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Cloning of a Bacteriophage Polysaccharide Depolymerase Gene and Its Expression in *Erwinia amylovora*

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A bacteriophage gene encoding a depolymerase specific for the surface polysaccharides of *Erwinia amylovora* was cloned and expressed in *Escherichia coli*. When overlaid with a lawn of *E. amylovora*, an *E. coli* colony harboring this plasmid was surrounded by a large halo as well as a shallow depression, characteristics of the margins of plaques produced by infection of *E. amylovora* with this bacteriophage. Hybridization experiments confirmed the phage origin of the cloned DNA. Introduction of the cloned DNA into *E. amylovora* by transformation altered colony morphology. Chemical analysis showed that the cells transformed with the plasmid encoding the depolymerase produced less ethanol precipitable slime and capsular polysaccharides and that the polysaccharides were of lower molecular weight than the polysaccharides from untransformed cells or cells transformed with the vector plasmid. *E. amylovora* cells carrying the plasmid produced necrotic lesions in pear fruit during pathogenicity studies. However, ooze production, a classic symptom of the disease, was eliminated. This indicated that tissue death, but not the characteristic symptom of ooze production, occurred in the presence of polysaccharide depolymerase.

Additional key words: extracellular polysaccharide, fire blight, virulence

Erwinia amylovora (Burrill) Winslow *et al.*, the fireblight pathogen of rosaceous plants, produces copious extracellular polysaccharides in culture. Mutant strains without detectable extracellular polysaccharides are avirulent and fail to multiply in host tissue (Ayers *et al.* 1979). The extracellular polysaccharide of *E. amylovora* has been reported to act as a host specific toxin during pathogenesis (Goodman *et al.* 1974), a claim that has been disputed (Sjulin and Beer 1978). Surface polysaccharides have also been reported to prevent agglutination of *E. amylovora* and *Pseudomonas solanacearum* in susceptible plants allowing dispersal of the pathogen within the tissue (Leach *et al.* 1982; Romiero *et al.* 1981). Therefore, the role of extracellular polysaccharides in plant disease is of considerable interest. Bacteriophages that produce clear plaques surrounded by translucent halos when grown on encapsulated *E. amylovora* have been described (Ritchie and Klos 1977). The clear plaque, which is the result of phage encoded lysin(s), does not contain viable cells; the translucent halo, which is the result of phage encoded polysaccharide depolymerase, contains viable but unencapsulated cells. The translucent halo, but not the clear plaque, continues to expand after the bacterial lawn has stopped growing, due to diffusion of the polysaccharide depolymerase through the soft agar overlay. The plaque morphology appears analogous to other systems in which a soluble capsular polysaccharide depolymerase is produced during phage infection (Bartel *et al.* 1968; Chakrabarty *et al.* 1967; Sutherland 1976; Yurewicz *et al.* 1971) and in which the soluble form of the enzyme results from production of polysaccharide depolymerases in excess of that which is packaged into phage particles (Bessler *et al.* 1975; Rieger *et al.* 1975).

We report the molecular cloning of the polysaccharide depolymerase gene from phage PEal(h) and its expression in *Escherichia coli* and *E. amylovora*. Expression of the phage gene in *E. amylovora* created a novel system for the study of bacterial extracellular polysaccharides in pathogenesis. Parts of this work have been presented previously (Hartung *et al.* 1984). An apparently similar enzyme has been partially purified from phage lysates of *E. amylovora* (Vandenburg *et al.* 1985) and a gene encoding the enzyme was cloned into *E. coli* (Vandenbergh and Cole 1986).

MATERIALS AND METHODS

Bacterial and phage cultures. The bacterial strains and plasmids used in this study are described in Table 1. The bacterial strains were stored at −20° C in 20 mM phosphate buffer pH 6.8 with 40% glycerol (w/v). Phages were stored over chloroform at 4° C in phosphate buffer.

Media. Strain Eall0R was grown on nutrient agar (Difco) supplemented with 0.5% glucose. Soft agar overlays (Adams 1959) for phage production consisted of 0.7% nutrient agar, 0.5% yeast extract (Difco), and 0.5% glucose. Strain JM105 was grown in a defined medium (DM-17), consisting of Davis Minimal (Lederberg 1950) supplemented with thiamin and niacin to 1 mg/L, glucose to 2 g/L, and all essential amino acids except methionine, proline, and glycine at 20 mg/L. Strain HB101 was grown in LB (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) or in DM-17 with 20 mg of l-proline per liter. Ampicillin, chloramphenicol, streptomycin, and rifampin were added to autoclaved media to final concentrations of 200, 170, 50, and 50 mg/L, respectively.

Purification of phages and phage DNA. Bacteriophages were harvested from confluently lysed soft agar overlays by scraping the overlays into an equal volume of cold

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phosphate buffer containing 1% chloroform. Phages were purified by differential centrifugation followed by sucrose density gradient centrifugation (Ritchie and Klos 1977). The sucrose density gradient was scanned at 260 nm, and the single sharply defined peak that contained PEal(h) was collected. Purified phage PEal(h) obtained from 20 soft agar overlays was pelleted at 42,000 × *g* and resuspended in 3.0 ml of lysis buffer (20 mM Tris, 5 mM EDTA, 1% $NaDodSO_4$, pH 7.6) (Davis *et al.* 1980). The suspension was incubated at 50° C for 20 min, then cooled to room temperature before sequential extraction with equal volumes of buffer-saturated phenol (0.1 M Tris/HCl, pH 7.7), phenol/chloroform (1:1), and chloroform, followed by ethanol precipitation.

Molecular cloning of the phage polysaccharide depolymerase gene. Restriction endonucleases, T-4 DNA ligase, X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactoside) and IPTG (isopropylthio-β-d-galactoside) were obtained from BRL (Gaithersburg, MD). Reaction conditions suggested by the manufacturer or described elsewhere (Ruther 1980) were followed. Phage DNA was digested with endonuclease *Sau*3A. and genomic pieces less than 2 kilobase pairs (kbp) in length were collected after centrifugation of the pooled partial digests in a linear 10–40% sucrose density gradient at 100,000 × *g* for 24 hr.

Plasmid pUC8 was isolated from cultures after overnight amplification, purified in a CsCl/ethidium bromide density gradient, and digested with *Bam*H1. Aliquots of digested pUC8 and the sized PEal(h)/*Sau*3A pieces were mixed and treated with T-4 DNA ligase.

A CaCl technique was used to transform *E. coli* JM105 to ampicillin resistance with the ligation mixture (Maniatis *et al.* 1982). Because we were concerned that induction of the phage genes in *E. coli* might be lethal, the transformants were transferred to DM-17 with and without X-gal and IPTG, the inducer of the lac promoter region. However, transformants grew equally well on either medium so this precaution proved to be unnecessary. Blue colonies were discarded and white colonies containing DNA insertions were transferred to a master plate. This plate was used to inoculate DM-17 with and without IPTG. To screen for clones producing soluble polysaccharide depolymerase, the plates were incubated at 37 or 27° C for 48 hr before the bacteria were killed with chloroform vapor. The plates were then overlaid with *E. amylovora* Eall0R in 3.0 ml of DM-17 containing 1% glucose (w/v), which enhances extracellular polysaccharide production by *E. amylovora*. The same CaCl technique was later used to introduce plasmids pJH94 into *E. coli* HB101 and pUC8 and pJH94 into *E. amylovora* Eall0R.

Table 1. Bacteria, plasmids, and bacteriophage

Designation	Genotype/phenotype	Reference
Escherichia coli		
HB101	F⁻, *hsdS20*(r_B^-, m_B^-), *recA13, ara-14, proA2, lacY1, galK2, rpsL20*(Sm^r), *xy-15, mt-1, supE44*	Ayers *et al.* 1979
JM105	(*lac pro*), *supE, thi, strA, endA, sbc*B15 *hsdR4*, F′*traD36 proAB, lacI*^{q}, *Z M15*	Messing *et al.* 1981
Erwinia amylovora		
Eall0R	rif^r	Ritchie and Klos 1977
Ea8	Acapsular	
Plasmids and bacteriophage		
pUC8	amp^r, lacZ$^+$	Messing *et al.* 1981
pJH94[a]	amp^r, lacZ$^-$, pdp$^+$	This work
PEal(h)	pdp$^+$	Ritchie and Klos 1977

[a] pdp$^+$ produces polysaccharide depolymerase specific for *Erwinia amylovora* extracellular polysaccharide and capsular polysaccharide.

Extraction of total DNA from *E. amylovora* Eall0R. Two 10-ml cultures of Eall0R were grown overnight at 31° C, pooled, and centrifuged at 3,000 × *g* for 5 min. The cells were lysed in 50 mM Tris/HCl, pH 8.0, in the presence of $NaDodSO_4$, EDTA, and proteinase K, phenol extracted, and ethanol precipitated (Davis *et al.* 1980).

Southern blotting and hybridization. DNA samples that were loaded in duplicate were electrophoresed for 4.5 hr at 5 V/cm in a 1% agarose gel in 0.08 M Tris-phosphate, 0.002 M EDTA buffer (Maniatis *et al.* 1982). DNA was transferred from the gel to nitrocellulose and probed with fragments of pJH94 liberated by double digestion of pJH94 with *Eco*RI and *Sal*I and purified by agarose gel electrophoresis. The two probe fragments (900 and 2,300 bp) were labeled with a nick translation kit (BRL 8160 SB) with biotin-11-dUTP (BRL 9507SA). Hybridization and posthybridization washes were done according to standard procedures (Maniatis *et al.* 1982) at 74° C. Biotinylated DNA was detected on the filters using a DNA detection kit (BRL 8239SA) and photographed using Polaroid Type 55 film with a blue filter.

***In vitro* and *in vivo* production of polysaccharide by *E. amylovora*.** Slime and capsular polysaccharide were prepared from bacterial cultures as described by Hollingsworth *et al.* (1984). Extracellular polysaccharides were removed by washing cells in phosphate buffer, pH 6.8, which contained 150 mM NaCl. The cells were pelleted and resuspended in the same buffer containing 500 mM NaCl. Polysaccharides that were precipitated with two volumes of 95% ethanol from low and high salt buffers were designated ethanol precipitable extracellular (EPS) and capsular (CPS) polysaccharides, respectively. For the *in vivo* production of polysaccharides, immature pears (approximately 2.5 cm in diameter) were surface-sterilized by immersion in 0.05% sodium hypochlorite for 10 min. They were rinsed with distilled water, sliced, and placed in sterile enamel pans lined with wet paper towels. A 10 μl drop of a culture of Eall0R grown in nutrient broth (Difco) supplemented with 0.05% glucose was placed on each slice and the slices were incubated at 27° C for 4 days. The bacteria and EPS were obtained by washing the fruit with phosphate buffer supplemented with 150 mM NaCl. The bacteria were removed by centrifugation and two volumes of 95% ethanol were added to the supernatant, which was stored at −20° C overnight. The EPS was collected by centrifugation at 16,000 × *g* and redissolved in a minimal volume of distilled water.

Preparation of soluble polysaccharide depolymerase from HB101 (pJH94) and Eall0R (pJH94) and from phage lysates of *E. amylovora*. Soluble polysaccharide depolymerase was obtained from overlays of PEal(h) lysed Eall0R that had been suspended in cold phosphate buffer. The debris was removed from the suspension by centrifugation at 10,000 × *g* and the supernatant was dialyzed against 20 mM Tris/HCl, pH 8.0 at 4° C.

HB101 (pJH94) was used to inoculate 250 ml of LB containing ampicillin. After 24 hr of growth at 27° C, the bacteria were pelleted by centrifugation and a sarkosyl lysate was prepared (Schwinghamer 1980). This lysate was fractionated with 0, 30, and 80% ammonium sulfate. The crude lysates were dialyzed against 20 mM Tris/HCl for 2 days at 4° C before being assayed for polysaccharide depolymerase.

Biochemical assays. Total carbohydrates in the bacterial EPS and CPS were quantified with the phenol/sulfuric acid method (Herbert *et al.* 1971), and reducing sugars were quantified with the bicinchoninate reagent (McFeeters 1980), both against galactose standards. Uronic acids (Blumenkrantz and Asboe-Hansen 1973) and proteins (Lowry *et al.* 1951) were also quantified. A unit of polysaccharide depolymerase was defined as the amount of enzyme that liberated 1 μMole of galactose equivalents per hour in a reaction containing 25 μl EPS (12 mg/ml), 12.5. μl distilled water, 50 μl 200 mM acetate buffer, pH 5.0, and 12.5 μl of a dilution of enzyme containing solution. The reactions were incubated at 42° C.

Effect of pH on the activity of soluble polysaccharide depolymerases. Serial dilutions of enzyme-containing solutions (12.5 μl) were added to reaction mixes that contained 200 mM buffer (50 μl), EPS produced in pear fruits (25 μl of 12 mg carbohydrate per milliliter), and distilled water (12.5 μl). The 100-μl reaction mixtures were incubated in triplicate at 42° C for 3–5 hr. The concentration of reducing sugars in each reaction was determined. A unit of enzyme activity produced 1 μMole of galactose equivalents per hour in this assay. Reaction mixtures that had distilled water substituted for either substrate or enzyme were also incubated and assayed as controls. Acetate buffer was used at pH 4.0 and 5.0, potassium phosphate buffer was used at pH 6.0 and 7.0, and Tris/HCl buffer was used at pH 8.0.

Comparison of polysaccharides as substrates for polysaccharide depolymerase. EPS prepared from Eall0R, Eall0R (pUC8), and Eall0R (pJH94) were adjusted to concentrations of 0.3 mg of carbohydrate per milliliter each and were used as substrates in an *in vitro* assay for polysaccharide depolymerase. Each reaction mixture contained 100 μl of polysaccharide depolymerase prepared from Eall0R (pJH94), 200 μl of EPS, and 200 μl of 200 mM acetate buffer, pH 5.0. Reactions were incubated in triplicate at 42° C for 5 hr before aliquots were removed and assayed for reducing sugars. Reaction mixtures that had water substituted for the polysaccharide depolymerase or for the EPS substrate were used as controls. CPS from the same strains were adjusted to 0.2 mg carbohydrate per milliliter and assayed as substrate for polysaccharide depolymerase in the same manner.

Pathogenicity study. The pathogenicity of strain Eall0R (pJH94) was compared to that of strains Eall0R, Eall0R (pUC8), and Ea8. Cultures were grown to late exponential phase in DM-17, pelleted at 12,000 × *g* for 5 min, and resuspended in phosphate buffer or in phosphate buffer with ampicillin to an OD_{600} of 0.05. Immature pear fruits (Pugashetti and Starr 1975) approximately 3 cm in diameter were surface disinfested in a solution of 5% bleach and 0.1% Triton X-100 for 10 min, rinsed in distilled water, and placed in styrofoam egg cartons. Three 10-μl drops of inoculum or buffer were placed on each pear fruit and the fruit was stabbed through each droplet with a 20 gauge syringe needle. The pears (four per treatment) were incubated at 27° C.

RESULTS

Screening of *E. coli* JM105 transformants and assay of polysaccharide depolymerase. Forty-eight hours after transformed JM105 cells were overlaid with *E. amylovora*, one of 140 colonies was surrounded by a very large translucent halo that appeared similar to, but larger than, the halos surrounding PEal(h) plaques associated with infection of Eall0R. The halo size was not affected by IPTG but was larger when the recombinant clones were grown at 27 than 37° C. As with phage-induced halos, the halo around transformed JM105 continued to expand after the bacterial lawn had ceased growing. Bacteria within this halo were viable and grew with normal morphology when cultured on DM-17 plates supplemented with glucose.

This clone contained a new plasmid, designated pJH94, which was approximately twice the size of the vector pUC8. pJH94 had an apparent size of 5.8 kilobases (kb) when digested with *Sal*I, which does not cleave phage PEal(h) DNA (data not shown). The phage DNA insert was 3.1 kb and included a single *Eco*RI site (Fig. 1, lane 3).

Southern blot analysis demonstrated that the cloned insert found in pJH94 was of phage origin (Fig. 1). Fragments of pJH94 hybridized to genomic fragments of PEal(h) but not to genomic fragments of Eall0R or to the vector plasmid pUC8.

Culture supernatants from JM105 (pJH94) and JM105 (pUC8) were spotted in dilution series on mature lawns of

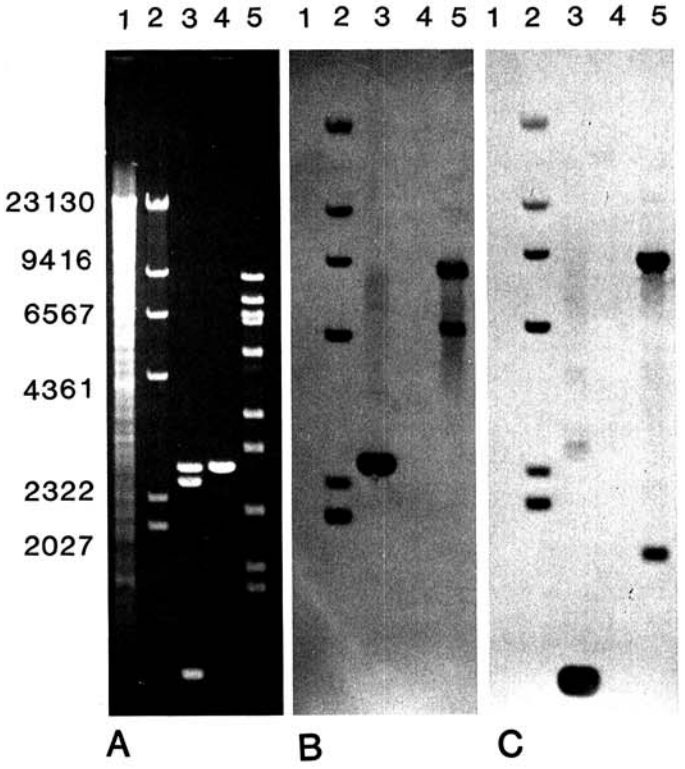

Fig. 1. Agarose gel and Southern blot analysis demonstrates the bacteriophage origin of the DNA cloned in pJH94. **A,** 1% agarose gel stained with ethidium bromide before blotting: Lane 1, Eall0R genomic DNA digested with *Eco*RI (4 μg); Lane 2, biotinylated lambda/*Hind*III fragments (300 ng); Lane 3, pJH94 DNA digested with *Eco*RI and *Sal*I (580 ng); Lane 4, pUC8 DNA digested with *Eco*RI (230 ng); Lane 5, PEal(h) DNA digested with *Bgl*II (1.6 μg). **B,** Southern blot of (A) using the 2,300 base pair *Eco*RI fragment of pJH94 as the biotinylated hybridization probe. **C,** Southern blot of (A) using the 900 bp *Eco*RI fragment of pJH94 as the biotinylated hybridization probe. Lambda/*Hind*III size standards in base pairs are in the left margin.

Eall0R. Culture supernatants from JM105 (pJH94) made distinct translucent clearings in the lawns where 10-μl droplets were applied. Such clearings also included slightly sunken areas termed craters, which are typical of polysaccharide depolymerase-containing fluids (Adams and Park 1956; Humphries 1948). No depolymerase activity was found in the supernatants from JM105 (pUC8) (data not shown).

Plasmid pJH94, purified by passage through a CsCl/ethidium bromide density gradient (Maniatis *et al.* 1982), was introduced into *E. coli* HB101 by transformation. When expressed as units of polysaccharide depolymerase produced per liter of growth medium, HB101 (pJH94) produced more soluble polysaccharide depolymerase than Eall0R infected by phage PEal(h) when assayed with *E. amylovora* EPS as substrate (Table 2). The specific activity of polysaccharide depolymerase was also greater in HB101 (pJH94) when compared to PEal(h) lysis of Eall0R (Table 2).

No differences were observed in the effect of pH on the activity of polysaccharide depolymerase isolated from phage PEal(h) lysates of Eall0R and from sarkosyl lysates of HB101 (pJH94) when assayed using *E. amylovora* EPS as substrate (Fig. 2). Enzymes from each source had optimal activity at pH 5.0 and no activity at pH 8.0. The activities were nearly equal for each enzyme at pH 4.0 and 7.0. These results suggest that the enzymes were identical.

Expression of polysaccharide depolymerase in *E. amylovora*. Plasmids were detected in lysates of Eall0R (pJH94) and Eall0R (pUC8) as ethidium bromide-stained bands of 5.8 and 2.7 kbp, respectively, after digestion with *Sal*I and electrophoresis through 0.9% agarose. No resident plasmids were detected in strain Eall0R (data not shown). When grown on solid DM-17 containing 2% glucose, strains Eall0R and Eall0R (pUC8) produced fluid colonies typical of *E. amylovora*. The acapsular strain Ea8 also had a fluid colony morphology under these conditions, although not nearly as pronounced as the capsule-producing strains. In contrast, Eall0R (pJH94) was not fluidal (Fig. 3). Polysaccharide depolymerase activity was found in the culture filtrates and cell lysates of Eall0R (pJH94) but not in the culture supernatants or lysates of strain Eall0R (pUC8).

Table 2. Polysaccharide depolymerase activity[a] recovered from phage PEal(h) lysates of Eall0R and from sarkosyl lysates of HB101(pJH94)

Source	Units/liter[b]	Specific activity (units/mg protein)
PEal(h) lysate of Eall0R		
Phage pellet[c]	36.5	n.d.[d]
Soluble	81.5	0.63
Total	118.0	
Sarkosyl lysate of HB101 (pJH94)		
Crude dialysed lysate	7,140	n.d.
Dialysed supernatant		
30% $(NH_4)_2SO_4$	6,800	n.d.
80% $(NH_4)_2SO_4$	0	n.d.
Pellet, 80% $(NH_4)_2SO_4$	6,240	12.5

[a] μMole galactose equivalents per hour, in a reaction containing 25 μl EPS (12 mg/ml), 12.5 μl of distilled water, 50 μl of acetate buffer, 200 mM, pH 5.0, and 12.5 μl of a dilution series of polysaccharide depolymerase containing solution. Reactions were incubated at 40°C, HB101 (pJH94) for 3 hr and Eall0R/PEal(h) for 5 hr.

[b] Units per liter of growth medium used to prepare the lysates. PEal(h) lysate of Eall0R prepared from soft agar overlays confluent lysis of Eall0R. Sarkosyl lysate of HB101 (pJH94) prepared from cells harvested at stationary phase.

[c] Phage pelleted by centrifugation.

[d] n.d. = not determined.

reducing ends present per unit of carbohydrate was much

Strain Eall0R (pJH94) produced less EPS and CPS per cell than either Eall0R or Eall0R (pUC8) (Table 3). EPS and CPS produced by Eall0R (pJH94) also differed qualitatively from that of the other two strains. The concentration of higher with strain Eall0R (pJH94) than with either of the other strains (Table 3). This suggested that the average polymer length of EPS and CPS was shorter when isolated from Eall0R (pJH94) than from the other strains. The EPS and CPS recovered from this strain also contained less uronic acid per weight of carbohydrate (Table 3). This may have been due to an unexpected modification of the polysaccharides in strain Eall0R (pJH94).

Incubation of EPS from both Eall0R and Eall0R (pUC8) with polysaccharide depolymerase resulted in a sharp increase in the concentration of reducing equivalents compared to controls incubated without polysaccharide depolymerase. In contrast, incubation of EPS from Eall0R (pJH94) with polysaccharide depolymerase resulted in only a barely detectable increase in the concentration of reducing equivalents in the same assay (Table 4). Results were similar with CPS obtained from the three strains (Table 4), indicating that these polysaccharides had been completely degraded by the polysaccharide depolymerase produced by Eall0R (pJH94).

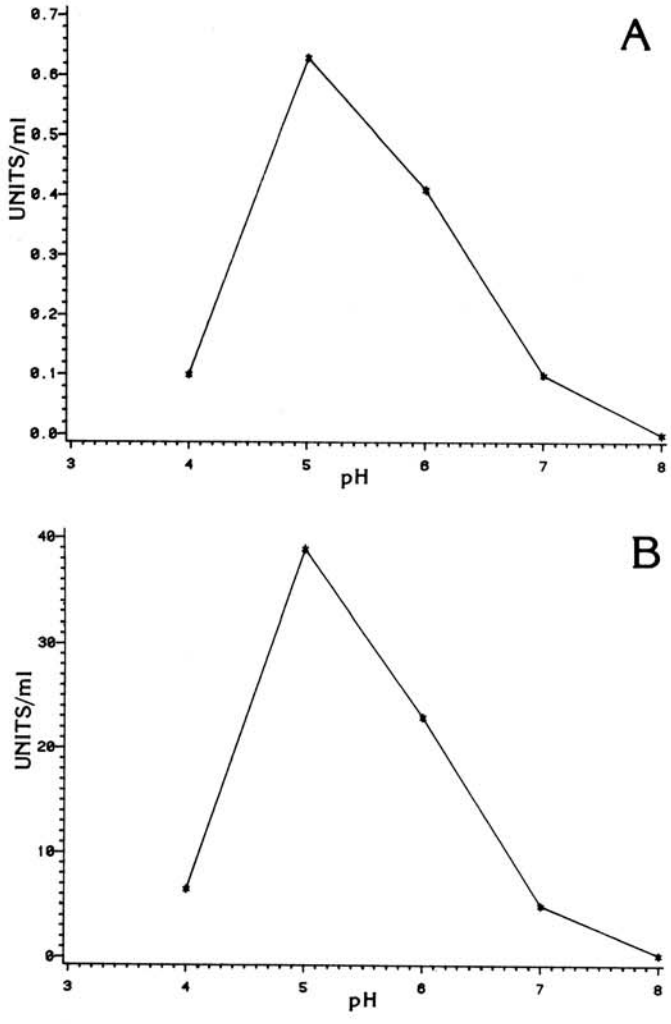

Fig. 2. Effect of pH on the activity of polysaccharide depolymerase in phage PEal(h) lysates of *Erwinia amylovora* Eall0R (**A**) and in sarkosyl lysates of *Escherichia coli* HB101 (pJH94) (**B**).

Pathogenicity. Symptoms developed rapidly in fruits inoculated with Eall0R, Eall0R (pUC8), or Eall0R (pJH94) (Fig. 4). Symptoms caused by Eall0R and Eall0R (pUC8) included extensive necrosis and copious ooze, which are diagnostic of fireblight infections in apple shoots and blossoms. Strain Eall0R (pJH94) caused necrosis but, in marked contrast to the other isolates, failed to produce ooze in inoculated pear fruit. In a few instances, ooze was produced by pear fruits inoculated with strain Eall0R (pJH94). However, the appearance of the ooze was delayed, compared with ooze on fruits inoculated with strain Eall0R, and the bacteria in the ooze were sensitive to ampicillin, indicating that they had lost plasmid pJH94. Strain Ea8 and the buffer controls produced only slight necrosis or no symptoms.

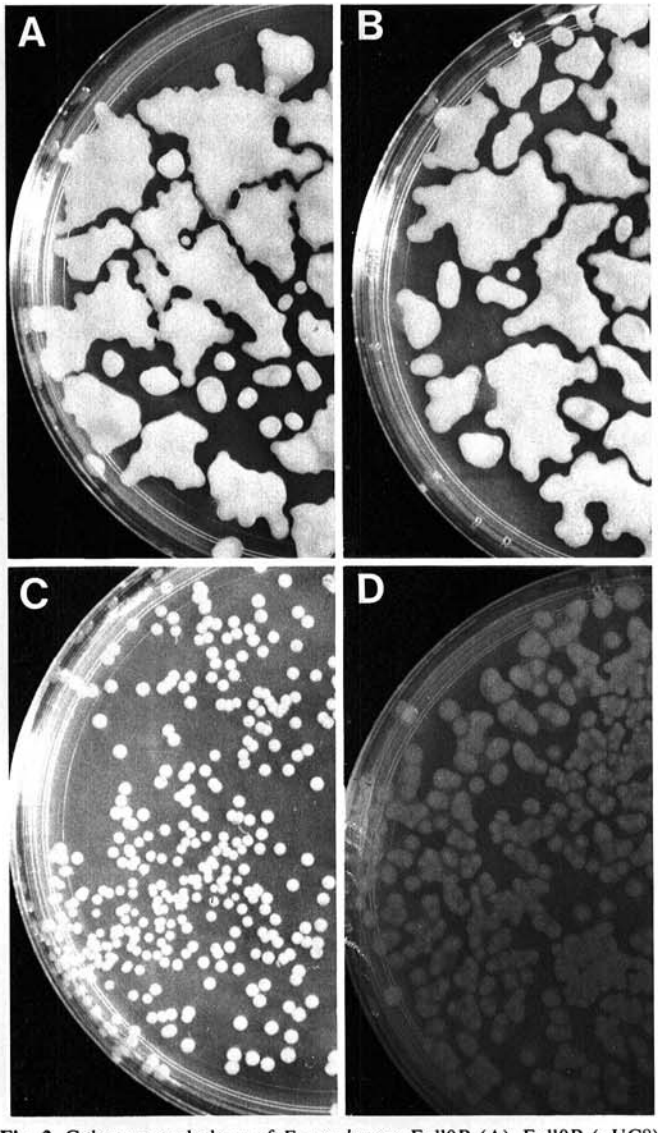

Fig. 3. Colony morphology of *E. amylovora* Eall0R (**A**), Eall0R (pUC8) (**B**), Eall0R (pJH94) (**C**), and Ea8 (**D**). The bacteria were grown for 5 days at 27° C on DM-17 medium that contained 2% glucose.

Table 3. Chemical characterization of ethanol precipitable extracellular (EPS) and capsular (CPS) polysaccharides of *Erwinia amylovora* strains

Strain		Carbohydrates ($mg/10^{11}$ cells)[a]	Reducing equivalents ($\mu g/mg$)[b]	Uronic acid (mg/mg)[c]
Eall0R	EPS	0.79 ± .08	15.7 ± .69	0.23 ± .01
Eall0R (pUC8)	EPS	0.67 ± .12	16.8 ± 2.25	0.21 ± .03
Eall0R (pJH94)	EPS	0.40 ± .01	50.7 ± 8.37	0.12 ± .01
Eall0R	CPS	0.30 ± .04	18.6 ± 1.03	0.18 ± .01
Eall0R (pUC8)	CPS	0.31 ± .05	19.3 ± 1.05	0.18 ± .01
Eall0R (pJH94)	CPS	0.25 ± .02	37.0 ± 3.41	0.12 ± 0

[a] Carbohydrates quantified with the phenol sulfuric acid method (Herbert *et al.* 1971) using a galactose standard. Cells enumerated with a Petroff-Hausser bacterial cell counter. Mean of three experiments, three replicates each, followed by standard deviation of the mean.

[b] Reducing equivalents determined with the bicinchoninate assay (McFeeters 1980) with a galactose standard. Mean of three experiments, three replicates each, followed by standard deviation of the mean.

[c] Determined as glucuronic acid equivalents (Blumenkrantz and Asboe-Hansen 1973). Mean of two experiments, three replicates each, followed by standard deviation of the mean.

Table 4. Comparisons of polysaccharides produced by strains of *E. amylovora* as substrates for polysaccharide depolymerase (PD)

Strain	Polysaccharide[a]	Galactose equivalents[b] released by PD (μg)
Eall0R	EPS	7.1 ± .016
Eall0R (pUC8)	EPS	6.8 ± .016
Eall0R (pJH94)	EPS	0.4 ± .017
Eall0R	CPS	1.9 ± .023
Eall0R (pUC8)	CPS	1.3 ± .092
Eall0R (pJH94)	CPS	0

[a] EPS was used at 60 μg/500 μl reaction; CPS was used at 40 μg/500 μl reaction.

[b] The bicinchoninate assay (McFeeters 1980) was used to quantify reducing sugars released by PD from the polysaccharides using a galactose standard. Mean ± standard deviation of three replicate determinations.

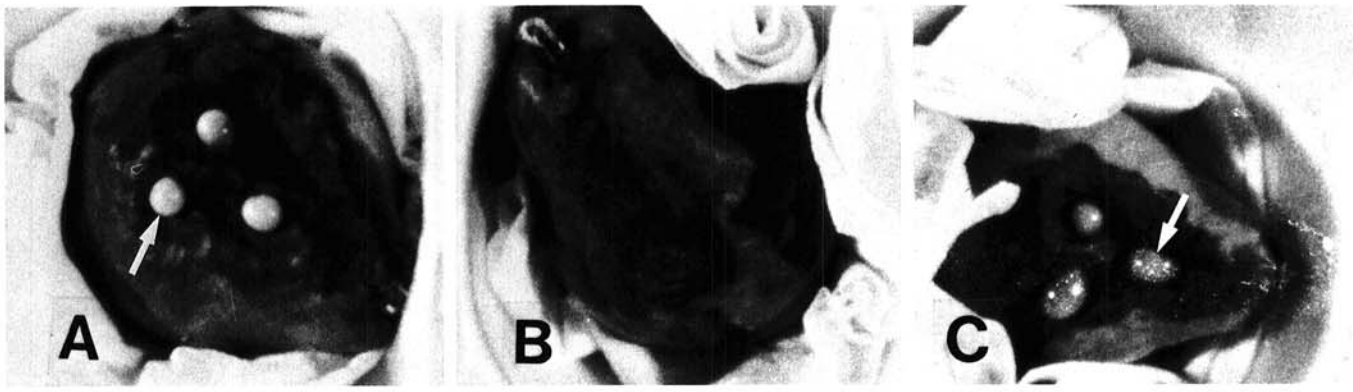

Fig. 4. Symptoms caused by *E. amylovora* strains inoculated on immature pear fruits, 3 days after inoculation. Arrows indicate ooze. **A,** Eall0R; **B,** Eall0R (pJH94); **C,** Eall0R (pUC8).

DISCUSSION

The biological and biochemical data together demonstrate that *E. coli* strains JM105 (pJH94) and HB101 (pJH94) produced an enzymatic activity not present in either JM105 or HB101. This enzyme was indistinguishable from the soluble form of the phage-associated polysaccharide depolymerase that caused halos to appear around plaques of PEal(h)-infected Eall0R, judged by the effect of pH on the relative activities of the enzyme collected from the two sources and assayed with EPS as substrate.

pJH94 was introduced into *E. amylovora* Eall0R by a CaCl-mediated transformation procedure. The resulting strain, Eall0R (pJH94) was resistant to ampicillin and produced polysaccharide depolymerase that was detected in culture supernatants and in cell lysates of Eall0R (pJH94). No native plasmids were found in lysates of Eall0R. The fluid colony morphology of Eall0R was lost when the strain was transformed with pJH94. Eall0R (pJH94) was nonfluid, compared to the acapsular mutant strain.

The development of extensive necrosis when pear fruits were inoculated with Eall0R (pJH94) suggested that intact extracellular polysaccharides may not be required for pathogenesis by *E. amylovora*. However, the lack of ooze in these tests suggested that ooze is composed largely of intact bacterial polysaccharides. This is in agreement with a chemical analysis of ooze polysaccharide and EPS, which has shown very similar molar ratios of several sugars found in each polysaccharide (Bennett and Billing 1980), chromatographic evidence, and serological data (Sijam *et al.* 1983). It is unknown if EPS and CPS were completely degraded by polysaccharide depolymerase when strain Eall0R (pJH94) was grown in pear fruits, and the possible presence of small amounts of intact EPS and CPS cannot be completely excluded.

The role of bacterial exopolysaccharides in pathogenesis has been investigated enzymatically in previous studies. Avery and Dubos (1931) enzymatically removed the EPS of a virulent strain of *Pneumococcus* before injecting it, with additional polysaccharide depolymerase, into the mouse peritoneum. The enzymatically decapsulated bacteria were rendered avirulent. The EPS was thought to interfere with the recognition of the bacteria by the phagocytes. In a model similar to this, EPS of *E. amylovora* and *Pseudomonas solanacearum* are thought to prevent agglutination of bacteria by basic plant proteins interacting with a component of the bacterial lipopolysaccharide (Leach *et al.* 1982; Romiero *et al.* 1981). The polysaccharide fragments produced by Eall0R (pJH94) could still retain this activity.

Recently, another polysaccharide depolymerase, produced by *E. amylovora* cells infected with bacteriophage ERA103 has been partially purified and characterized (Vandenburg *et al.* 1985). A pH of 6.0 was optimum for activity, unlike the polysaccharide depolymerase from PEal(h) infected cells, which has an optimum of pH 5.0 (Fig. 2). Phage ERA103 appears unrelated to PEal(h) since the genome size of ERA103 is reported as 19 kb (Vandenbergh and Cole 1986) whereas the genome size of PEal(h) is about 47 kb as can be seen by the summation of the *Bgl*II fragments (Fig. 1, lane 5). When the polysaccharide depolymerase gene of ERA103 was cloned in *E. coli* (Vandenbergh and Cole 1986), a decreased specific activity was noted, compared with phage lysates; in our study, the lysates of HB101 (pJH94) had a markedly greater specific activity than PEal(h) lysates of Eall0R (Table 2). In this recent study, *E. amylovora* was not transformed.

In our study, the gene encoding a polysaccharide depolymerase was introduced into a fully pathogenic strain of *E. amylovora*, and the enzyme was produced by the bacterium. It is important to emphasize that pathogenicity, the ability to incite disease, was separated from the symptoms of infection. This has not been possible in previous studies that relied on avirulent, acapsular mutant strains (Ayers *et al.* 1979). This novel approach to the study of exopolysaccharides could complement studies designed to examine the role of polysaccharides in microbe-plant interaction. It could presently be applied to the many bacterial species for which appropriate bacteriophages are already available.

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