature-induced Peh⁻ derivatives and the parental strain. Alkaline Southern transfer of plasmid DNA from gels was performed as described by Reed and Munn (1985). Purified plasmid pRZ102 (ColE1 replicon containing Tn5) was labeled with [^{32}P] d-ATP by the random primer method (Sambrook et al. 1989). The 2.8-kb *Eco*RI-*Bam*HI insert from pPEC325 that contains *pehA* was isolated from low melt agarose, using Bio 101 (LaJolla, CA) Geneclean II kit according to the manufacturer protocol, and labeled in a similar manner.

Cloning, subcloning, and restriction mapping.

CsCl-ethidium bromide-purified plasmid pPEC320 DNA was digested with restriction enzyme *Sst*I (Life Technologies, Gaithersburg, MD), and the resulting fragments were ligated into cosmid pUFRO34 (DeFeyter et al. 1990). The cosmid library was packaged in vitro into lambda-phage particles, using the Packagene system (Promega Corp., Madison, WI) according to the manufacturers instructions. A fraction of the phage suspension was used to infect *E. coli* DH5αMCR cells and Km^r transductants selected on LB agar supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma), and Km. Transductants containing recombinant cosmids were used as donors in triparental matings with pRK2013 and strain BCS20 as the recipient. Transconjugants were selected on VBG me-

dium amended with Km. The transconjugants were assayed for Peh activity on HA and M9GP media and for maceration of plant tissue as described above. Restriction mapping of the cosmids and plasmid was accomplished by standard methods (Sambrook et al. 1989).

Transposon and marker-exchange mutagenesis.

Transposon mutagenesis was accomplished with pHoKmGus by the procedure of Bonas et al. (1989). The mutagenized cosmids were mobilized into strain BCS20, using pRK2013, and tested for Peh activity. Production of β-glucuronidase activity by pHoKmGus was measured by the method of Jefferson (1987). Restriction mapping of the pHoKmGus inserts was accomplished by standard methods (Sambrook et al. 1989). For marker exchange, derivatives of pPEC325 containing the pHoKmGus insertions in the structural gene were mobilized into strain ATCC25416. To facilitate marker exchange, transconjugants were transferred for several cycles on TN agar without antibiotic selection. Marker-exchanged isolates were Km^r and Gm^s. The mutants were assayed for Peh activity with HA medium, tested for activity on plant material, and used as donors in self-transfer experiments. The location of the insertion was confirmed by Southern blot analysis of plasmid DNA prepared from the mutants with a Tn*5* probe.

Secretion analysis.

Supernatants of 18-h cultures of *B. cepacia* strains grown in M9GP medium at 28°C and shaken at 250 rpm (model 4710, Queue Systems) were filtered (0.22 μm), and a 10-μl sample was spotted on a pectate-agarose gel (50 mM potassium acetate, pH 4.5) (Reid and Collmer 1985). After 1 h at 28°C, the gel was stained with ruthenium red. A clear zone was indicative of Peh activity. For analysis of excretion by *P. aeruginosa*, PAO25 and PAO25(pPEC324) cells were grown at 37°C (200 rpm; model G76, New Brunswick Scientific) in M9GP medium supplemented with leucine and arginine (0.50 mM). After 18 h, the cultures were concentrated by centrifugation ($7,000 \times g$ at 5°C for 10 min), and the supernatant was saved. The cells were washed twice in 10 mM Tris-HCl (pH 7.0) containing 20% sucrose. The washed cells were resuspended in one-half volume of the same buffer containing 5 mM EDTA and held on ice for 10 min. The cells were centrifuged as described previously and resuspended in $^1/_{20}$ volume of 0.5 mM MgCl$_2$. The suspension was held on ice for 10 min, centrifuged, and supernatant (osmotic shock fluid) collected. Extracellular and periplasmic enzyme activities were determined from the culture supernatant and osmotic shock fluid, respectively, using the pectate gel assay. Strains PAO25, BCP251, and BCP252 (negative controls) were analyzed under the same conditions.

Nucleotide sequence.

The 2.85-kb *Eco*RI-*Bam*HI fragment from pPEC324 was cycle sequenced with SequiTherm polymerase (Epicentre Technologies, Madison, WI) as described by the manufacturer. Both strands were sequenced. The sequence was determined with oligonucleotide primers overlapping previously sequenced portions of the fragment. The primers used were constructed by the Gene Technology Laboratory, Texas A&M University, College Station. Similar sequences were identified by the BLAST network service (Atschul et al. 1990) at the National Center

for Biotechnology Information, Bethesda, MD. Sequence comparisons were done with the BESTFIT program and the multiple sequence alignment with the PILEUP program from the University of Wisconsin Genetics Computer Group (Devereux et al. 1984).

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Erratum, Vol. 10, no. 7, 1997

An article by C. F. Gonzalez et al. (10:840–851) entitled "Mobilization, Cloning, and Sequence Determination of a Plasmid-Encoded Polygalacturonase from a Phytopathogenic *Burkholderia* (*Pseudomonas*) *cepacia*" contained errors in the footnotes of Table 3. The corrected table appears below.

Table 3. Onion tissue maceration and/or necrosis by *Burkholderia cepacia* strains

Strain	Inoculum (CFU/bulb scale)	
	10^3	10^5
ATCC25416	$26 \pm 3.2^{a,b}$	$29 \pm 4^{a,b}$
BCP252	8.3 ± 1^c	10.3 ± 2^c
BCC10	$0^{a,c}$	7.7 ± 1.1^c
BCC101	$9.5 \pm 3^{a,b}$	$16 \pm 5^{a,b}$
BCS20	$0^{a,c}$	$0^{a,c}$
BCS201	$0^{a,c}$	$0^{a,c}$

[a] Number indicates the average macerating diameter (in millimeters) plus/minus the standard deviation at 48 h for four replicates.

[b] No necrosis was measurable due to masking by tissue maceration.

[c] Number indicates the average necrosis diameter (in millimeters) plus/minus the standard deviation at 48 h for four replicates.