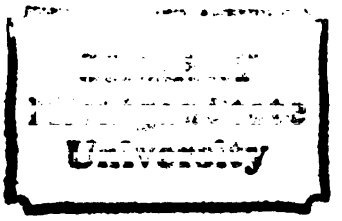

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CLONING OF THE POLYSACCHARIDE DEPOLYMERASE GENE OF BACTERIOPHAGE PEa1(h) AND ITS EXPRESSION IN *ERWINIA AMYLOVORA*

By

John Stephen Hartung

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ABSTRACT

CLONING OF THE POLYSACCHARIDE DEPOLYMERASE GENE FROM BACTERIOPHAGE PEa1(H) AND ITS EXPRESSION IN ERWINIA AMYLOVORA

By

John Stephen Hartung

A bacteriophage gene which encoded a polysaccharide depolymerase (PD) specific for the surface polysaccharides of Erwinia amylovora was cloned and expressed in E. coli. The cloned gene was also used to study the controversial role of these surface polysaccharides in pathogenesis of E. amylovora.

Bacteriophage PEa1(h) produced clear plaques surrounded by translucent haloes when it infected encapsulated strains of Erwinia amylovora. The haloes were caused by a soluble polysaccharide depolymerase (PD) associated with phage infection. The PD gene was cloned in Escherichia coli from the ds DNA phage genome using the JM105(pUC8) system. A new 5.85 kbp plasmid, pJH94, was found in JM105 clone 94. The production of PD by strain JM105(pJH94) was confirmed with an in vitro assay which used purified extracellular polysaccharides (EPS) prepared from Ea110R as substrate. Southern blotting experiments confirmed that the cloned PD gene was of phage, not bacterial, origin.

Purified plasmid pJH94 transformed Ea110R to ampicillin resistance, with a concomitant loss of fluidal colony morphology. Production of PD by Ea110R(pJH94) was confirmed

with the *in vitro* assay. Chemical analysis showed that Ea110R(pJH94) produced less slime and capsular polysaccharides, and that the polysaccharides were of lower molecular weight than the polysaccharides produced by Ea110R or Ea110R(pUC8). The necrotic lesions incited by Ea110R(pJH94) in immature pear fruits did not produce ooze as did lesions incited by Ea110R or Ea110R(pUC8). The results suggest that the disease, but not the ooze production characteristic of the disease, can occur in the presence of an enzyme which can depolymerize the EPS. It must be emphasized however that we do not know if the extracellular polysaccharides are totally degraded *in vivo*, nor do we know whether or not the oligosaccharide products of the depolymerase enzyme have biological activity. These questions must be addressed before firm conclusions regarding the role of extracellular polysaccharides in pathogenicity and virulence can be made.

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LITERATURE REVIEW

Literature Review

The phytopathogenic bacteria represent a diverse group of prokaryotes which includes the genera Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, Xanthomonas and Streptomyces. A common feature of these genera is the production of polysaccharide capsules or extracellular polysaccharides in vitro (21,50,78,85,99) and the appearance of bacteria in a polysaccharide matrix in vivo (56,85,103,125). The role(s) of bacterial surface polysaccharide in plant disease is an example of a subject much studied, yet poorly understood.

When Duguid (51) suspended cultured bacterial cells in water and negatively stained them with India ink, two types of extracellular polysaccharides were observed: Those which remained tightly adherent to the cells (capsular polysaccharides or CPS) and those which were dispersed into the water (slime polysaccharides or EPS). The terminology of Duguid will be followed in this work except where explicitly noted.

The presence of capsules and slime polysaccharides on phytopathogenic bacteria is not unique since capsules were found surrounding bacteria in such diverse habitats as animal blood (6), bovine rumen fluid (44) and freshwater

streams (44). It has been proposed that these polysaccharide capsules aid the bacteria in adhering to surfaces and forming colonies (44) and act as ion exchangers to concentrate nutrients near the bacterial surface (44.) Capsular polysaccharides could also protect the bacteria from infection by bacteriophages which require specific membrane proteins or lipopolysaccharides for recognition (89,135).

The slime polysaccharide of Xanthomonas phaseolicola produced in vitro was shown to prevent dessication, absorb U.V. light, and increase bacterial viability at high temperatures (85). These results reinforced conclusions of earlier researchers who studied Erwinia amylovora polysaccharides produced in vivo (70,110). It is evident therefore that capsular and extracellular polysaccharides can perform a great variety of ecologically useful and adaptive functions.

Avery and his colleagues (6) showed that the capsule of Pneumococcus was required for pathogenicity in mice because the presence of the capsule prevented phagocytosis of the bacteria by the white blood cells. There has also been a long association of encapsulation with pathogenicity in phytopathogenic genera, particularly Pseudomonas (50,75,78) and Erwinia (7,21) although the nature of the association has remained controversial.

A very strong correlation between virulence and the presence of EPS exists in Pseudomonas solanacearum (75,78). Sequeira and his colleagues reported that avirulent, acapsu-

lar strains of P. solanacearum were rapidly attached to and enveloped by cell walls in tobacco leaf mesophyll while virulent, encapsulated strains were not attached or enveloped. The attachment was proposed as a trigger for the induction of host resistance (115). It was also reported that all avirulent, acapsular strains were bound via their lipopolysaccharide (LPS) to a potato lectin. This in vitro agglutination was blocked by the presence of EPS on virulent isolates, or when EPS was added to suspensions of avirulent cells prior to the potato lectin (116). It was proposed that resistance could be induced in the host after binding of a component of the bacterial LPS by lectin in vivo, and that this binding could be prevented by the presence of bacterial EPS (116). However, when the bacterial agglutinin was purified to apparent homogeneity and chemically characterized, the red blood cell agglutination activity characteristic of potato lectin was lost (83).This work demonstrated that the bacterial agglutinin present in potato tubers was not potato lectin, but rather a hydroxyproline-rich glycoprotein similar to the cell wall protein extensin (83,84).

A different role for capsular polysaccharides has been established for Rhizobium trifolii. Studies have demonstrated an early step in the establishment of a symbiotic infection of the root hair by R. trifolii. Trifoliin A, a lectin present on the root hair surface, specifically bound the capsular polysaccharides of Rhizobium trifolii (42) or

its oligosaccharide fragments (2), as well as its lipopolysaccharide (73). In the R. trifollii/clover system however, recognition of the potential symbiont leads to infection; recognition of avirulent P. solanacearum via its LPS induces resistance to the bacterium. In the models proposed for P. solanacearum and R. trifolii, EPS blocks recognition (116) and participates in the recognition event (46,2), respectively.

The specificity of the attachment and envelopment of heterologous or saprophytic as opposed to homologous, virulent bacteria in the leaf mesophyll was challenged by Hildebrand et. al. (69) who reported that the observed "entrapment films" were not the result of a specific binding event. These workers studied the fate of various Pseudomonas syringae pathovars and Pseudomonas fluorescens in bean, Phaseolus vulgaris, leaf mesophyll. They concluded that the "entrapment films" were formed non-specifically at air-water interfaces which resulted from dessication following the introduction of bacteria into the mesophyll by infiltration. If the mesophyll remained watersoaked until fixation for electron microscopy, no differential binding between homologous and heterologous bacteria was observed (69). They also observed larger entrapment films in younger leaves and proposed that the presence of such entrapment films in younger leaves could be correlated to increased susceptibility to bacterial pathogens, by providing a water soaked environment

conducive to bacterial multiplication (69,76).

El-Banoby and Rudolph (54) reported that EPS produced in vitro by several Pseudomonas syringae pathovars and from Xanthomonas campestris pv.malvacearum induced water soaking when infiltrated into leaves of homologous host plants. No water soaking was induced when EPS was infiltrated into leaf mesophyll of heterologous host plants (54). The EPS acted as a host selective agent which induced water soaking. These results were later extended to explain host resistance at the cultivar level with P. syringae pv.phaseolicola (55,56). It was concluded that the resistance of bean leaves to the persistant water soaking effect of EPS was due to the enzymatic breakdown of EPS infiltrated into resistant leaves (56). Susceptible leaves were unable to enzymatically degrade EPS from a compatible bacterium, and the region of infiltration remained watersoaked because the EPS in the intercellular spaces captured water carried there via the transpiration stream (56).

The possible involvement of polysaccharides produced by Erwinia amylovora in the symptomology of fire blight has been controversial. Hildebrand (70) studied the polysaccharides found in fire blight ooze and found that a non-specific vascular plugging mechanism induced wilt in pear shoots. When this topic was re-investigated, it was concluded that ooze polysaccharide induced wilt in rosaceous but not non-rosaceous plants in the manner of a host-selec-

tive toxin (61). The ooze polysaccharide was given the trivial name of "amylovorin". The role of "amylovorin" as a host-selective toxin has been disputed by others who showed that it acted as a non-specific vascular plugging agent as was originally suggested (69,119). Furthermore, it was reported that sensitivity of several plant species to wilting in solutions of "amylovorin" was correlated to shoot flexibility, but not to the susceptibility or resistance of the cultivars tested to E. amylovora (18).

EPS produced in vitro by numerous isolates was quantified and positively correlated with the virulence of the isolates on various susceptible hosts (7), but the quantification of the EPS was later partially retracted (118). EPS has been reported to prevent agglutination of virulent isolates by a factor which was isolated from apple seeds and stems (109). There was no evidence that the factor was a lectin but rather a very basic protein which was postulated to interact with acidic polysaccharides based on an electrostatic interaction as has recently been shown to be the case in the P. solanacearum/potato interaction (83,84). An avirulent, acapsular strain was more strongly agglutinated in vitro than was a virulent, encapsulated strain (109). The authors proposed that this was due to the EPS of the virulent strain binding the agglutination factor and removing it from solution, leaving the encapsulated bacteria in solution. The avirulent strain, producing no EPS, was agglu-

tinated via its LPS and removed from solution.

This model is basically the same as proposed by Sequeira for P. solanacearum, in which agglutination of cells is prevented by agglutination of EPS. It was said to be consistent with earlier work from the same laboratory which showed that the avirulent strain, but not the virulent strain, was agglutinated in xylem vessels after artificial inoculation (124,125). However, the same lab reported earlier that after natural infection, the pathogen moved exclusively in the phloem (63,87), which would make the significance of the xylem agglutination in these experiments moot. The "anti-agglutination" role for EPS proposed above is also proposed for E. stewartii EPS in corn plants (30). However, the factor which agglutinated avirulent isolates E. stewartii was isolated from seed of a corn variety which was very susceptible to the pathogen.

Encapsulation can not be the sole virulence determinant for E. amylovora since an avirulent, fully encapsulated strain has been reported (19,20,25). When this strain was co-inoculated into apple shoots with an avirulent, acapsular strain, typical disease symptoms were observed (19). Similar results were obtained using an in vitro pear fruit assay in which the acapsular, avirulent strain induced cell leakage, but not ooze (25). The authors concluded that virulence in E. amylovora was associated with at least two factors: a cell leakage inducing agent and EPS (19,25).

The research on bacterial slime and capsular polysaccharides in plant disease reviewed above is an example of a subject much studied, but surrounded with controversy. In the case of E. amylovora, EPS has been reported to function as a toxin (61), vascular plugging agent (70,119) and anti-agglutination factor (109). The role of capsular polysaccharides in pathogenesis of Pneumococcus was established, in part, by using an enzyme to remove the capsular polysaccharides from a virulent strain prior to a virulence assay (6,49). Analogous experiments performed with plant pathogens could be equally rewarding. It is also interesting to note that resistance to P. syringae pv. phaseolicola has been associated with the presence of polysaccharide degrading enzymes in the leaf mesophyll (56).

Enzymes which remove the capsular and slime polysaccharides of bacteria have been isolated from heterologous bacteria (6,47). They are frequently associated with bacteriophage infection of encapsulated bacteria (4,8,10,53,71, 121,137). The presence of such a polysaccharide depolymerase (PD) typically results in the appearance of expanding, translucent haloes surrounding true plaques when bacteriophage are grown in soft agar overlays. The halo has been observed to result from the action of a diffusable enzyme which decapsulates, but does not kill, the bacteria (4,9,74). Polysaccharide depolymerases have been observed and studied from phage infected Klebsiella pneumoniae

(4,22,74,106), Escherichia coli (23,121), Aerobacter aerogenes (137), P. aeruginosa (10),and R. trifolii (8,67,71).

Early workers observed that when PD containing lysates were spotted on mature lawns of susceptible bacteria, haloes or shallow "craters" developed. An assay which was used in purification of these enzymes consisted of spotting aliquots of such lysates on lawns in dilution series and observing the development of craters (4,74). Other workers developed in vitro assays based on the liberation of reducing sugars (10,137), or hexoseamines (10) from polysaccharide substrate upon incubation with the depolymerases. In cases where increases in reducing sugar or hexoseamines were not detectable, purification was achieved by quantifying the rate at which enzyme containing aliquots reduced the viscosity of polysaccharide substrate solutions (8,67,71).

The enzymes have been shown to be endo-glycanases (which cleave the glycosidic bond by the addition of a molecule of water) in the majority of cases (10,22,23,106,137), but a lyase (which cleaved the glycosidic bond by the removal of a molecule of water) has recently been reported (71). The endo cleavage catalysed by these enzymes has been generally shown to procede until the resulting oligosaccharides consisted of 1-3 repeating unit oligosaccharides (106,137). This explained the concominant increase in reducing sugars and decrease in viscosity observed when polysaccharides were incubated with such enzymes. Enzy-

matic activity was stimulated by the presence of divalent cations in some studies (67,71). The enzymes have been shown to be very substrate specific, generally hydrolysing only polysaccharide from strains on which the phage associated with the enzyme could reproduce (106,127) although exceptions have been noted in the Rhizobiaceae (126). In some cases complete hydrolysis of homologous polysaccharide required prior deacetylation (71).

The enzymes which have been purified and characterized had molecular weights ranging from 155,000 to 550,000 (10,23,71,105,137) and were composed of several subunits (23,105,137). Enzymatic activity with identical characteristics has been found bound to the phage particles in many systems (4,16,74,137) and genetic linkage data indicated that one depolymerase was encoded by the bacteriophage (33). Stirm and his colleagues have shown that the soluble depolymerase associated with several E. coli K phages consisted of phage tail spikes which were not assembled into the phage particles themselves (23,105). The same conclusion was reached by Sutherland who studied a Klebsiella/phage system (127). He observed polysaccharide depolymerase activity with purified phage particles which made plaques without haloes when grown on an encapsulated strain of K. aerogenes (127).

Polysaccharide depolymerases have been exploited for structural determinations of their substrate polysaccharides (71,132,136). The presence of polysaccharide depolymerase

activity bound to tail spikes of E. coli K phage particles resulted in "tunnels" through the capsule (16). "Tunneling" through the bacterial capsule allowed phage particles to attach and inject their genomes at the sites of membrane adhesion to the cell wall of E. coli (13,14,16). These membrane adhesion sites have been shown to be the site of capsular polysaccharide synthesis (15). The role of the polysaccharide depolymerases in nature was therefore proposed to be binding to the polysaccharide capsule and digesting it to reach the membrane adhesion sites where the phage genome was injected (16). The E. coli K phages were shown to only infect encapsulated bacteria; spontaneous acapsular mutants were not infected (121). Thus the capsule, which prevents infection by phages which require receptors located on the outer membrane (44,89,135), is a required receptor for this group of phage.

Bacteriophage which attack phytopathogenic Pseudomonads have been used to select mutants with altered surface components which were related to virulence (60), or to identify surface components related to virulence (68). Bacteriophage which attacked phytopathogenic bacteria have been isolated from rice paddy water (62), orchard soils (57), and apple foliage which had been killed by E. amylovora (108). Lysogenic strains of P. syringae pv. morsprunorum have been observed (59), but little is known about how (or whether) bacteriophage overwinter in orchards (57,107). Civerolo

explored the possibility of using a bacteriophage to control X. pruni damage to peach foliage, but was only able to achieve limited success (36,37,38,39,40). Ritchie (108) isolated from Erwinia amylovora infected apple tissue several phage which produced clear plaques surrounded by translucent haloes in vitro. The haloes observed by Ritchie (108) resembled those produced by a polysaccharide depolymerase associated with phage infection.

One of these phage, PEa1(h), was studied further. PEa1(h) lysates of E. amylovora were shown to contain an enzymatic activity in vitro which degraded slime polysaccharides extracted from E. amylovora grown on pear fruits (64), thus confirming the analogy with phage associated polysaccharide depolymerases found in other systems. A gene was cloned from PEa1(h) which encoded a polysaccharide depolymerase which was produced in both E. coli and E. amylovora (64). The molecular cloning of the polysaccharide depolymerase gene of PEa1(h) and preliminary experiments directed towards the use of the cloned polysaccharide depolymerase gene in the study of bacterial exopolysaccharides in plant disease developement are reported in this dissertation.

CHAPTER I

MOLECULAR CLONING OF THE POLYSACCHARIDE DEPOLYMERASE GENE OF BACTERIOPHAGE PEa1(h) IN *ESCHERICHIA COLI*

Introduction

Ritchie (107,108) reported the isolation and characterization of a series of phage which produced clear plaques surrounded by translucent haloes when grown on encapsulated Erwinia amylovora. One of these phage, PEa1(h), was selected for further study, since the phage/bacterial interaction appeared entirely analogous to those previously characterized in Escherichia coli, Klebsiella aerogenes, Pseudomonas aeruginosa and Aerobacter aerogenes (10,22,110,137). Molecular cloning of the polysaccharide depolymerase gene from purified PEa1(h) and expression of the gene in E. coli would directly demonstrate that the polysaccharide depolymerase is phage encoded as has been suggested in other systems (111,127). It would also allow production of large amounts of the enzyme for further characterization and study.

There does not seem to be any insurmountable barrier to heterologous gene expression between E. coli and E. amylovora, since drug resistance plasmids transferred from one species to the other resulted in full expression of markers in each species (34). Therefore expression of the polysaccharide depolymerase gene of PEa1(h) could be expected in E. coli, which is not a host for the phage. The

cloning vector pBR322 (28) contains a colE1 replicon (66) and so replicates independently of the chromosome. This property is useful since it allows amplification of the plasmid with chloramphenicol prior to purification (42,94). The small size of the plasmid (4.3 kb) also facilitates purification. The plasmid contains two selectable antibiotic resistance genes, either of which can be inactivated by the insertion of foreign DNA in one of several unique restriction endonuclease cleavage sites.

Derivitives of pBR322, pUC8 and pUC9, have been constructed (95). In these plasmids the tetracycline resistance gene of pBR322 has been replaced by the *lac* z gene of *E. coli*, leaving the ampicillin resistance gene as the selectable marker. The *lac* z gene encodes a B-galactosidase which cleaves lactose and which can be conveniently assayed because it also cleaves the colorless substrate analog 5-bromo-4-chloro-indolyl-B-D-galactoside (X-Gal) to yield a bright blue pigment (96). The *lac* z gene through operator and promoter functions can be specifically induced by the addition of isopropylthiogalactoside (IPTG) to the growth medium (95,96). The *lac* z gene on pUC8 can *trans*-complement *E. coli* JM83 which contains a deletion in the chromosomal *lac* z gene. The presence of pUC8 in this strain results in ampicillin resistant colonies which are blue colored when grown in the presence of X-Gal and IPTG. Several unique restriction endonuclease cleavage sites have been introduced

into pUC8 at the junction of the transcriptional promoter and the structural *lac* z gene (95). Insertion of foreign DNA into these sites, followed by transformation into JM83 results in clones which are ampicillin resistant but *lac* z^- due to insertional inactivation of the *lac* z gene. Such clones grow as white colonies in the presence of X-Gal and IPTG because the inserted DNA is transcribed instead of the *lac* z gene.

The *E. coli lac* I gene encodes a specific repressor of the lactose operon which prevents transcription of the operon (94). Strains of *E. coli* with a constitutively expressed mutation of *lac* I, *lac* I^q (94), are constiutively repressed and inducible only when IPTG is added exogenously. *E. coli* strain JM105 was derived from strain JM83 by the introduction of an F'episome which contains the *lac* I^q gene. Therefore transcription of the *lac* z gene of pUC8 or of any gene inserted distal to the *lac* z promoter of pUC8 is constitutively repressed in JM105, and specifically inducible with IPTG. Plasmid pUC9 completes the cloning system, and differs from pUC8 only in the reverse orientation of multiple cloning sites (95). This allows transcription of genes encoded on either strand of a given fragment of DNA depending upon its insertion into pUC8 or pUC9.

This cloning system was chosen for this study because expression of the phage polysaccharide depolymerase gene in *E. coli* was required for detection of the cloned gene. The

promoter region of the phage gene, which might not be present on the DNA fragment which contained the PD gene, could be substituted for with the lac z promoter region. Furthermore, transcription of the phage gene could be either constitutive (JM83) or repressed and specifically inducible (JM105). This latter property was desirable since uncontrolled transcription of the phage gene in E. coli might be deleterious to the bacterium (32) or cause plasmid instability (123).

Materials and Methods

Bacterial and phage cultures- The bacterial strains used in this study are described in Table 1. After streaking to single colonies and genotyping on appropriate media the strains were stored at -20C in 20 mM phosphate buffer pH 6.8 (PB) with 40% glycerol (w/v). Phage were stored over chloroform at 4C in 20 mM phosphate buffer, pH6.8 (96).

Media- Ea110R was grown in nutrient agar (Difco) supplemented with 0.5% glucose (NGA). Soft agar overlays (3) for phage production consisted of 0.7% nutrient agar, 0.5% yeast extract (Difco) and 0.5% glucose (NGAYE). JM83 and JM105 were grown in a defined medium consisting of Davis Minimal (86) 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 (DM-17). HB101 was grown in LB (tryptone 10 gm/l, yeast extract 5 gm/l, NaCl 5 gm/l) or in DM-17 with L-proline at 20 mg/l. Ampicillin was added after autoclaving to a final concentration of 200 mg/l. Chloramphenicol was added to a final concentration of 170 mg/l. Streptomycin sulfate and rifampin were used at 50 mg/l.

Table 1. *E. coli* strains used in this study

Strain	Relevant Genotype	Reference
JM83	*ara*, *lac pro*,*thi*, *str*A, 80d,*lac* Z,M15	(95)
JM105	*lac pro*,*sup*E,*thi*,*str*A, *end*A,*sbc*B15,*hsd*R4 F'*tra*D36, *pro*AB,*lac*Iq,Z M15	(95)
HB101	F-,*hsd*S20(rB-,mB-),*rec*A13,*ara*14, *pro*A2,*lac*Y1,*gal*K2,*rps*L20(Smr), *xyl*5,*mtl*1,*sup*E44,lambda-	(29,95)

Source of enzymes and reagents- Restriction endonucleases and T-4 DNA ligase were obtained from BRL, Gaithersburg, MD. Reaction conditions were those suggested by the manufacturer. Calf intestinal alkaline phosphatase grade 1 was obtained from Boeringer/Mannheim, Indianapolis, IN. X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside and IPTG (isopropylthio-B-D-galactoside) were purchased from BRL and used as described by Ruther (112,113). Eggwhite lysozyme, ampicillin, streptomycin sulfate, chloramphenicol, rifampicin, isoamyl alcohol, 2-mercapto ethanol, ethidium bromide and triton X-100 were obtained from Sigma, St. Louis, MO. Crystalline phenol was obtained from Mallinkrodt, St. Louis, MO., chloroform from Fisher Scientific, and 8-hydroxyquinoline from J.T. Baker Company. Cesium chloride was from Kawecki-Berylco, Inc.

Purification of Phage and Phage DNA-Bacteriophage were harvested from confluently lysed soft agar overlays by scraping the overlay into an equal volume of cold phosphate buffer containing 1% chloroform. After homogenization, the agar and bacterial debris were removed by centrifugation at 12,000g for 15 minutes. Phage were centrifuged at 42,000g for 90 minutes, resuspended in phosphate buffer overnight, and centrifuged at 50,000g on linear 10-40% sucrose density gradients in a Beckman SW 25.1 rotor. After fractionation with an ISCO U.V. gradient fractionator the phage were again pelleted and resuspended in phosphate buffer.

Sucrose density gradient purified PEA1(h) obtained from 20 soft agar overlays were pelleted at 42,000g and resuspended in 3.0 ml of lysis buffer (Tris 20 mM, EDTA 5 mM, SDS 1%, pH 7.6) (45). The suspension was incubated at 50C for 20 minutes then cooled to room temperature before being extracted with an equal volume of buffer saturated phenol prepared as described (94). The aqueous phase was extracted with phenol:chloroform (1:1) one time then with chloroform two times. The phage genome was precipitated at -20C with two volumes of ethanol and 1/10 volume sodium acetate pH5.2. After centrifugation for 10 minutes at 13,000g the pellet was dried in vacuo and resuspended in TE (Tris 10 mM EDTA 1 mM, pH 8.0) at a concentration of 50 ug/ml and stored at -20C until use.

Purification of plasmid pUC8 DNA- E. coli JM83(pUC8) was grown overnight at 37 C in LB containing ampicillin. A 1/100 dilution of this culture into fresh media was shaken until the $O.D._{600}$ reached 0.6. At that time chloramphenicol was added to a final concentration of 170 mg/l (94). The culture was shaken an additional 16 hours at 37 C before being centrifuged at 10,000g for 15 minutes. The pellet was resuspended in 125 ml of TE and centrifuged as before. After the supernatant had been decanted, the pellet was resuspended in 8 mls of 10mM Tris buffer, pH 8.0 containing 25 % sucrose (w/v). A 1.5 ml aliquot of 250 mM Tris buffer pH 8.0 containing freshly dissolved lysozyme (15 mg/ml) was

added to the cell suspension in a 50 ml SS-34 centrifuge tube which was then incubated in an ice water bath for 5 minutes. A 3.0 ml aliquot of 250 mM EDTA, pH 8.0 was added, the contents of the tube were gently mixed by inversion and returned to the ice water bath for 10 minutes. Lysis was completed by the addition of 11 mls of a solution composed of 62.5 mM EDTA, 50 mM Tris, and 1% Sarkosyl (v/v). The contents of the tube were mixed by gentle inversion and placed in a water bath at 45 C for 3 minutes. The extremely viscous lysate was again mixed gently by inversion before being centrifuged at 36,000g in a Sorvall SS-34 rotor for 70 minutes.

The viscous plasmid containing supernatant was decanted into a cellulose nitrate TI-40 centrifuge tube. Care was taken so that the lower, gelatinous chromosomal DNA layer and the hard pellet would not contaminate the plasmid preparation. Ground $CsCl_2$ was added to the plasmid solution in the tube (8 gm $CsCl_2$/8.3 mls solution) and dissolved by stirring with a glass rod. An aliquot of 0.5 ml of ethidium bromide (10 mg/ml dH_2O) was then mixed into the solution. The tubes were weighed and balanced to within 20 mg with a few drops of a $CsCl_2$ solution prepared in TE. After balanced tubes were capped, the air space above the solution was filled with mineral oil. The tubes were balanced again to within 20 mg and centrifuged at 34,000 RPM in a TI-40 rotor for 40 hours at 15 C in a Beckman L5-2B ultracentrifuge with

the brake turned off.

The ethidium bromide stained DNA bands were visualized with a hand held UV light source and the lower, plasmid containing band was removed by puncturing the tube just below the band with a 20 G syringe needle using a piece of transparent tape as a gasket (94). The plasmid containing solution (1.5 ml) was extracted with an equal volume of isoamyl alcohol 8 times. The solution was then dialysed for 24 hours at 4 C against 2 l of 5 mM Tris, 0.25 mM EDTA, pH 8.0. The dialysis solution was changed one time. The dialysed solution was then extracted with phenol, phenol:chloroform, and chloroform before the plasmid DNA was precipitated with ethanol as described for the phage DNA above and resuspended in TE at a concentration of 250 ug/ml.

Restriction endonuclease digestion of phage PEa1(h) DNA - Restriction endonuclease Sau3A was used to digest the phage genome in a dilution series in order to create a series of partial genomic digests. The presence of a series of partial genomic digests was confirmed by agarose gel electrophoresis. The digestions were stopped by adding EDTA to 25 mM and extracted with phenol, phenol:chloroform, and chloroform. Genomic pieces less than 2 kbp in length were removed by centrifuging the pooled partial digests (35 ug DNA) through a linear 10-40% sucrose density gradient at 100,000g for 24 hours (94). Fractions of 0.5 ml were collected and the sizes of the genomic pieces in each frac-

tion was determined by agarose electrophoresis. Fractions containing fragments larger than 2 kbp were pooled.

5' Terminal dephosphorylation of pUC8 - Calf intestinal alkaline phosphatase was assayed immediately before it was used. The assay substrate was 1 mM p-nitrophenylphosphate in 1 M Tris/HCl, pH8.0. A 100 ul aliquot of enzyme was added to 3 ml of substrate and the A_{410} nm was read after 60 seconds against a substrate blank which received no enzyme. The assay was performed in triplicate on enzyme diluted to 0.05, 0.005, 0.0005 mg/ml. The enzyme units/mg protein were calculated as follows:

$$\text{U/mg protein} = \frac{A_{410} \times 1000}{(1.62 \times 10^4)(\text{mg protein/reaction volume})}$$

Plasmid pUC8 (5 ug) was digested to completion with restriction endonuclease BamH1, extracted with equal volumes of phenol, phenol:chloroform, chloroform and ether. The DNA was precipitated with 2 volumes of ethanol and 1/10 volume 2.5 M sodium acetate, pH 5.2 and resuspended in 10 ul of 10 mM Tris/HCl, pH 8.0.

Five ul of 10x CIP buffer (94), (0.5 M Tris/HCl, pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, and 10 mM spermidine) were added to the reaction tube. Water was added to 48 ul, and then 0.1 U CIAP was added to the reaction in a volume of 2 ul. The reaction was incubated at 37 C for 70 minutes before the enzyme was inactivated by the addition of 40 ul dH_2O, 5 ul of 10% SDS, and 10 ul of 10x STE followed by heating in a

68 C block for 15 minutes. 10x STE consisted of 100 mM Tris/HCl, pH 8.0, 1 M NaCl, and 10 mM EDTA. Enzyme inactivation was confirmed by withdrawing 2 ul from the reaction mix and adding it to p-nitrophenylphenol in the assay described above.

Ligation of pUC8 and PEa1(h) fragments - The 5' dephosphorylated pUC8 DNA was extracted with phenol, phenol:chloroform, and chloroform and then precipitated with ethanol and resuspended in TE. An aliquot (0.5 ug) was added to an aliquot of the sized PEa1(h)/Sau3A pieces (1.5 ug) and ethanol precipitated. The precipitate was resuspended in 30 ul of ligation buffer (66 mM Tris/ HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 66 uM ATP). T-4 DNA ligase was added (0.75 Units) and the reaction was incubated in a water bath with decreasing temperature (12-4 C) for 36 hours.

Transformation of bacterial strains with plasmid DNA - A $CaCl_2$ technique (93) similar to that described by Maniatis et. al. (94) was used. The E. coli strains were grown overnight at 37 C in LB then diluted 1/100 into fresh LB in SS-34 centrifuge tubes and grown until the $O.D._{600}$ reached 0.6. The cultures were then centrifuged at 5,000 RPM and the pellets resuspended in 1/2 the growth volume of ice cold 50 mM $CaCl_2$, centrifuged again and resuspended in 1/10 the original culture volume of ice cold 50 mM $CaCl_2$. These competent cells were then transformed.

The DNA to be used for transformation (2 ug) was dissolved in 100 ul of TE. A 200 ul aliquot of a solution of 10 mM Tris/HCl, pH 8.0, 10 mM $CaCl_2$, and 10 mM $MgCl_2$ was added to the DNA solution. The 300 ul DNA solution was added to an equal volume of the competent cells in a 1.5 ml microfuge tube and incubated in an ice water bath for 25 minutes. The tubes were then transferred to a 37 C water bath for 3 minutes and then to a 25 C water bath for 10 minutes. The cell suspensions were then added to 1.0 ml of LB in 13 x 100 mm culture tubes and incubated with shaking at 37 C for 45 minutes. Aliquots of 10, 25, 50, 100, and 200 ul were added to LB top agar at 45 C, mixed, then poured over LB basal medium. Top agar contained 0.7% bacto agar (w/v) and the basal agar had 1.5 % agar. Both were supplemented with ampicillin (200 ug/ml) to select for transformants. After 18 hours at 37 C, colonies which grew were transferred to replica plates of the same medium. As a control 300 ul of competent cells were diluted with 300 ul of the buffer mix containing no DNA and treated just as the true transformation mix.

Screening of E. coli transformants- Transformants were replica plated to DM-17 media amended with X-GAL and IPTG and to non-amended media. Clones which were blue in color were discarded since they contained intact pUC8.

Clones from the non-amended master plate were transferred to make a second master plate containing only

pUC8/PEa1(h) recombinants. This master was used to inoculate replica plates of the same media with and without IPTG, the lac operon inducer. The plates were incubated at 37C and 27C for 48 hours before the bacteria were killed with chloroform vapor. The replica plates were then overlayed with E. amylovora Ea110R in 3.0 ml of DM-17 containing 1% glucose (w/v) to enhance extracellular polysaccharide production.

Effect of chloroform vapor on the detection of haloes surrounding JM105 clone 94 - Ten replicate colonies of JM105 clone 94 were grown for 2 days at 27 C DM-17 amended with proline and ampicillin. Even numbered colonies were excised before the plate was inverted over chloroform for 10 minutes. The plate was then overlayed with E. amylovora Ea110R in DM-17 which contained 1 % dextrose and incubated at 27 C for 2 days.

Rapid, small scale isolation of plasmid DNA and detection of plasmid pJH94 in JM105 clone 94- "Mini-preps" of plasmid DNA were prepared as follows (94). A 5 ml culture of the strain was grown overnight at 37 C (E. coli) in 5 ml of LB which contained ampicillin. The cultures, which had been grown in SS-34 tubes, were pelleted by centrifugation at 7700g and resuspended in 0.5 ml of LB. The concentrated culture was transferred to a 1.5 ml microfuge tube and pelleted at 13,000g for 1 minute before being resuspended in 350 ul of STET buffer (8% nuclease-free sucrose, 2.5% Triton X-100, 50 mM EDTA and 50 mM Tris-HCl pH 8.0). Fresh lysozyme

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(25 ul of a 10 mg/ml solution) was added and the tube was placed in a boiling water bath for 40 seconds. The tube was then centrifuged for 10 minutes at room temperature before the top-most 200 ul of the supernatant was transferred to a clean microfuge tube which contained 200 ul of isopropanol. The DNA was precipitated for 1 hour at -80 C or at least 6 hours at -20 C. The precipitate was collected by centrifugation at 13,000g for 10 minutes at 4 C, drained, dried *in vacuo*, and resuspended in 100 ul of TE buffer. Aliquots of 10 ul were sufficient for restriction enzyme digestion followed by agarose electrophoresis. Plasmid "mini-preps" were prepared from five 10 ml replicate cultures of JM105 clone 94 which had been grown overnight in LB. A "mini-prep" was also prepared from JM105(pUC8). Aliquots from the "mini-preps" were digested with EcoR1 and electrophoresed with lambda/HindIII size standards in 0.9% agarose (94).

Preparation of extracellular polysaccharide produced in vitro and in vitro assay for polysaccharide depolymerase - Extracellular polysaccharide from Ea110R was prepared by washing cells grown for 4 days on DM-17 amended with 2% glucose with phosphate buffer containing 0.15 M NaCl and 1 mM Mg SO4 (71). After centrifugation to remove bacteria, 3 volumes of 95% ethanol were added to the supernatant to precipitate extracellular polysaccharides. The polysaccharides were quantified by the phenol/sulfuric acid method (48,65), and used as substrate for an *in vitro* assay for

polysaccharide depolymerase (137). EPS at a concentration of 1.5 mg/ml was incubated at 42 C in 100mM Acetate buffer pH 5.0 with a polysaccharide depolymerase containing aliquot. An aliquot was removed and assayed for increased reducing sugars using a galactose standard with the bicinchoninate assay (98).

Preparation of EPS from immature pear fruits infected with E. amylovora strain Ea110R - Immature pear fruits approximately 2.5 cm in diameter were surface disinfested with 0.05 % sodium hypochlorite for ten minutes, rinsed with distilled water, sliced, and placed in sterile enamel pans lined with wet paper towels. A 10 ul drop of a culture of Ea110R grown in nutrient broth (Difco) supplemented with .05 % glucose was placed on each slice and the fruits were incubated at 27 C for 4 days. The bacteria and slime polysaccharides were obtained by washing the fruit with 20 mM potassium phosphate buffer, pH 6.8 supplemented with 150 mM NaCl. The bacteria were removed by two cycles of 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, redissolved in a minimal volume of distilled water, and quantified with the phenol/sulfuric acid method (60).

Preparation of soluble polysaccharide depolymerase from phage lysates of Ea110R and from HB101(pJH94) - Soluble polysaccharide depolymerase was obtained from 16 overlays of

Ea110R which had been confluently lysed by phage PEa1(h). These lawns were prepared and the lysates processed as described above for the isolation of bacteriophage PEa1(h). After bacterial debris, agar and bacteriophage particles were removed from the suspension by centrifugation, the supernatant was dialysed against 50 volumes of 20 mM Tris/HCl, pH 8.0 at 4 C. The concentration of protein in this "crude polysaccharide depolymerase" was determined by the method of Lowry et. al. (43,90).

Plasmid pJH94 was introduced into E. coli strain HB101 (29) by calcium chloride transformation with "rapid mini-prep" DNA prepared from JM105(pJH94). A single colony of E. coli HB101 was used to inoculate 250 ml of LB medium which contained ampicillin. After 24 hours growth at 27 C, the bacteria were pelleted by centrifugation at 10,000 g for 20 minutes and resuspended in 125 ml of TS (50 mM Tris/HCL, pH 8.0, 50 mM NaCl). Sarkosyl was added to 0.1 % (w/v) and the suspensions were vortex mixed for 30 seconds (114). The bacteria were again pelleted by centrifugation. The pellet was resuspended in 16 ml of TES (TS + 10 mM EDTA) and then 14 ml of sucrose mix (1.6 M sucrose, 0.55 M Tris/HCl pH 8.0, 0.1 M EDTA) was added to the mixture which was incubated at 5 C for 20 minutes before 6.0 ml of lysozyme (2.5 mg/ml, in 50 mM Tris/HCl, pH 8.0) was added. After incubation at 5 C for 20 minutes, 100 ml of 2.5 % sarkosyl was added and the lysate was stirred on ice for 1 hour at 100

RPM.

An aliquot from this lysate was set aside and ammonium sulfate was added to the remaining lysate to 30 % saturation with stirring on ice. The precipitate was collected by centrifugation (12,000 g for 30 minutes) and resuspended in 20 mM Tris/HCl, pH 8.0. Ammonium sulfate was added to the 30 % supernatant to 80 % saturation and the precipitate was collected and dissolved in Tris buffer. The crude lysate, and the 30 % and 80 % ammonium sulfate supernatants as well as the 80 % precipitate were dialysed against 20 mM Tris/HCl for 2 days at 4 C before being assayed for polysaccharide depolymerase.

Effect of pH on the activity of soluble polysaccharide depolymerases - Serial dilutions of enzyme containing solutions (12.5 ul) were added to reaction mixes wich contained 200 mM buffer (50 ul) and E. amylovora EPS obtained from infected pear fruits (25 ul of 12 mg carbohydrate/ml) and distilled water (12.5 ul). The 100 ul reaction mixes were incubated at 42 C for 3-5 hours before being diluted to 1.0 ml with distilled water. The concentration of reducing sugars present in each reaction was then determined with the bicinchoninnate assay (98) using a galactose standard. Reaction mixes which 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. All assays were performed in triplicate.

Purification of pJH94 - HB101(pJH94) was grown in 500 ml of DM-17 containing ampicillin, and amplified as was pUC8 above. After centrifugation at 10,000g for 10 minutes the pellet was resuspended in 10 ml of a solution containing 25 mM Tris/HCl pH 8.0, 50 mM glucose, 10 mM EDTA, and freshly dissolved lysozyme (5 mg/ml) (94). The suspension was transferred to a Beckman SW-27 polycarbonate centrifuge tube and allowed to stand at 25 C for 5 minutes before the addition of 20 ml of a freshly made solution of 0.2 N NaOH and 1% SDS (w/v). The contents of the tube were mixed by gentle inversion and placed in an ice water bath for 10 minutes before the addition of 15 ml of an ice cold solution of 5M potassium acetate, pH 4.8, prepared as described (94). The contents of the tube were mixed by sharp inversion and returned to the ice water bath for 10 minutes. The tube was then centrifuged at 72,000g for 20 minutes and the supernatant decanted into two, 30 ml Corex tubes before 0.6 volumes of isopropanol were added to each tube. The contents of the tubes were mixed by inversion and allowed to stand at room temperature before being centrifuged at 12,000g for 30 minutes at room temperature in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet was washed with 70% ethanol at room temperature before being dried briefly in a vacuum dessicator at room temperature.

The pellet was dissolved in a volume of 8.3 ml TE, purified through $CsCl_2$/ethidium bromide density gradients and further processed as described above for pUC8. After the final ethanol precipitation, pJH94 was resuspended in TE at a concentration of 660 ug/ml.

Extraction of total DNA from E. amylovora Ea110R - Two 10 ml cultures of Ea110R were grown overnight at 31 C, pooled, then centrifuged at 3000g for 5 minutes in a Sorvall SS-34 rotor. The pellet was resuspended in 5 ml of 50 mM Tris/HCl, pH 8.0, containing 50 mM EDTA before lysozyme was added to a final concentration of 1 mg/ml (45). The suspension was incubated in an ice bath for 30 minutes before 1 ml of STEP (0.5% SDS (w/v), 50 mM Tris/HCl pH 7.5, 400mM EDTA, and proteinase K (Sigma) (1 mg/ml), added immediately before use) was added to the suspension. The lysate was incubated at 50 C for 15 minutes with gentle mixing by inversion before an equal volume of Tris/HCl buffered phenol (pH 7.8) was added and mixed for 5 minutes. After a 5 minute centrifugation in a clinical centrifuge the upper (aqueous) phase was transferred to a clean teflon capped glass tube and 0.6 ml of 3 M sodium acetate, pH 5.2 was mixed into the lysate. Two volumes of 95% ethanol were added to the tube which was mixed by inversion. The precipitate was spooled out of the mixture with a glass micropipette and transferred to a clean tube containing 5 ml of 50 mM Tris/HCl pH 7.5, 1 mM EDTA, and 50 ugRNAse/ml. The precipitate was

dissolved by gentle inversion for 30 minutes and then incubated at 37 C with occasional inversion for 30 minutes. Five ml of chloroform was added, mixed to an emulsion, and the tube centrifuged for 5 minutes as before. The upper layer was transferred to a clean tube to which 2 volumes of 95% ethanol were added and mixed. The DNA precipitate was spooled out of the tube and dissolved in 6 ml of 50 mM Tris/HCl pH 7.5 containing 1 mM EDTA. This procedure (45) yielded 660 ug of DNA with an $A_{260/280}$ of 1.6 which was stored at 4 C.

Southern Blotting - A 0.9% agarose (w/v) gel was cast in a 200 X 145 mm electrophoresis apparatus using a 15 well comb. Samples were loaded in duplicate at opposite sides of the gel. Samples included 70 ng of pUC8 which had been digested with EcoR1, 70 ng of pJH94 digested by EcoR1, 2.25 ug of lambda DNA digested with HindIII (BRL), 940 ng of PEA1(h) DNA digested with BglII, and 3.6 ug of Ea110R DNA digested by EcoR1. Electrophoresis was for 20 hours at 20 mA and 14 V in E buffer (77). DNA bands were visualized after staining with ethidium bromide.

The gel was cut to separate the two replica gels, and the unused portions of the gel were removed. The gels were transferred to a glass baking dish and soaked for 10 minutes in 0.25% HCl. The gels were drained and then soaked in 3 volumes of a solution consisting of 1.5 M NaCl and 0.5 M NaOH. The solution was changed 4 times in the course of 1

hour. The gels were then soaked in 3 volumes of 1 M Tris/HCl and 1.5 M NaCl for 1 hour. The solutions were changed 4 times (84,109).

A glass plate was wrapped in a sheet of Whatman 3MM filter paper and placed over a glass baking dish which contained 10X SSC which had been diluted from a 20X stock. 20X SSC (pH7.0) contained 175.3 g of NaCl and 88.2 g of sodium citrate per liter of dH_2O. A second piece of 3MM paper was cut longer than the glass plate and its ends were placed in the 10X SSC to act as a wick. The gels were inverted on the wet filter papers and air bubbles were removed. Nitrocellulose filters (Schleicher & Schuell BA 85) were cut 1 mm longer in each dimension than the gels. They were wet by soaking in 2X SSC for 3 minutes then put on top of the gels. Two pieces of 3 MM paper cut to the exact dimensions of the gel were soaked in 2X SSC and put on each filter. Paper towels cut 1 mm smaller in each dimension than the gels were put on top of the filter paper and stacked 8 cm high. A glass plate was put on top of the stacks and a 1 liter flask of water was put on top of the plate to apply pressure to the gels. Transfer was allowed to proceed for 18 hours (94,120).

After transfer had been completed the positions of the gel slots were marked on the nitrocellulose filter with a ball point pen and the filter was then soaked for 5 minutes in 6X SSC. The filter was then air dried on a sheet of 3MM

paper before being dried for 2 hours in a vacuum oven at 80 C.

Alpha ^{32}P-Labeling of DNA by Nick Translation - Purified plasmid pUC8 and pJH94 DNA were labled with ^{32}P by nick translation using E. coli DNA polymerase I as follows.

A buffer consisting of 50 mM Tris/HCl, pH7.5, 10 mM $MgSO_4$, 1mM dithiothreitol and 50 % glycerol (v/v) was prepared. DNAse (Sigma D-4763) was added to a final concentration of 1 mg/ml, dissolved, and stored at -20 C. Prior to use the DNAse solution was diluted 1/200 two times with a buffer consisting of 50 mM Tris/HCl, pH 7.5, 10 mM $MgSO_4$, 1 mM dithiothreitol and 50 ug BSA (Fraction V, Sigma)/ml.

dNTP's were obtained from PL Biochemicals, Inc. 2'-Deoxyadenosine 5'-triphosphate, disodium (dATP), 2'-deoxycytidine 5'-triphosphate, sodium (dCTP), 2'-deoxyguanosine 5'triphosphate, sodium, (dGTP), and 2'-deoxythymidine 5'triphosphate, sodium (dTTP) were separately dissolved in and then diluted with sterile water to final concentrations of 500 uM. A 2x dNTP mix was prepared which consisted of 100 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 50 uM dATP, 50 uM dGTP, 50 uM dTTP, and 10 uM dCTP.

For each reaction, 100 uCi of ^{32}P-dCTP (Amersham) was placed in sterile 1.5 ml microfuge tubes containing 25 ul of the 2x dNTP reaction mix and 0.5 ug purified plasmid DNA in a total volume of 48 ul. The reaction tubes were incubated for 15 minutes in an ice bath and then 1 ul of the diluted

DNAse solution and 1.2 ul of E. coli DNA polymerase I (BRL , Inc) were added to each tube. The reaction mixes were then incubated for 4.5 hours at 14 C before being stopped by heating for 10 minutes at 65 C.

An aliquot of 1 ul was removed from each nick translation reaction mix and added to 9 ul dH_2O for determination of the percent ^{32}P incorporation into DNA. Aliquots of 2.5 ul from each 1/10 dilution of the reaction mixes were spotted onto duplicate 2.4 cm diameter Whatman DE-81 filters which had been moistened with 20 ul of d H_2O. One filter of each pair was washed 5 times with 5% Na_2PO_4 (w/v). Each wash consisted of a a 5 ml volume and lasted for 5 minutes. The other filter of each pair was not washed. Each filter was then placed in a scintillation vial to which 10 ml of dH_2O was added and the radioactivity contained on each disc was then quantified with a scintillation counter. The percent ^{32}P incorporation was then determined by dividing the counts per minute retained by the washed filters (incorporated) by the cpm found on the unwashed filters (total radioactivity)

^{32}P-Labled plasmid DNA was separated from unincorporated ^{32}P-dCTP by chromatography of the reaction mixes on Bio-Gel P-60 columns. Bio-Gel P60 was prepared by boiling in a buffer consisting of 10 mM Tris/HCl, pH 7.5, 1 mM EDTA. After the gel was allowed to settle the fines were decanted and the gel was resuspended in a volume of buffer equal to the volume of the swelled gel. The buffer consisted of 10 mM

Tris/HCl, pH 7.5, 1 mM EDTA, and 0.4% SDS(w/v). The resuspended gel was poured into a sterile disposable 5 3/4" Pasteur pipette which contained a plug of sterile glass wool. The reaction mixes were loaded on top of the columns which were then washed with a buffer consisting of 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.2% SDS (w/v). Nine fractions of 200 ul each were collected and monitored for radioactivity with a hand-held Geiger counter. The four tubes which comprised the first (incorporated) peak were pooled, and the radioactivity contained in the peak was quantified by scintillation counting.

Hybridization of Southern blots- The baked nitrocellulose filters were soaked for 2 minutes in 6X SSC and then placed in heat sealable bags (Sears' Seal-n-Save). Prehybridization solution at 68 C was added to the bags at the rate of 0.2 ml/cm^2 of filter. The prehybridization solution consisted of 6X SSC, 0.5% SDS, 5X Denhardt's solution, and 100 ug/ml denatured salmon sperm DNA. The Denhardt's solution was prepared as a 50X stock consisting of 5 gm Ficoll, 5 gm polyvinylpyrrolidone, and 5 gm BSA (Fraction V) in 500 ml dH_2O. The salmon sperm DNA (Sigma Type III, sodium salt) was prepared as a 1 mg/ml stock, denatured by boiling for 10 minutes and then placed in an ice bath for 15 minutes immediately before use (94).

The filters were sealed in their bags and incubated submerged for 2 hours at 68 C. Care was taken to remove air

bubbles. The prehybridization solution was removed by cutting a corner of the bags and hybridization solution was put in the bags at the rate of 0.05 ml/ cm^2 of filter. Hybridization solution was the same as the prehybridization solution except that it also contained 10mM EDTA. Air bubbles were removed from the bags and then $4x10^6$ CPM of ^{32}P-pUC8 or ^{32}P-pJH94 were added to the bags which were resealed and incubated at 68 C overnight (94).

The filters were removed from the bags and separately submerged for 5 minutes at room temperature in 2x SSC which contained 0.5% (w/v) SDS. The filters were then submerged in a solution of 2x SSC which contained 0.1% (w/v) SDS for 15 minutes at room temperature. The filters were then transferred to flat bottomed plastic boxes which contained 0.1 x SSC and 0.5% SDS (w/v) and incubated at 68 C for 2 1/2 hours. The buffer was changed once. The filters were then removed from the buffer and air dried on a sheet of 3MM paper before autoradiographs were prepared (94).

Autoradiography of nitrocellulose filters - The air dried, nitrocellulose filters were taped at one end to a glass plate. The filter and plate were then wrapped in Saran wrap and placed in an X-Ray film casette. In the darkroom, a piece of Kodak X-Omat AR film was placed on top of the filters and a Dupont Cronex Lightning-Plus intensifying screen was placed on top of the film. The casette was closed, wrapped in foil, and an alligator clamp was attached

to each edge of the casette. Different pieces of film were exposed in the same casette for 15, 45 and 180 minutes at -80 C. The photographic images were developed by soaking the film in Kodak liquid X-Ray developer for 3 minutes, in Kodak rapid fixer for 3 minutes, and in dH_2O for 30 minutes, before the film was air dried at room temperature.

RESULTS

Restriction endonuclease digestion of PEa1(h)- Aliquots containing 1 ug of PEa1(h) DNA partially digested with different amounts of Sau3A were electrophoresed through 0.9% agarose and stained with ethidium bromide (Figure 1). As expected, there was an inverse relationship between the amount of enzyme used and the average size of the fragments produced.

After centrifugation over an alkaline sucrose density gradient and fractionation, aliquots of the fractions were removed and electrophoresed through 0.8% agarose. Fractions which contained only fragments larger than 2 kb were pooled. An aliquot was removed and electrophoresed against a size standard (Figure 2), to confirm that only fragments larger than 2 kb had been retained.

Screening of E. coli transformants - After 48 hours at 27 C, clone 94 was surrounded by a very large translucent halo which appeared similar to the haloes surrounding plaques of PEA1(h) except that it was much larger. The halo size was not effected by IPTG, but was much larger when the recombinant clones had been grown at 27 C than when they were grown at 37 C (Figure 3 A,B). Since 27 C is the optimal

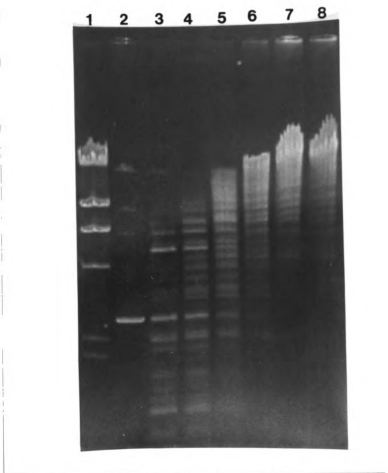

Figure 1. PEa1(h) DNA digested by restriction endonuclease Sau3A. Lane 1 - lambda/HindIII size standards, Lane 2 - plasmid pUC8/BamH1,Lanes 3-8 - PEa1(h)/Sau3A at 1U/ug,0.5U/ug,0.25U/ug,0.125U/ug, 0.063U/ug and 0.031U/ug DNA. Reactions were incubated for 3 hrs, at 37 C, and 1 ug DNA per lane was electrophoresed in 0.8% agarose and stained with ethidium bromide.

Figure 2. PEa1(h)/Sau3A fragments which were larger than 2kb, pooled after fractionation over an alkaline sucrose density gradient. Lane 1 - PEa1(h)/Sau3A fragments, Lane 2- lambda/HindIII size standards. Fragments were electrophoresed through 0.8% agarose and stained with ethidium bromide. The arrow indicates a 2 kb fragment.

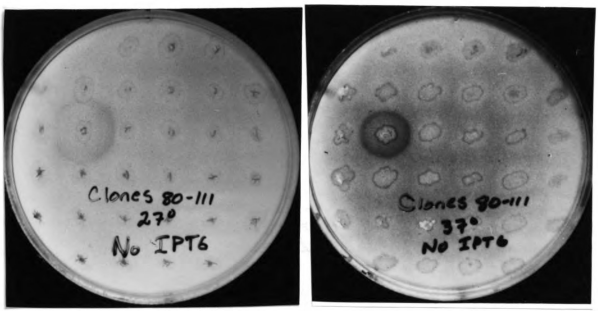

Figure 3. Screening of recombinants for polysaccharide depolymerase activity. JM105 clones containing pUC8 derivitives with PEa1(h) DNA inserts were grown 2 days at 27 C (A) and 37 C (B) prior to being exposed to chloroform vapor and overlayed with E. amylovora Ea110R. No IPTG was included in this medium. Note the large halo which surrounds one clone.

growth temperature for both E.amylovora and PEA1(h), these observations suggested that clone 94 contained the intact polysaccharide depolymerase gene as well as its promoter.

Effect of chloroform vapor on the detection of haloes surrounding JM105 clone 94 - Excision of colonies of JM105 clone 94 prior to exposing the plate to chloroform vapor did not prevent the appearance of haloes surrounding the site of the bacterial colony (Figure 4), demonstrating that membrane damage caused by chloroform vapor was not responsible for the liberation of PD from the colonies and the appearance of haloes. This suggested that PD was being either excreted into the growth medium or was appearing there as the result of lysis of a portion of the cells which contained pJH94.

Detection and size estimation of plasmid pJH94 in JM105 clone 94 - All five replicate cultures of JM105 clone 94 contained a new plasmid, designated pJH94, which was approximately twice the size of the vector pUC8 (Figure 5).

Purified pJH94 was completely digested with restriction endonucleases Sal1 and Eco R1 and sized by agarose electrophoresis against standards (Figure 6). pJH94 had an apparent size of 5850 base pairs. The phage DNA insert is therefore 3150 base pairs and has a single EcoR1 site.

Detection of polysaccharide depolymerase activity in culture supernatants - Culture supernatants from JM105(pJH94) and JM105(pUC8) were spotted in dilution series on mature lawns of Ea110R grown on DM-17 + 1% glucose.

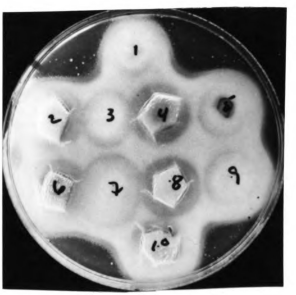

Figure 4. Effect of chloroform vapor on the appearance of haloes around JM105(pJH94). Ten replicate colonies were grown for 2 days at 27 C. Even numbered colonies were excised before plate was exposed to chloroform vapor. Plate was overlayed with E. amylovora Ea110R and incubated for 2 days at 27 C.

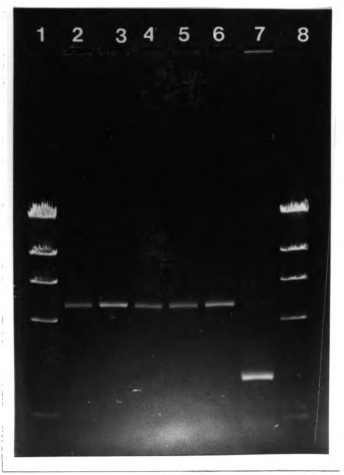

Figure 5. Plasmid pJH94 detected in JM105 clone 94. The lanes contained: "Mini-Prep" DNA from 5 replicate cultures digested with EcoR1 (Lanes 2-6), pUC8 digested with EcoR1 (Lane 7), Lambda/HindIII size standards (Lanes 1,8).

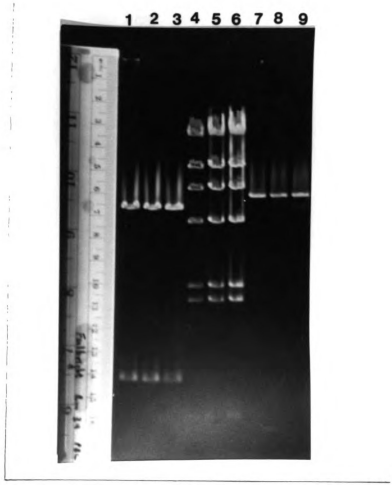

Figure 6. Estimation of the size of pJH94. Purified pJH94 was digested with EcoR1 (Lanes 1-3), and Sal 1 (Lanes 7-9). Lambda DNA digested with HindIII as size standards (Lanes 4-6). DNA was electrophoresed through 0.8 % agarose and stained with ethidium bromide.

Culture supernatants from JM105(pJH94) made distinct craters where 10 ul droplets were applied. Such craters are typical of PD containing fluids and have been used as an assay for studying PD (4,53,74). No activity was found in the supernatants from JM105(pUC8).

Preparation of polysaccharide depolymerase from PEa1(h) lysates of Ea110R and from E. coli HB101(pJH94) - The 400 ml of nutrient medium used to prepare 16 lawns of E. amylovora confluently lysed by PEa1(h) produced 42.6 units of polysaccharide depolymerase, which was in two forms (Table 2). About two thirds of the activity was in the soluble form with a specific activity of about 0.63 units/ ug protein. The remaining activity pelletted with the bacteriophage and was presumably bound to the phage particles, but possibly could have included enzyme bound to intact phage tail plates (16,122).

The 250 ml of nutrient medium used to grow E. coli HB101(pJH94) produced 1560 units of soluble polysaccharide depolymerase which were assayed after the cells were lysed and the protein precipitated with ammonium sulfate (Table 2). When the data were expressed as units of polysaccharide depolymerase produced per liter of growth medium, HB101(pJH94) produced 75 times as much soluble polysaccharide depolymerase as did Ea110R infected by phage PEa1(h) (Table 2).

Effect of pH on the activity of soluble polysaccharide

Table 2. Polysaccharide depolymerase activity[1] recovered from phage PEa1(h) lysates of Ea110R and from Sarkosyl lysates (112) of HB101(pJH94).

Source	Units/liter[2]	Specific Activity[3]
PEa1(h) lysate of Ea110R		
Phage Pellet	36.5	n.d.[4]
Soluble	81.5	0.63
Total	118.0	
Sarkosyl lysate of HB101(pJH94)		
80% $(NH_4)_2SO_4$ Pellet	6240	12.5

1. uMole galactose equivalents/hr, in a reaction containing 25 ul EPS (12 mg/ml), 12.5 ul distilled H_2O, 50 ul of acetate buffer, 200 mM, pH 5.0, and 12.5 ul of a dilution series of polysaccharide depolymerase containing solution. The reaction was incubated at 42 C for 3 hours {HB101(pJH94)} and 5 hours {Ea110R/PEa1(h)}.

2. Units/liter of growth medium used to prepare the lysates.

3. Units/mg protein (43,90)

4. n.d. = not determined

depolymerases - No differences were observed in the effect of pH on the activity of polysaccharide depolymerase isolated from phage PEa1(h) lysates of Ea110R and from sarkosyl lysates of HB101(pJH94) (Figures 7 and 8). 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.

Hybridization of ^{32}-P labled pUC8 and pJH94 to Ea110R and PEa1(h) genomes - The BglII digestion of PEa1(h) (Figure 9;lane 4) appeared complete and the bands were well separated, although some band smearing was observed. Summation of the sizes of the PEa1(h) fragments after BglII digestion gave a genomic size estimate of 47 kbp. A continuous size distribution of Ea110R genomic fragments was produced by EcoR1 (Figure 9;lane 6).

When ^{32}-P labled pUC8 was used as the probe for a blot of this gel (Figure 10) it hybridized with itself, pJH94, and with a region of the Ea110R/EcoR1 genomic digest. Hybridization with either PEa1(h) or lambda genomes were not detected even after prolonged exposure of the autoradiogram.

When ^{32}-P labled pJH94 was used as the probe for a blot of this gel (Figure 10), it hybridized with itself, pUC8, and the same region of the Ea110R/EcoR1 digest as pUC8. In contrast to pUC8, pJH94 strongly hybridized to a region of the PEa1(h)/BglII genomic digest. No hybridization with lamda DNA was detected even after prolonged exposure of the

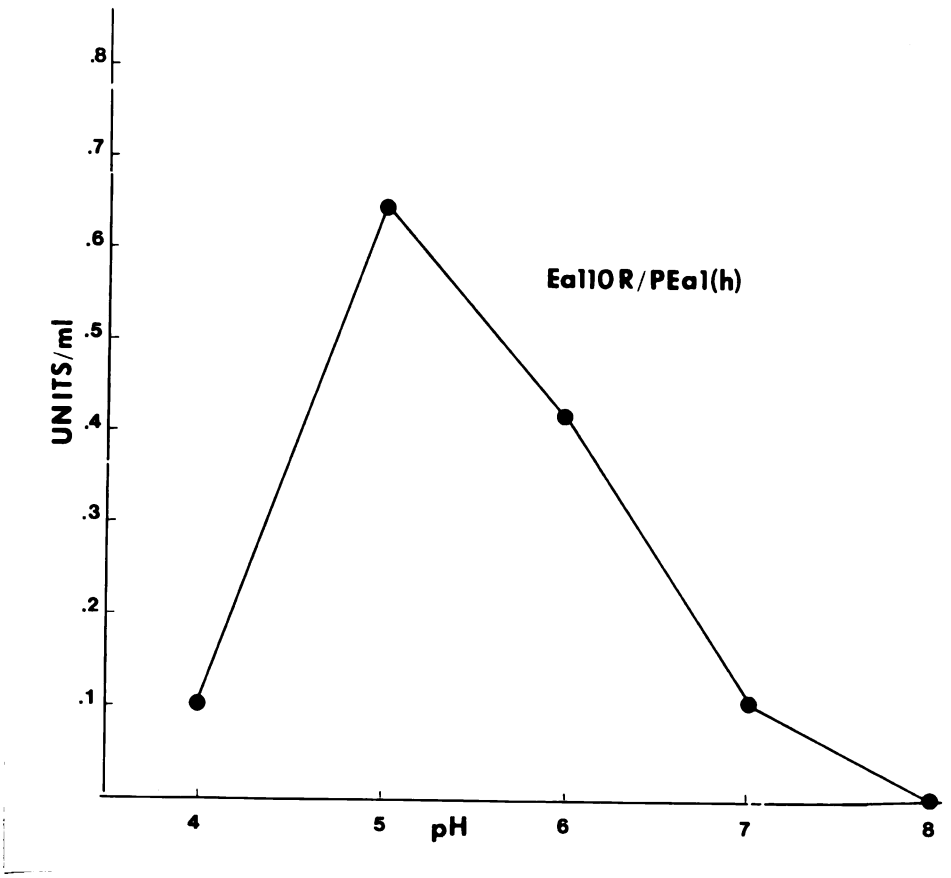

Figure 7. The effect of pH on the activity of soluble polysaccharide depolymerase isolated from PEa1(h) lysates of E. amylovora Ea110R. A unit of activity produced one uMole of galactose equivalents per hour.

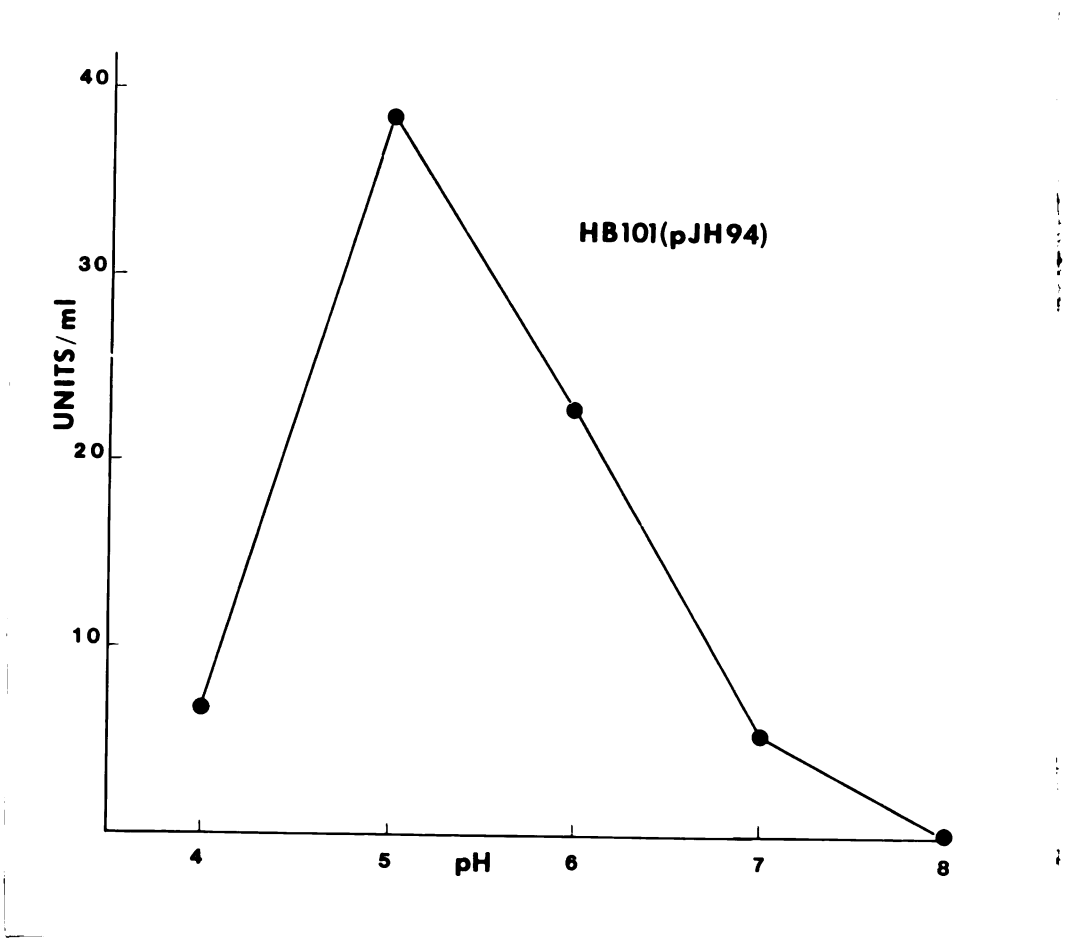

Figure 8. The effect of pH on the activity of polysaccharide depolymerase isolated from sarkosyl lysates of E. coli HB101(pJH94). A unit produced one uMole of reducing equivalents per hour.

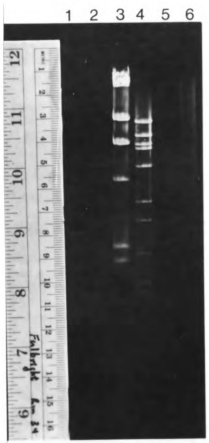

Figure 9. Agarose electrophoresis gel stained with ethidium bromide prior to Southern blotting. The lanes contained pUC8/EcoR1,0.13 ug (Lane 1),pJH94/EcoR1, 0.13 ug (Lane 2), lambda/hindIII standards (Lane 3), PEa1(h)/BglII (Lane 4), Ea110R genome/EcoR1 (Lane 6).

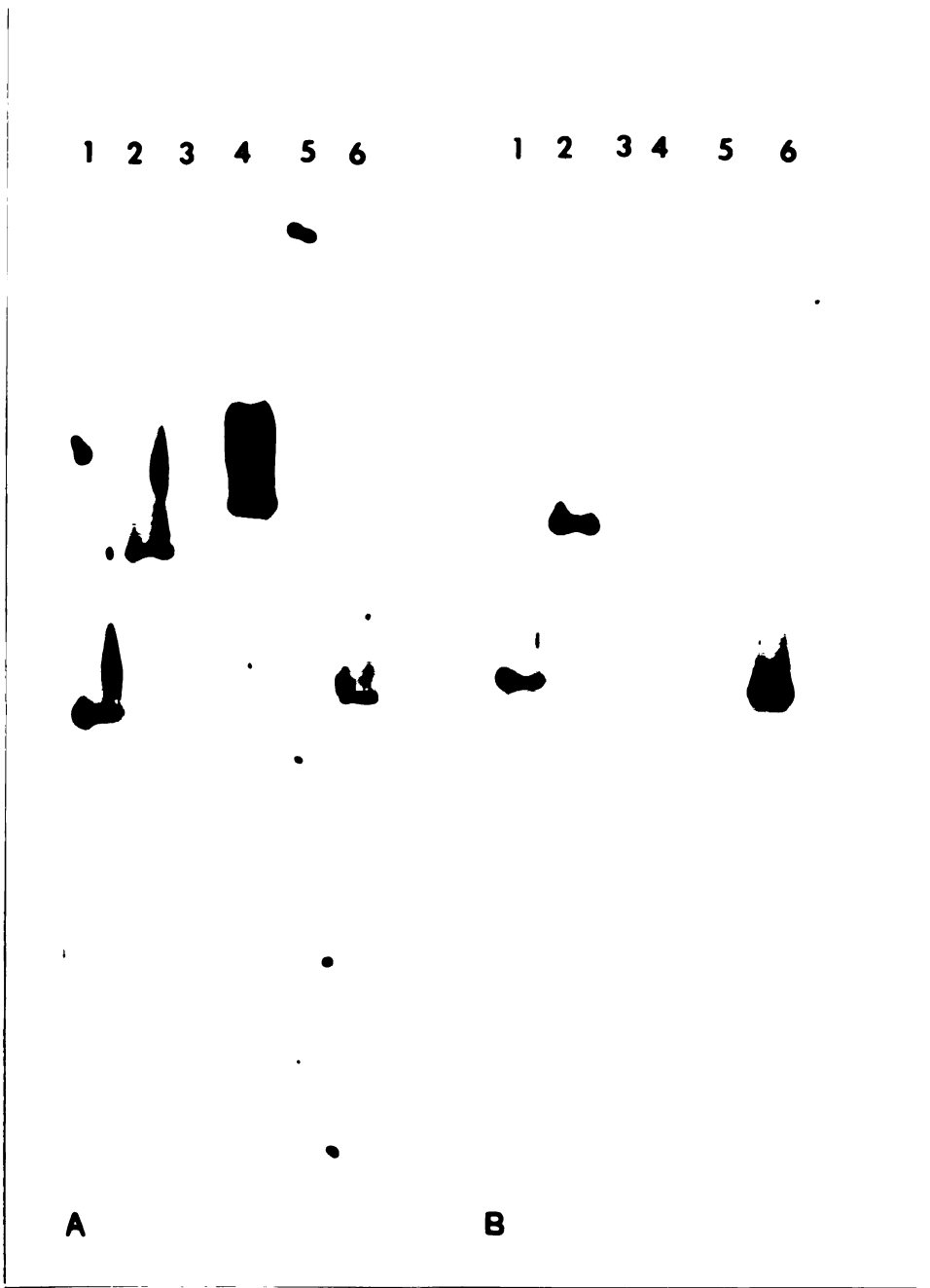

Figure 10. Autoradiogram of Southern blot which confirms that polysaccharide depolymerase is phage encoded. ^{32}P-labled pJH94 was the probe (A), and ^{32}P-labled pUC8 was the probe (B). Lane 1, pUC8, Lane 2,pJH94, Lane 3, Lambda/HindII, Lane 4, PEa1(h)/Bg1II, Lane 5, blank, Lane 6, Ea110R genome/EcoR1. Autoradiogram exposed for 15 minutes at -80 C.

autoradiogram. The results demonstrate that the 3150 BP insert in pJH94 encoding PD activity is of phage, not bacterial origin.

Discussion

E. coli JM105 was transformed with a recombinant DNA library of phage PEa1(h) in plasmid pUC8, replica plated and grown 48 hours on a medium containing X-Gal and IPTG. After the recombinant clones were killed with chloroform vapor and overlayed with a lawn of *E. amylovora*, clone number 94 was surrounded by a translucent halo when viewed with transmitted light. The region of the halo appeared as a shallow crater when viewed with reflected light and continued to expand after growth of the lawn was complete. The bacteria within the halo were fully viable. These characteristics are identical with those of the haloes produced by PEa1(h) infection of *E. amylovora* (107,108) and of haloes produced by phage which infect other encapsulated bacteria (4,74).

A dilution series of the culture filtrate of clone 94 spotted on mature lawns of *E. amylovora* resulted in the appearance of craters, just like those used to assay for polysaccharide depolymerases in other systems (4,53,74) and identical to those produced by spotting dilutions of PEa1(h) lysates on *E. amylovora* (107). When culture filtrates and cell lysates of JM105 clone 94 were incubated with purified EPS from Ea110R, the concentration of reducing sugars in-

creased with time. This was similar to the results obtained when PEa1(h)/Ea110R lysates were incubated with the same EPS. No craters or haloes were observed when culture filtrates from JM105 or JM105(pUC8) were spotted on mature lawns of Ea110R, nor were reducing sugars increased when they were incubated with EPS. When the effects of pH on the relative activities of the soluble polysaccharide depolymerases found in phage PEa1(h) lysates of E. amylovora Ea110R and in sarkosyl lysates of E. coli HB101(pJH94) were compared, no differences were observed; the curves which related pH to enzymatic activity were identical (Figures 7 and 8). The biological and biochemical data together demonstrated 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 which caused haloes to appear around plaques of PEa1(h) on Ea110R.

A plasmid of about 5.8 kb was found in the polysaccharide depolymerase producing strains and designated pJH94. Plasmids pJH94 and pUC8 were ^{32}P-labled and used as hybridization probes to determine if the insert DNA was of phage or bacterial origin. Southern blots clearly showed that pJH94, but not pUC8, hybridized to the PEa1(h) genome (Figure 10). Both plasmids hybridized to the same low molecular weight region of the E. amylovora genomic digest, indicating a

region of homology between the vector, pUC8, and a region of the E. amylovora genome. The results demonstrate that the insert DNA carried by pJH94 is of phage, not bacterial, origin. The hybridization of pJH94 to several bands in the PEa1(h) genomic digest lane is unexplained. One explanation is that the multiple band hybridization pattern is due to incomplete digestion of the PEa1(h) genome by BglII. However this seems unlikely since the digestion was carried out with excess enzyme for a prolonged period of time. Furthermore, the ethidium bromide stained gel (Figure 9) showed no sign of any bands resulting from partial digestion which can be detected by apparent non-stoichiometric binding of ethidium bromide by the fragments.

The size of the phage DNA insert in pJH94 was determined to be 3.15 kb by restriction endonuclease digestion followed by agarose gel electrophoresis with standards of known size. The molecular masses of similar well characterized polysaccharide depolymerase enzymes (10,137) allowed a rough approximtion that about 2 kb of DNA would be required to encode the entire enzyme. Production of polysaccharide depolymerase by JM105(pJH94) did not require IPTG in the growth medium, as it would have if transcription of the gene was under lac z operator control. It is apparent that the transcriptional promoter of the polysaccharide depolymerase gene was cloned as well as the polysaccharide depolymerase gene itself. This conclusion is reinforced by the observa-

tion that polysaccharide depolymerase production was enhanced when E. coli JM105(pJH94) was grown at 27 C as compared to 37 C (Figure 3), since 27 C is the optimum temperature for PEa1(h) multiplication in E. amylovora (107).

It has been proposed (111,127) that soluble depolymerases found in phage lysates are due to overproduction of phage base plate spikes which contain the activity. Ritchie (107) observed that non-halo making mutant phage were always found when PEa1(h) was grown on Ea110R in broth. Phage PEa1(nh) may have polysaccharide depolymerase activity associated with its particles as was observed on a non-halo making Klebsiella phage (127). The cloning of the polysaccharide depolymerase gene with an intact transcriptional promoter may provide insight on of the regulation of this gene.

Whenever JM105(pJH94) was grown on solid or in liquid medium, polysaccharide depolymerase activity was found in the growth medium. It is unclear whether the enzyme is excreted by the bacteria into the medium or if a portion of the cells lyse and thereby release enzyme into the medium. Proteins naturally excreted from bacterial cells typically have a very hydrophobic NH_2-terminal sequence which associates with the cell membrane during translation (17). It is not known if this is the case with this protein. It is clear that the destruction of membranes with chloroform is not required for the enzyme to appear in the growth medium

(Figure 4).

It was not possible to amplify and purify pJH94 in E. coli strain JM105. Uncontrolled transcription of plasmid encoded genes has led to both plasmid instability and cell lysis in other systems (32,123). Uncontrolled transcription from the phage polysaccharide depolymerase gene promoter could explain both the plasmid instability observed in strain JM105(pJH94) and the appearance of polysaccharide depolymerase in the growth medium.

The molecular cloning of the polysaccharide depolymerase gene of PEa1(h) and its expression by strains of E. coli provides a direct demonstration that the gene is phage encoded, and not a bacterial gene which is induced by phage infection as may be the case in other systems (67,71). The appearance of polysaccharide depolymerase in very high concentration in cell lysates of HB101(pJH94) could be a great aid in the purification of this enzyme. The same cloning strategy should be applicable in other phage/bacterial systems which have been demonstrated to produce polysaccharide depolymerases (22,23,71,100,106,127).The presence of a phage promoter which controls transcription of this gene, may make possible its use to study the role of extracellular polysaccharides in the pathogenesis of Erwinia amylovora (Chapter 2).

CHAPTER II

EXPRESSION OF THE CLONED POLYSACCHARIDE DEPOLYMERASE GENE OF BACTERIOPHAGE PEa1(h) IN *ERWINIA AMYLOVORA*

Introduction

The roles of extracellular and capsular polysaccharides of phytopathogenic bacteria in disease development have been studied using two basic approaches. The first approach has been to compare disease development incited by an acapsular mutant strain to that incited by a fully encapsulated parent strain in a susceptible host. This approach has been pursued vigorously with E. amylovora (7,19,20,25, 125), E. stewartii (30) and P. solanacearum (115,116). A concensus has developed that the primary role of the EPS in these systems is to prevent agglutination of the bacteria by the susceptible host (30,109,116,125). Mutants which lack EPS seem to be rapidly agglutinated by either lectins (30,116), or by basic proteins (83,84,109) which bind to a moiety in the bacterial LPS. The interpretation of electron micrographs which purport to demonstrate this agglutination in vivo has been vigorously challenged (69).

A different approach has been to study the fate of EPS from several phytopathogenic bacterial species in leaf mesophyll of plant species susceptible and resistant to the several pathogens (54). The EPS induced persistant water soaking only in plants susceptible to the bacteria from which it had been obtained (54). The experiments were exten-

ded to compare the fate of EPS from P. syringae pv. phaseolicola in bean cultivars susceptible and resistant to several strains of the pathogen. These workers concluded that the role of EPS in vivo was to maintain a hydrated gel in the leaf mesophyll (55,56). The hydrated gel presumably enhanced bacterial growth (69,76). The workers further concluded that resistance of bean leaves to this persistant water soaking was due to enzymatic degradation of the EPS in an incompatible, but not in a compatible interaction (56).

Still another approach was used by Avery and Dubos in a study of the role of EPS in pathogenicity of Pneumococcus in mice (6,49). These workers enzymatically removed the extracellular polysaccharides of a virulent strain of Pneumococcus prior to injecting it, with enzyme, into the mouse peritoneum. The enzymatically decapsulated bacteria were rendered avirulent, presumably because they were more susceptible to phagocytosis (6). The EPS interfered with the recognition of the bacteria by the phagocytes, a model similar to that proposed for E. amylovora, E. stewartii and .P. solanacearum EPS today.

The cloning of the polysaccharide depolymerase gene of phage PEa1(h) reported in chapter 1 of this work made possible a fourth approach to the study of EPS and CPS in disease development. The intact polysaccharide depolymerase gene was introduced into a fully virulent, encapsulated strain of E. amylovora by transformation with plasmid pJH94. The result-

ing strain, Ea110R(pJH94), produced polysaccharide depolymerase and was characterized with respect to its surface polysaccharides and pathogenicity.

Materials and Methods

Transformation of E. amylovora Ea110R with plasmids pJH94 and pUC8 - The $CaCl_2$ procedure (94) described in chapter 1 for E. coli was used to transform E. amylovora Ea110R, except that bacterial growth was at 27 C. Ampicillin resistant transformants, designated Ea110R(pJH94) and Ea110R(pUC8) were selected for further study.

Physical detection of plasmids in E. amylovora strains- Strain Ea110R(pJH94) was grown in DM-17 containing ampicillin. Amplification of the two 250 ml cultures with chloramphenicol (42,94) and alkaline lysis (26,94) were carried out as described for HB101(pJH94) in chapter 1. The plasmid containing pellet obtained after alkaline lysis was used as substrate for restriction endonuclease Sal1 after it was sequentially extracted with phenol, phenol:chloroform, and chloroform, precipitated with ethanol, and resuspended in TE buffer. The $A_{260/280}$ of the plasmid preparation was 1.9. Plasmid preparations were made in the same manner from strain Ea110R and Ea110R(pUC8). Aliquots of 20 ul from these preparations were digested for 3 hours at 37 C with 30 U of Sal1. An aliquot of pJH94 isolated from HB101(pJH94) and purified through cesium chloride density gradients was also

digested with Sal1 as a control. Samples were electrophoresed through 0.9% agarose at 50 mA and 100V for 90 minutes before bands were visualized with ethidium bromide.

Production of polysaccharide by E. amylovora in vitro - Plasmid containing strains were grown in medium containing ampicillin at 200 mg/ml. Overnight cultures of Ea110R, Ea110R(pUC8), and Ea110R(pJH94) in DM-17 were diluted 1/10 in fresh medium. After 1 hour incubation at 25 C the cultures were diluted 1/1000 in phosphate buffer and aliquots of 0.1 ml were spread on Petri plates containing DM-17 supplemented with 2% dextrose (w/v). The Petri plates were sealed in a plastic bag and incubated for 4 days at 27 C.

The plates were washed twice with 2.5 ml of 10 mM potassium phosphate buffer pH 7.2 which contained 150 mM NaCl and 1 mM $MgSO_4$ (PBSM) (71). The suspension was centrifuged for 10 minutes at 7,700g in a SS-34 rotor, the supernatant retained, and the pellet washed 2 times with the same buffer. The supernatants from the first two centrifugation steps were pooled, and centrifuged 2 times at 12,000g for 15 minutes. Three volumes of 95% ethanol were added to the final supernatant which was then stored overnight at -20 C. The precipitate was considered to be slime polysaccharide or EPS (51,71).

The cell pellets from the previous steps were pooled and resuspended in 10 mM potassium phosphate buffer pH 7.2 which contained 500 mM NaCl and 1 mM $MgSO_4$ (PBSSM) (71). The

suspension was stirred vigorously for 1 hour at room temperature before the cells were removed by 2 cycles of centrifugation at 12,000g for 20 minutes. Three volumes of 95% ethanol were added to the supernatants which were then stored overnight at -20 C. The precipitate was considered to be capsular polysaccharide (CPS) (51,71).

The polysaccharide precipitates were collected by centrifugation at 16,000g for 1 hour, resuspended in 20 ml of dH_2O with rotary shaking for 22 hours. The supernatants were concentrated to 1/3 their original aqueous volume by rotary evaporation.

The initial cell suspension was diluted 1/50, 1/75, and 1/100 in PBSM and the cell numbers were determined with a Petroff-Hauser counting chamber after staining with 1/10 volume of a solution of 0.005% crystal violet (w/v).

Chemical analysis of polysaccharides and oligosaccharides -Carbohydrates which were precipitated with 3 volumes of ethanol and those which were not preciptated were studied. Total carbohydrate content of the samples was quantified using the phenol/sulfuric acid method (48,65). The concentration of reducing ends in the samples was quantified with the bicinchoninnate method (98). Uronic acids were quantified using the method of Blumenkrantz (27).

Preparation of polysaccharide depolymerase from Ea110R(pJH94) culture supernatants - A 10 ml overnight culture of Ea110R(pJH94) in DM-17 containing ampicillin at 200

ug/ml was used to inoculate 500 ml of the same medium. The culture was shaken for 48 hours at 27 C before the cells were removed by two cycles of centrifugation. Ammonium sulfate (enzyme grade) was added to 80% saturation and stirred at 0 C for 3 hours. The precipitate was collected by centrifugation at 10,000g in a GSA rotor and resuspended in 30 ml of phosphate buffer before dialysis against two 2 liter changes of phosphate buffer at 4 C.

Comparison of polysaccharides produced by E. amylovora strains as substrates for polysaccharide depolymerase - EPS prepared from Ea110R, Ea110R(pUC8), and Ea110R(pJH94) were adjusted to concentrations of 0.3 mg carbohydrate/ml each and used as substrate in an in vitro assay for polysaccharide depolymersase. Each reaction mix contained 100 ul of PD prepared from Ea110R(pJH94), 200 ul of EPS, and 200 ul of 200 mM acetate buffer pH 5.0, and was incubated at 42 C for 5 hours. Aliquots were removed and assayed for reducing sugars with the bicinchonninate assay. Assays were performed in triplicate. Reaction mixes which had dH_2O substituted for the polysaccharide depolymerase or for the EPS substrate were used as controls. CPS from the same strains was adjusted to 0.2 mg carbohydrate/ml and assayed as substrate for PD in the same manner.

Test of polysaccharide depolymerase from Ea110R(pJH94) for lyase activity- Polysaccharide lyases cleave the glycosidic bond by an elimination reaction, which creates an

unsaturated carbon-carbon bond in the product which can be quantified spectrophotometrically if the product is an uronic acid (73,79). Depolymerases which cleave the glycosidic bond by hydrolysis do not create such a bond. Three tubes were prepared each containing 1 mg EPS from Ea110R, 500 ul buffer, and 500 ul dH_2O. Two of the tubes received a 200 ul aliquot of crude PD and were incubated at 42 C. After 7 hours, PD was added to the third tube, and the A_{235nm} of the incubated reaction mixes was determined using the unincubated reaction mix as the blank. As a control the incubated reaction mixes were assayed for reducing sugars by the bicinchonninate method using the unincubated reaction mix as the blank.

Quantification of polysaccharide depolymerase retained by Ea110R(pJH94) and found in LB medium - Three 10 ml cultures of Ea110R(pJH94) were grown 20 hours at 27 C in LB containing ampicillin (200 mg/l). The cultures were pooled, an $O.D._{600}$ of 1.3 was measured, and three 10 ml aliquots were removed. The aliquots were centrifuged (10,000g, 5 minutes, SS-34 rotor), and the cell pellets resuspended in 10 ml each of ice cold TS (50 mM Tris/HCl, pH 8, 50 mM NaCl). Sarkosyl was added to 0.1% (w/v) final concentration (114), and the cultures were vortex mixed for 30 seconds and centrifuged as before. Each pellet was resuspended in 0.4 ml TES (TS + 10 mM EDTA) and then 0.35 ml of sucrose mix (1.6 M sucrose, 0.55 M Tris, pH 8, 10 mM EDTA) was added to

each tube. Tubes were incubated at 5 C for 20 minutes before 0.15 ml lysozyme (5 mg/ml in 50 mM Tris pH 8) was added and mixed by inversion. This was followed by the addition of 3.6 ml of 10 mM EDTA, pH 8. After the suspensions were mixed they were incubated at 5 C for 20 minutes. At this point one culture received 2.5 ml of 2.5% sarkosyl (w/v); one culture was vortex mixed at high speed for 30 seconds before receiving 2.5 ml dH_2O and one culture was sonicated and then received 2.5 ml dH_2O. All cultures received 3.0 ml dH_2O to bring their volumes to 10 ml each.

The original culture supernatant and each lysate was assayed for PD in vitro in triplicate. Reducing sugars were quantified in each reaction mix with the bicinchoninate assay.

Analysis of proteins in culture supernatants of E. amylovora strains - Single colonies of strains Ea110R, Ea110R(pUC8), and Ea110R(pJH94) were used to initiate 10 ml cultures in LB medium or in LB medium supplemented with ampicillin. Cultures were grown overnight at 24 C before they were diluted 1/50 in fresh medium and grown a further 24 hours. A fourth overnight culture, strain Ea110R, was inoculated with phage PEa1(h) at a M.O.I. of 1/1, 3 hours after it was diluted 1/50 into fresh LB medium. Bacteria were removed from all cultures by two cycles of centrifugation at 8,000 g for 15 and 30 minutes. All culture supernatants were spotted in 2-fold dilution series on mature lawns

of strain Ea110R and examined after 6 hours for the presence of craters typically produced by polysaccharide depolymerases (4,74,107). Ammonium sulfate was added to the supernatants to 80 % saturation with stirring for 30 minutes at 0 C. A fifth culture, of Ea110R, was grown in the manner of the first three cultures and the cells collected by centrifugation. These cells were used to prepare sarkosyl lysates (114 and above). After dialysis against 20 mM Tris/HCl, pH 8.0, proteins were precipitated by the addition of ammonium sulfate to 80 % saturation at 0 C. Precipitates were collected by centrifugation at 12,000 g for 20 minutes and resuspended in and dialysed against 20 mM Tris/ HCl, pH 8.0. The concentration of protein in each of the five preparations was quantified by the method of Lowry et. al. (43,90).

A denaturing 7.5 % polyacrylamide gel was prepared according to the procedure of Laemmli (82). Samples which contained 16.5 ug of protein of each of the five preparations and a sixth sample which contained 3 ug of protein size standards were denatured by boiling for 3 minutes in the presence of SDS and electrophoresed through the polyacrylamide gel. Electrophoresis was for 7 hours at 45 mA and 110 V. After electrophoresis the protein bands were visualized by silver staining as described by Morrissey (97).

Determination of the minimium inhibitory concentration of $CuSO_4$ and antibiotics with strains Ea110R(pUC8) and Ea110R(pJH94) - The bacterial strains were grown overnight

at 27 C on LB agar which contained 0.5% dextrose to enhance encapsulation and ampicillin to maintain selection for the plasmids in the strains. The bacteria were suspended in 5 mls of phosphate buffer and diluted 1/100. Aliquots of 0.1 ml was used to inoculate a 3.0 ml top agar NGAYE overlay which was immediately poured over a NGA plate which contained ampicillin. After the top agar had solidified, 3 filter paper discs were put on the surface of each plate. The discs had previously received 5 ul each of $CuSO_4$ solutions at the following concentrations (mM): 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39. The plates were incubated at 27 C for 40 hours before the greatest dilution at which the bacteria grew up to the edge of the discs was recorded.

The sensitivities of the two strains to several antibiotics were compared in the same manner. Strains were grown overnight at 25 C in DM-17 which contained 1 % dextrose to enhance encapsulation and ampicillin to maintain selection for the plasmids contained in the strains. The $O.D._{600}$ was adjusted to 0.24 with phosphate buffer. Aliquots of 0.1 ml were added to 3.0 ml top agar overlays (DM-17) which were poured over plates of the same medium which contained ampicillin. The antibiotics naladixic acid, streptomycin sulfate, and tetracycline hydrochloride were diluted with distilled H2O to concentrations of (mg/ml): 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005. Aliquots of

2.5 ul were applied to each of three filter paper discs which were placed on the surface of the overlay after it had hardened. Plates were incubated for 40 hours at 27 C and the greatest dilution at which bacteria were able to grow to the edge of the disc was determined.

Pathogenicity study- The pathogenicity of strain Ea110R(pJH94) was compared to that of strains Ea110R, Ea110R(pUC8), and Ea8 (61). Cultures were grown to late exponential phase in DM-17, pelleted at 12,000g for 5 minutes, and resuspended in phosphate buffer or in phosphate buffer with ampicillin to an $O.D._{600}$ of 0.05. Immature pear fruits (25,104) approximately 3 cm in diameter were surface disinfested in a solution of 5% bleach and 0.1% triton X-100 for 10 minutes, rinsed in distilled water and placed in styrofoam egg cartons. Three 10 ul drops of inoculum or buffer were placed on each pear fruit and the fruit were stabbed through the droplet with a 20 G syringe needle. The pears (4/treatment) were incubated at 27 C while being observed daily for symptom development.

RESULTS

Strains of Erwinia amylovora - The genotypes of the four E. amylovora strains are given in Table 3. Plaques with haloes were observed on all four strains tested when PEa1(h) was streaked on basal DM-17 supplemented with 1 % dextrose and overlayed with bacteria. Even the "acapsular" strain Ea8(61) produced plaques surrounded by haloes in this experiment. Although strain Ea110R(pJH94) produced haloes when infected with PEa1(h), the haloes were very faint and indistinct as compared with either haloes produced on strains Ea110R, Ea110R(pUC8), or Ea8 (Figure 11).

When grown on solid DM-17 containing 2% dextrose, strains Ea110R and Ea110R(pUC8) produced very fluidal colonies typical of E. amylovora. Strain Ea8 also had a fluidal colonial morphology under these conditions, although not nearly as pronounced as the typical strains. In contrast to these strains, Ea110R(pJH94) was not at all fluidal (Figure 12). Polysaccharide depolymerase activity was found in the culture filtrates and cell lysates of Ea110R(pJH94) but not in the culture supernatants or lysates of strain

Table 3. Strains of *E. amylovora* used in this study

Strain	Relevant Genotype	Reference or Source
Ea110R	rif^r	107,108
Ea110R(pUC8)	rif^r(amp^r,*lac* z)	This work (95)
Ea110R(pJH94)	rif^r(amp^r,*lac* z^-)	This work
Ea8		(61,124,124)

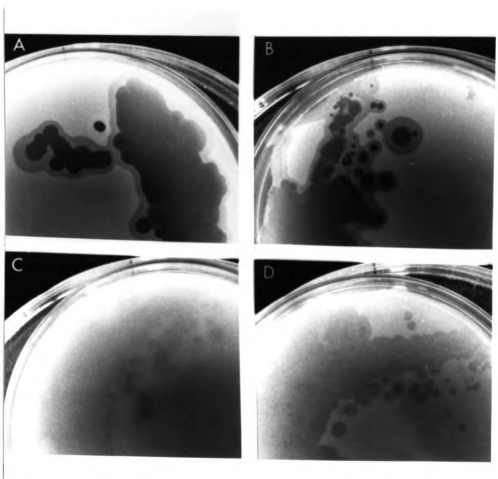

Figure 11. Plaque morphology of PEa1(h) on E. amylovora. Ea110R (A),Ea110R(pUC8) (B), Ea110R(pJH94) (C), and Ea8 (D). Bacteria were grown in DM-17 overlays which contained 1% glucose for 5 days at 27 C.

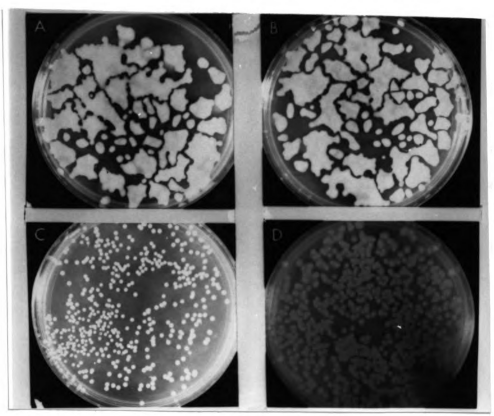

Figure 12. Colonial morphology of E. amylovora. Ea110R (A), Ea110R(pUC8) (B), Ea110R(pJH94) (C), and Ea8 (D). The bacteria were grown on DM-17 medium which contained 2 % glucose for 5 days at 27 C.

Ea110R(pUC9).

Physical detection of plasmids in E. amylovora strains Plasmid pJH94 was detected in lysates of Ea110R(pJH94) after digestion with Sal1 and electrophoresis through 0.9% agarose (Figure 13). Plasmid pJH94 purified from E. coli HB101 was completely digested by Sal1, but pJH94 prepared from Ea110R was apparently only partially digested by Sal1 (Figure 13; lanes 1 vs 2). Plasmid pUC8 was detected in strain Ea110R(pUC8) (Figure 13; lane 3). No resident plasmids were detected in strain Ea110R (Figure 13; lane 4). The preparation of pJH94 DNA isolated from Ea110R(pJH94) (Figure 13; lane 2) was used to transform E. coli HB101 to ampicillin resistance. All transformants tested (80/80) produced polysaccharide depolymerase in vitro, which indicated that the ampicillin resistance and polysaccharide depolymerase markers remained linked after passage in E. amylovora.

Chemical analysis of polysaccharides and oligosaccharides -The quantification of total carbohydrates produced by the bacterial strains is presented in Table 4. Strain Ea110R(pJH94) produced less ethanol precipitable EPS and CPS than either Ea110R or Ea110R(pUC8) on a per cell basis.

The ethanol precipitable EPS and CPS produced by Ea110R(pJH94) also differed qualitatively from that of the other two strains. The concentration of reducing ends present per mg of carbohydrate was much higher with strain Ea110R(pJH94) than with either of the other strains (Table

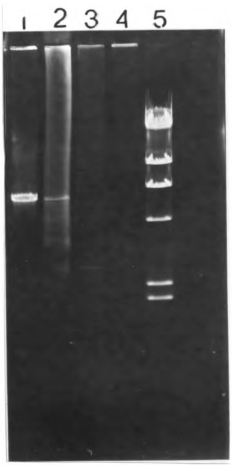

Figure 13. The physical detection of plasmids in alkaline lysates of E. amylovora strains. pJH94 purified from HB101(pJH94) lane 1,lysates of Ea110R(pJH94), lane 2, Ea110R(pUC8), lane 3, and Ea110R, lane 4, lambda/HindIII size standards, lane 5, Lanes 1-4 electrophoresed after digestion with SalI.

Table 4. Quantification of ethanol precipitable polysaccharides produced by E. amylovora strains.

	mg EPS[1,2]/10^{11} cells	mg CPS/10^{11} cells
STRAIN		
Ea110R	0.79	0.30
Ea110R(pUC8)	0.68	0.31
Ea110R(pJH94)	0.41	0.24

1. Carbohydrates quantified with the phenol sulfuric acid method (48,65) using a galactose standard. Cells enumerated with a Petroff-Hausser bacterial cell counter.
2. Mean of 3 experiments, 3 replicates each.

Table 5. Quantification of reducing sugars in ethanol precipitable polysaccharides produced by E. amylovora strains

	ug Reducing equiv./mg EPS[1,2]	ug Reducing Equiv./mg CPS
Strain		
Ea110R	15.1	18.1
Ea110R(pUC8)	16.1	19.3
Ea110R(pJH94)	49.4	35.7

1. Reducing equivalents determined with the bicinchonninate assay (27), with a galactose standard. Carbohydrates quantified with the phenol/sulfuric acid assay (48,65), with a galactose standard. Each assay used a galactose standard. Cells enumerated with a Petroff-Hauser bacterial cell counter.
2. Mean of 3 experiments, 3 replicates each.

5). This demonstrated that the average polymer length was shorter for EPS and CPS recovered from Ea110R(pJH94) than for the other strains. The EPS and CPS recovered from this strain also contained less uronic acid per weight of carbohydrate (Table 6). Thus both the ethanol precipitable EPS and CPS of strain Ea110R(pJH94) differed quantitatively and qualitatively from that of strains Ea110R and Ea110R(pUC8).

Much larger amounts of carbohydrate soluble in three volumes of ethanol were recovered from the EPS of strain Ea110R(pJH94) than from the other strains (Table 7). This ethanol soluble carbohydrate probably included oligosaccharide products of the enzymatic hydrolysis of EPS andCPS of strain Ea110R(pJH94). However, the very high proportion of reducing equivalents to carbohydrate in this material as well as that from the other strains may suggest that it also contained a simple sugar, possibly glucose from the growth medium. No differences were observed among the ethanol soluble sugars recovered from the CPS of these strains (Table 7).

Comparison of polysaccharides produced by E. amylovora strains as substrates for polysaccharide depolymerase - Incubation of EPS from both Ea110R and Ea110R(pUC8) with polysaccharide depolymerase resulted in a sharp increase in the concentration of reducing equivalents as compared to tubes incubated without EPS or without polysaccharide depolymerase. In contrast, incubation of EPS from Ea110R(pJH94)

Table 6. Quantification of uronic acids in ethanol precipitable polysaccharides produced by *E. amylovora*.

Strain		mg uronic acid[1]/mg carbohydrate[2]
Ea110R	EPS	0.23
Ea110R(pUC8)	EPS	0.21
Ea110R(pJH94)	EPS	0.12
Ea110R	CPS	0.18
Ea110R(pUC8)	CPS	0.18
Ea110R(pJH94)	CPS	0.12

1. Determined as glucuronic acid equivalents (27). Mean of two experiments, three replicates each.
2. Total carbohydrate, determined as galactose equivalents (48,65). Mean of two experiments, three replicates each.

Table 7. Quantification of ethanol soluble carbohydrates recovered from *E. amylovora* strains.

Strain		mg/10^{11} CFU[1]	ug reducing equivalent[2] / mg carbohydrate
Ea110R	EPS	0.52 + 0.1	630 + 45
Ea110R(pUC8)	EPS	0.39 + 0.03	640 + 190
Ea110R(pJH94)	EPS	2.42 + 1.9	953 + 108
Ea110R	CPS	0.10 + 0.06	278 + 29
Ea110R(pUC8)	CPS	0.08 + 0.03	327 + 85
Ea110R(pJH94)	CPS	0.06 + 0.02	371 + 126

1. Mean + standard deviation of 3 experiments. Determined with the phenol sulfuric acid (48,65) method against a galactose standard.
2. Mean + standard deviation of 3 experiments. Determined with the bicinchonninate method (98) against a galactose standard.

with polysaccharide depolymerase resulted in only a barely detectable increase in the concentration of reducing equivalents in the same assay (Table 8). The tube which contained EPS from Ea110R(pJH94) but no added PD showed a high concentration of reducing equivalents. These results suggested that EPS from Ea110R(pJH94) had few, if any, remaining sites for polysaccharide depolymerase cleavage.

Similar results were observed with CPS obtained from the 3 strains (Table 8). When incubated with polysaccharide depolymerase, CPS from Ea110R and Ea110R(pUC8) produced an increase in the concentration of reducing sugars in the reaction mix, which indicated that they had been cleaved by polysaccharide depolymerase. CPS from Ea110R(pJH94) produced no increase in reducing sugars when incubated with polysaccharide depolymerase. As was the case with EPS from this strain, the concentration of reducing equivalents measured in the reaction mix containing only CPS of Ea110R(pJH94) was very much greater than in the reaction mixes containing only CPS from Ea110R or Ea110R(pJH94). The results suggested that CPS from Ea110R(pJH94) was not substrate for polysaccharide depolymerase.

Test of crude polysaccharide depolymerase from Ea110R(pJH94) for lyase activity - When assayed spectrophotometrically there was no increase in the A_{235} in the tubes incubated with polysaccharide depolymerase for 7 hours as compared to the tube which had not been incubated. In con-

Table 8. Comparisons of polysaccharides produced by strains of *E. amylovora* as substrates for polysaccharide depolymerase.

Strain	Polysaccharide[1]	A_{565}[2]	ug Galactose Equiv.[3] Released by PD
Ea110R	EPS	.345+.008	7.1
Ea110R(pUC8)	EPS	.325+.008	6.8
Ea110R(pJH94)	EPS	.020+.009	0.4
Ea110R	CPS	.093+.001	1.9
Ea110R(pUC8)	CPS	.067+.004	1.3
Ea110R(pJH94)	CPS	-.003+.002	0

1. EPSwas used at 60 ug/500ul reaction. CPS was used at 40 ug/500 ul reaction.

2. Mean + standard deviation of three replicates. A_{565} of control reactions containing substrate or enzyme only have been subtracted from all values.

3. The bicinchonninate assay (98) was used to quantify reducing sugars released by PD from the polysaccharides using a galactose standard.

trast, there was a sharp increase in the concentration of reducing equivalents in the incubated as compared to the non-incubated reaction mixes.

Quantification of polysaccharide depolymerase retained by Ea110R(pJH94) and found in LB medium - A unit of polysaccharide depolymerase activity was defined as the amount of polysaccharide depolymerase which would produce 1 umole of reducing equivalents per hour in a standard reaction mix. The majority of polysaccharide depolymerase activity was retained within Ea110R(pJH94) in this experiment but a significant amount appeared in the culture supernatant (Table 9).

Analysis of proteins found in culture supernatants of E. amylovora strains - Craters were produced on mature lawns of E. amylovora when dilutions of culture supernatants obtained from Ea110R(pJH94) or from Ea110R/PEa1(h) were spotted on them. No craters were observed when supernatants from the other strains were spotted on them. Culture supernatants obtained from strains Ea110R or Ea110R(pUC8) contained few proteins which could be resolved in a 7.5 % polyacrylamide gel (Figure 14; lanes 2 and 3). This was in sharp contrast to the supernatant obtained from strain Ea110R(pJH94) which produced a very complex pattern which was virtually indistinguishable from the patterns produced by either phage or sarkosyl lysis of strain Ea110R (Figure 14; lanes 4-6). The results suggest that strain Ea110R(pJH94) partially lysed

Table 9. Quantification of PD retained by Ea110R(pJH94) and found in LB medium.

Source	Units[1] PD/ml	Total Units
Culture Supernatants	1.1	32
Sphaeroplasts lysed with sarkosyl	4.7	142
Sphaeroplasts lysed with sonication	4.7	142
Sphaeroplasts lysed with vortex mixing	1.6	50

1.1 Unit = 1uMole Galactose Equivalent/ml hr. The following is a sample unit calculation: A_{565} =.195 = 4 ug gal eq./50 ul aliquot assayed. 4 ug X 8 = 32 ug/400 ul reaction. 32ug/180 ug/uMole gal =.177 uMole Gal. eq./3 hr = .059 uMole/hr in 50 ul aliquot of PD assayed. .059 X 20 = 1.19 U/ml at 1/4 dilution or 4.74 U/ml undiluted. 4.74 U/ml X 30 ml = 142 U total.

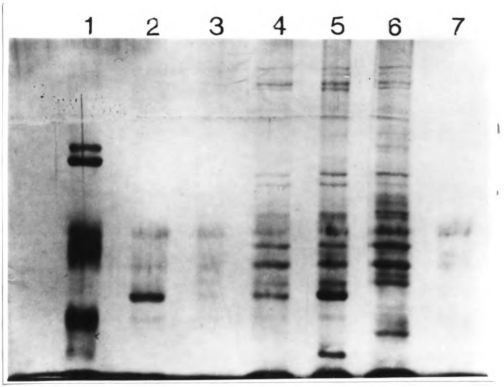

Figure 14. Analysis of proteins found in culture supernatants of E. amylovora strains. 16.5 ug of protein was loaded per lane in a 7.5 % polyacrylamide gel which was electrophoresed under denaturing conditions. Culture supernatants were from Ea110R (lane 2), Ea110R(pUC8) (lanes 3 and 7), and Ea110R(pJH94) (lane 4). Proteins from a PEa1(h) lysate of Ea110R and a sarkosyl lysate of Ea110R were in lanes 5 and 6. Protein size standards (3 ug) were loaded in lane 1.

either before or during the stationary phase of growth. Neither strain Ea110R or Ea110R(pUC8) seemed to have lysed under identical conditions. Polysaccharide depolymerase appeared in the growth medium most likely as the result of this partial lysis of the culture, either before or during the stationary growth phase, and probably not as the result of specific excretion of the enzyme by Ea110R(pJH94).

Determination of the minimum inhibitory concentrations of $CuSO_4$ and antibiotics on strains Ea110R(pUC8) and Ea110R(pJH94) - No differences in sensitivity to antibiotics or $CuSO_4$ were observed for the two strains. The minimum inhibitory concentrations for naladixic acid, streptomycin sulfate and tetracycline hydrochloride were 0.16 mg/ml, 0.04 mg/ml, and 0.31 mg/ml respectively for each of the two strains tested. The minimum inhibitory concentration of $CuSO_4$ was 50 mM for both strains.

Pathogenicity study- Symptoms developed rapidly in fruits inoculated with Ea110R, Ea110R(pUC8) or Ea110R(pJH94) (Figure 15). Symptoms caused by the first two strains included extensive necrosis and the copious production of ooze which are diagnostic of fireblight infections. Strain Ea110R(pJH94) caused necrosis but in marked contrast to the other isolates usually failed to produce any ooze. Strain Ea8 and the buffer controls produced only slight necrosis or no symptoms at all in this experiment which was repeated several times. Occasionally ooze was produced by pear fruits

Figure 15. Symptoms caused by *E. amylovora* strains on immature pear fruits. Pear fruits were inoculated five days earlier with *E. amylovora* strains Ea110R {left}, Ea110R(pJH94){center}, and Ea110R(pUC8){right}.


Ea110 rif/pJH94
A = 0.05
600

inoculated with Ea110R(pJH94), particularly after prolonged incubation. In these instances the bacteria present in the ooze were sensitive to ampicillin, indicating that they had lost pJH94.

DISCUSSION

The introduction of pJH94 into *E. amylovora* strain Ea110R by transformation dramatically altered the colonial morphology of Ea110R (Figure 12). On rich medium, or on defined medium containing excess glucose, the slimy or fluidal morphology typical of *E. amylovora* isolates was observed with strains Ea110R and Ea110R(pUC8) but not with strain Ea110R(pJH94). Evidence which shows that this altered morphology is due to the presence of the cloned polysaccharide depolymerase gene in Ea110R(pJH94) includes:

i. The isolation of pJH94 from Ea110R(pJH94).

ii. The presence of polysaccharide depolymerase activity in culture supernatants and cell lysates of this strain but not in culture supernatants or cell lysates from Ea110R or Ea110R(pUC8).

iii. The cleavage of both EPS and CPS from Ea110R by the polysaccharide depolymerease *in vitro*.

iv. The observation that EPS and CPS from Ea110R(pJH94) were not substrates for polysaccharide depolymerase *in vitro*

v. The recovery of quantitatively less ethanol precipitable EPS and CPS from Ea110R(pJH94).

vi.The increased concentration of reducing ends inboththe EPS and CPSisolatedfrom Ea110R(pJH94).

vii. The sharply reduced intensity of haloes surrounding plaques made by PEa1(h) on Ea110R(pJH94).

If Ea110R(pJH94) had excreted polysaccharide depolymerase into the growth medium, the complexity of the protein mixture found in its culture supernatant would have differed from that of Ea110R and Ea110R(pUC8) only by the addition of a band or bands corresponding to the polysaccharide depolymerase. If, on the other hand, polysaccharide depolymerase entered the growth medium as a result of lysis of Ea110R(pJH94), then the complexity of the protein mixture in the Ea110R(pJH94) supernatant would be similar to that observed for a phage infected culture or for a sarkosykl lysate of Ea110R. Culture supernatants of Ea110R(pJH94) contained a very complex mixture of proteins as revealed by SDS/acrylamide electrophoresis (Figure 14; lane 4). Supernatants of PEa1(h) infected Ea110R contained an extremely similar mixture of proteins, as did a sarkosyl lysate of Ea110R (Figure 14; lanes 5,6). Such a complex mixture of proteins was not observed in supernatants of Ea110R or Ea110R(pUC8) (Figure 14; lanes 2,3). It is most likely that polysaccharide depolymerase appeared in the growth medium at or before stationary phase apparently due to lysis of a

portion of the cultures of Ea110R(pJH94). There was no evidence that polysaccharide depolymerase was specifically excreted by Ea110R(pJH94).

The sensitivities of strain Ea110R(pUC8) and Ea110R(pJH94) to antibiotics and $CuSO_4$ were compared because it had been reported that treatment of Ea110R with a crude preparation of polysaccharide depolymerase resulted in increased sensitivity to streptomycin sulfate (107). Ea110R(pJH94) was no more sensitive than Ea110R(pUC8) to any of three antibiotics or to $CuSO_4$. Perhaps the earlier result (107) was due to the presence of another, unidentified factor in the crude enzyme prep used in that study. If so, identification of that factor could prove to be rewarding.

The development of extensive necrosis when pear fruits were inoculated with Ea110R(pJH94) suggests that intact extracellular polysaccharides may not be required for pathogenesis by E. amylovora. The lack of ooze produced by pear fruits infected with Ea110R(pJH94) suggests 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 (21), chromatographic evidence (21,118) and serological data (118). The pathogenicity study should be repeated using intact plants and using a wide range of inoculum concentrations on immature pear fruits to gain a better understanding of the

effect of the lower molecular weight extracellular and capsular polysaccharides on disease development.

While care must be taken when interpreting the results of the pathogenicity study which compared Ea110R, Ea110R-(pUC8), Ea110R(pJH94) and Ea8, some tentative conclusions can be made. Strains Ea110R and Ea10R(pUC8) did not differ quantitatively or qualitatively in the production of EPS or CPS *in vitro*. The pathogenicity of Ea110R was also not affected by pUC8, nor was the production of the ooze characteristic of fire blight disease. Plasmid pJH94 did alter colony morphology (Figure 12) and ethanol precipitable polysaccharides, both quantitatively and qualitatively *in vitro* (Tables 4-6). Neither EPS nor CPS isolated from Ea110R(pJH94) were good substrates for polysaccharide depolymerase, as compared to EPS and CPS isolated from Ea110R and Ea110R(pUC8) (Table 8, Figure 1). Plasmid pJH94 also prevented the production of ooze characteristic of fire blight disease although it did not prevent infection of, and multiplication in, pear fruit.

The results of this preliminary pathogenicity study can be reconciled with the proposed role of EPS and CPS as anti-agglutination factors (109,116), since polysaccharide fragments could still retain this activity. Experiments with intact plants will be required to determine how (or whether) the polysaccharide fragments influence the vascular plugging symptom associated with fire blight disease (70,119). Slime

polysaccharides of different molecular weights naturally produced by C. michiganense pv.insidiosum have been observed to accumulate at different locations in the transpiration stream of plants dipped in solutions of labled polysaccharides (99). Lower molecular weight polysaccharides moved further in the transpiration stream, to vessels of smaller capillary diameter, than did larger ones, consistent with the hypothesis that the vascular blockage was mainly mechanical, and not the result of agglutination as has been proposed for E. amylovora (109).

The EPS of P. syringae pv. phaseolicola has been reported to be enzymatically degraded in the leaf mesophyll of resistant, but not susceptible bean cultivars (56). Experiments with a polysaccharide depolymerase specific for P. syringae pv. phaseolicola EPS might prove especially rewarding in that system.

The introduction of pJH94 and pUC8 into E. amylovora by transformation demonstrates that ColE1 replicons (66) can be maintained in E. amylovora. Another ColE1 replicon, pBR322, was introduced into E. herbicola earlier by transformation (81). The ability of E. amylovora to maintain ColE1 replicons should greatly facilitate the genetic analysis of characters associated with pathogenicity and virulence in this organism.

The utility of strains Ea110R(pJH94) and Ea110R for studying the role of extracellular polysaccharides in patho-

genesis could be improved in several ways. One improvement would be to reduce the size of the phage DNA insert in pJH94. The size of the phage insert in pJH94 was shown to be 3150 bp. Based on the molecular weights of well characterized polysaccharide depolymerases purified from phage infected Aerobacter aerogenes (137), and Pseudomonas aeruginosa (10), about 2200 bp of DNA would be required to encode such an enzyme. The phage DNA insert in pJH94 is then perhaps 1000 bp larger than needed to encode PD, and the "extra" DNA could possibly encode another function which would affect the interpretation of experiments which involved pJH94. A double digestion of pJH94 with restriction endonucleases Sal1 and EcoR1 would remove the phage DNA insert from the pUC8 vector (95), since the BamH1 cloning site into which the phage DNA was inserted lies between unique sites for these two enzymes on the vector. The excised insert could then be "trimmed" and subcloned (94).

The appearance of ampicillin sensitive Ea110R in ooze produced by immature pear fruits which had been inoculated with Ea110R(pJH94) showed that pJH94 was being lost by the bacteria in the absence of continuous selection for ampicillin resistance. The possibility that these ampicillin sensitive bacteria were contaminants seems unlikely since fruit inoculated with buffer only did not become diseased. Although the appearance of ooze in these fruits was much delayed as compared to fruits inoculated with Ea110R or

Ea110R(pUC8), its appearance is still troublesome. The plasmid might be stabilized and the problem solved by introducing pJH94 into a Rec A^- derivitive of Ea110R. The behavior of pJH94 in E. coli strains suggests this approach. Despite repeated attempts, it was not possible to amplify and purify pJH94 in E. coli strain JM105, which is a $RecA^+$ strain. However, pJH94 was easily amplified in and purified from E. coli strain HB101, which is a $RecA^-$ strain (29). This observation could be explained in terms of the $RecA^-$ mutation which limits the ability of the HB101 genome to recombine with plasmid DNA (41,72). The introduction of a similar $RecA^-$ mutation into Ea110R might have a similar stabilizing effect on pJH94 in that organism.

The relative instability of pJH94 in $recA^+$ strains and its tendency to cause lysis of cells harboring it (Figure 14), are both probably due to the uncontrolled transcription of the phage polysaccharide depolymerase gene (Chapter 1, 32,123). The introduction of a strong transcriptional terminator at the 3' terminus of the phage gene could resolve both problems (32).

Final improvements of Ea110R(pJH94) for the study of exopolysaccharides in pathogenesis would involve further modifications of the polysaccharide depolymerase gene itself. The addition of a hydrophobic leader peptide at the amino terminus of the protein might facilitate excretion of the enzyme by the bacterial strain, since naturally excreted

proteins contain such sequences (17). This could be accomplished by the synthesis of a polynucleotide *in vitro* which encoded such a leader peptide and its addition to the 5' terminus of the polysaccharide depolymerase gene using standard recombinant DNA technology (94).

Lastly, the replacement of the phage gene's own transcriptional promoter with transcriptional promoters from other sources which differed in their "strength" (31,91) could create a series of plasmids which caused the bacterial strains which harbored them to produce quantitatively different amounts of polysaccharide depolymerase. Such a series of bacterial strains would represent a very useful tool for the study of exopolysaccharides in the pathogenesis of *E. amylovora*.

It is important to emphasize that in the present study pathogenicity, the ability to incite disease, was separated from symptomology. This has not been possible in previous studies which relied on avirulent, acapsular mutant strains. Although Ea110R(pJH94) was able to reproduce in pear fruits and cause extensive necrosis, it was unable to produce the ooze characteristic of fire blight disease.

APPENDIX

INTERACTION OF BACTERIOPHAGE PEa1(h) AND *ERWINIA AMYLOVORA* IN LIQUID MEDIA

Introduction

Ritchie (107) observed that when E. amylovora strain Ea110R was grown in NBGYE (0.8% nutrient broth, 0,5% yeast extract (Difco), 0.5% dextrose), bacteriophage PEa1(h) was unable to completely lyse the culture. The sharp decrease in turbidity associated with phage lysis of Aerobacter aerogenes (137) did not occur. Instead after an initial decrease in turbidity following the addition of phage to an exponentially growing culture, the turbidity of the culture increased to nearly the level of a culture not inoculated with phage. Although the phage had increased in titer and polysaccharide depolymerase became detectable in this medium, strain Ea110R was still able to grow to cell densities comparable to cultures not inoculated with phage.

PEa1(h)-r strains of Ea110R obtained from plaques were acapsular (107). The results of a fluctuation test (92) with PEa1(h) as the selective agent and Ea110R as the test strain demonstrated that the secondary growth in phage inoculated broth cultures was not due to the presence of spontaneous, acapsular mutants, but was instead an adaptational response

(107). When streaked on solid medium, the bacteria, which were acapsular in the presence of PEa1(h) in broth, again grew as with capsules and slime. Ritchie proposed that the resistance of the bacteria to the phage in broth was due to the removal of the casular binding sites by the PD associated with the phage infection (107). This situation, in which the bacterial capsule is required for phage infection, has been reported by Stirm (121) for a series of K-antigen specific E. coli phage, and investigated in detail by Bayer et. al. (16).

Efficient lysis of Ea110R by PEa1(h) in broth culture would make large amounts of crude polysaccharide depolymerase available for purification and study, as has been done in other systems (10,137). The interaction of phage PEa1(h) and strain Ea110R in broth was studied in order to determine if conditions could be found in which PEa1(h) would efficiently lyse Ea110R.

Materials and Methods-

Media- The rich media used in these experiments included NBGYE (107) and LB (94). The phosphate buffered medium of Yurewicz (137) and Davis broth (86) were used as semi-defined media. Davis medium was supplemented with all essential amino acids except methionine, proline and glycine at a final concentration of 20 mg/l of the L-form (DM-17). The components of the media used are given in Table 10. When media were to be used in petri plates, agar (Bacto) autoclaved separately was added to a final concentration of 1.5% for the basal medium and 0.7% for the top agar.

Purification of Phage- Phage PEa1(h) was purified from soft agar lysates of E. amylovora Ea110R through sucrose density gradients as described (Chapter 1). The phage stocks were titered by making 10-fold dilution series in PB and adding 0.1 ml aliquots to soft agar overlays containing 0.1 ml of an exponentially growing culture of E. amylovora strain Ea110R. Assays were always performed in triplicate.

Optical Density Measurements- Appropriately diluted samples of bacterial cultures, or bacterial cultures inoculated with phage were read against similarly diluted, non-inoculated media blanks in a Gilford Model 240 Spectropho-

Table 10. Media used to study interaction of PEa1(h)and Ea110R in broth

Yurewicz et. al.[1]		Davis[2]	
$(NH_4)_2SO_4$	76 mM	K_2HPO_4	40 mM
Na_2HPO_4	70 mM	KH_2PO_4	14 mM
KH_2PO_4	22 mM	Na*Citrate	2 mM
K_2SO_4	6 mM	$(NH_4)_2SO_4$	7.6 mM
NaCl	18 mM	$MgSO_4$	0.5 mM
Casamino Acids	0.5 gm/l	L-Amino Acids	20 mg/l
$MgSO_4*7H_2$	1 mM	Glucose	11 mM
$CaCl_2*2H_2O$	0.1 mM	Thiamine	2 mg/l
$FeSO_4*7H_2O$	4 uM	Niacin	2 mg/l
Glucose	56 mM		
Thiamine	2 mg/l		
Niacin	2 mg/l		

1. Reference 137

2. reference 86

tometer.

Growth of PEa1(h) in Rich Medium- Ea110R was grown overnight at 25 C from a single colony to an OD_{620} of 3.8, then diluted to an $O.D._{620}$ of 0.1. After 5 hours the culture was diluted into fresh medium at an $O.D._{620}$ of 0.08 in paired flasks. Phage Pea1(h) was added to the first flask after 1 hour and to the second flask after 3 hours incubation to a M.O.I.of approximately 1 PFU/7 CFU. Incubation was continued overnight and lysis was monitored spectrophotometrically.

Interaction of PEA1(h) and Ea110R in Defined Liquid Media - DM-17 and Yurewicz Broth (Table 1) were supplemented with 100 uM $ZnSO_4$. DM-17 was also supplemented with 100 uM $CaCl_2$ so that the concentrations of divalent ions would be comparable in each medium. Glucose which had been autoclaved separately was added to aliquots of each medium to final concentrations of 0.2% and 1% (w/v). After 2 overnight pregrowths in each of the four media, E. amylovora Ea110R was diluted to an $O.D._{600}$ of 0.1 in each of four flasks/medium and shaken at 160 RPM at 24 C. After 4.5 hours phage PEa1(h) was added to three flasks at M.O.I. of 1/40, 1/20, and 1/1. Lysis of the three cultures/medium inoculated with phage was monitored spectrophotometrically by comparison with the fourth culture which was not inoculated with phage.

Lysis of Ea110R by PEa1(h) in DM-17 Broth - Dextrose was added from a 20% stock to a final concentration of 0.2%

(w/v) in DM-17. Thiamine and niacin were added from a filter sterilized stock to a final concentration of 2 ug/ml. A single colony of Ea110R was used to start an overnight culture which was then diluted into fresh medium to an $O.D._{600}$ of 0.075. After 2, 4, and 6 hours of rotary shaking at 80 rpm at 24 C, purified phage were added at an MOI of approximately 0.6 PFU/CFU. Lysis was monitored spectrophotometrically.

Effect of Divalent Cations on Lysis of Ea110R by PEa1(h) - $CaCl_2$ was added to ice cold DM-17 from a 1 M stock to final concentrations of 0.1mM, 0.5mM, 1.0mM, and 2.5mM. A single colony of Ea110R was used to start an overnight culture which was diluted to an $O.D._{600}$ of approximately 0.045. After 2 hours of rotary shaking at 80 RPM and at 24 C purified phage were added at a M.O.I. of 6PFU/CFU. Lysis was monitored spectrophotometrically.

$MgSO_4$ was added to ice cold DM-17 medium prepared without $MgSO_4$ to final concentrations of 0, 0.5, 2.0, 4.0, and 8.0 mM. $CaCl_2$ was added to a final concentration of 0 or 1 mM. Overnight cultures were diluted to an $O.D._{600}$ of about 0.015, shaken at 80 RPM and 24 C for 6.5 hours, then inoculated with purified phage at a M.O.I. of 0.6 PFU/CFU. Lysis was monitored spectrophotometrically.

$ZnSO_4$ was added to ice cold DM-17 medium from a 1M stock to final concentrations of 0, 10, and 100 uM. Overnight cultures of Ea110R were diluted to an $O.D._{600}$ of

approximately 0.05 and shaken at 80 RPM at 24C for 4.5 hours before purified phage were added at a M.O.I. of 0.6PFU/CFU. Lysis was monitored spectrophotometrically.

Lysis of Ea110R by PEa1(h) in DM-17 broth was compared to lysis in DM-17 amended with $CaCl_2$ at 1 mM and $ZnSO_4$ at 100 uM. A petri plate culture of Ea110R grown 24 hours on D-17 medium was diluted to an initial $O.D._{600}$ of 0.12 in paired flasks of the two media. After 4 hours incubation at 31C and 80 RPM phage were added to one of each pair of flasks at a M.O.I. of 0.6PFU/CFU. Incubation was continued and lysis was monitored spectrophotometrically.

RESULTS

Growth of PEa1(h) in Rich Medium - PEa1(h) was not able to completely lyse the cultures of Ea110R grown in NBGYE. Shortly after the addition of PEa1(h) at a multiplicity of infection (M.O.I.) of 1/7, the turbidity of the inoculated culture decreased as compared to the culture not yet inoculated with phage. Turbidities never approached zero which would have indicated total lysis but instead maintained a relatively constant value before increasing in parallel with the culture not inoculated with phage (Figure 16). After incubation overnight, the optical densities of the phage infected cultures were 1.3, indicating large numbers of cells growing in the presence of PEa1(h). A culture of Ea110R not inoculated with phage typically had an $O.D._{600}$ of 3.6 after overnight incubation.

Growth of PEA1(h) in Defined Liquid Media - Phage PEA1(h) was unable to lyse Ea110R growing in Yurewicz Broth when added at several points during exponential growth at a M.O.I. of 1/40 (Figure 17). Varying either the glucose concentration or the M.O.I. did not affect the results (Figure 18). The resulting plots of $O.D._{600}$ vs time looked exactly

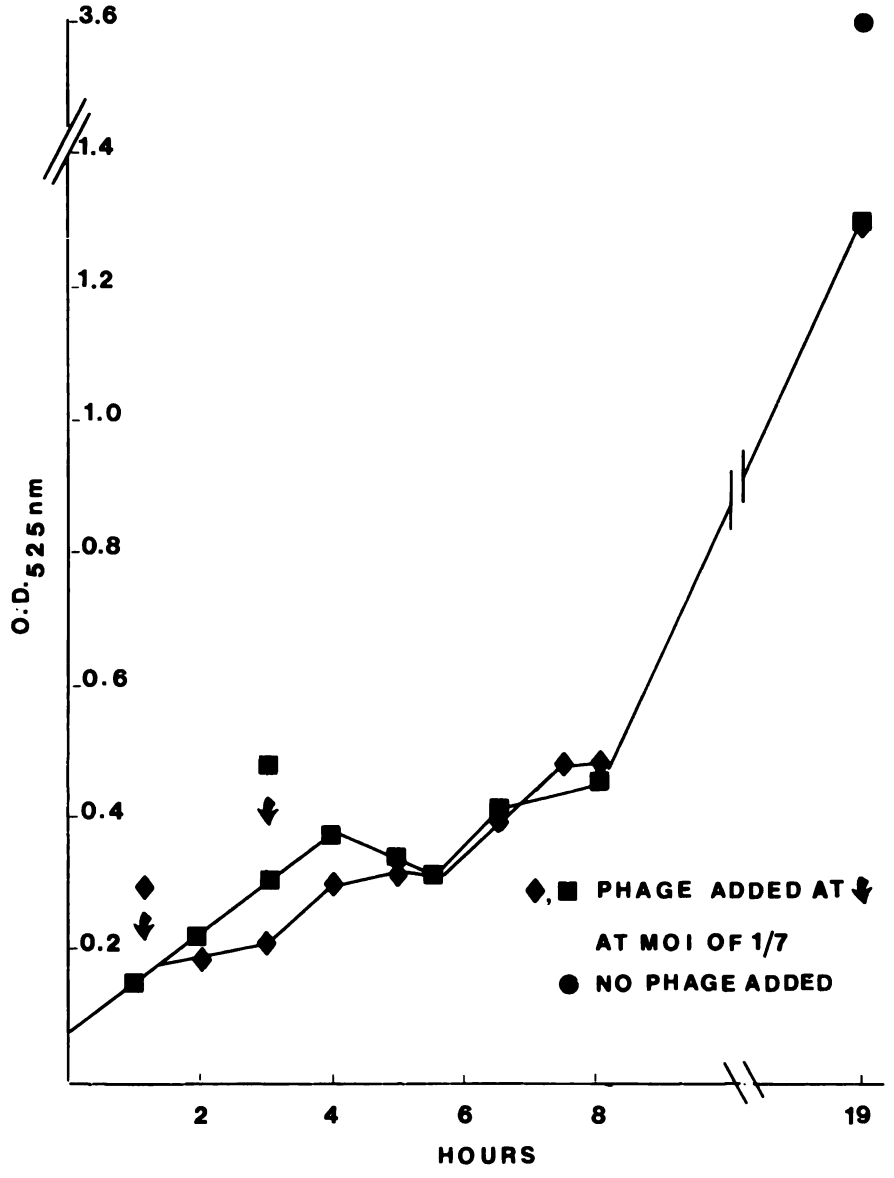

Figure16. Interaction of E. amylovora strain Ea110R and phage PEa1(h) in nutrient broth.

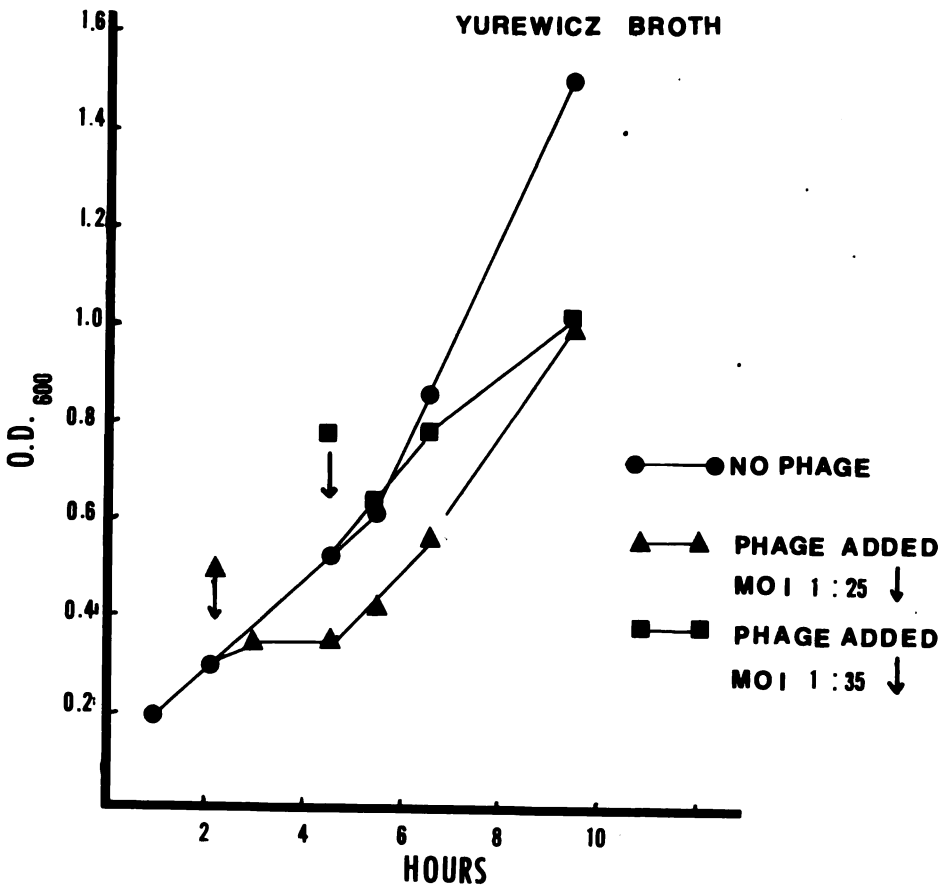

Figure 17. Interaction of strain Ea110R and phage PEa1(h) in Yurewicz broth.

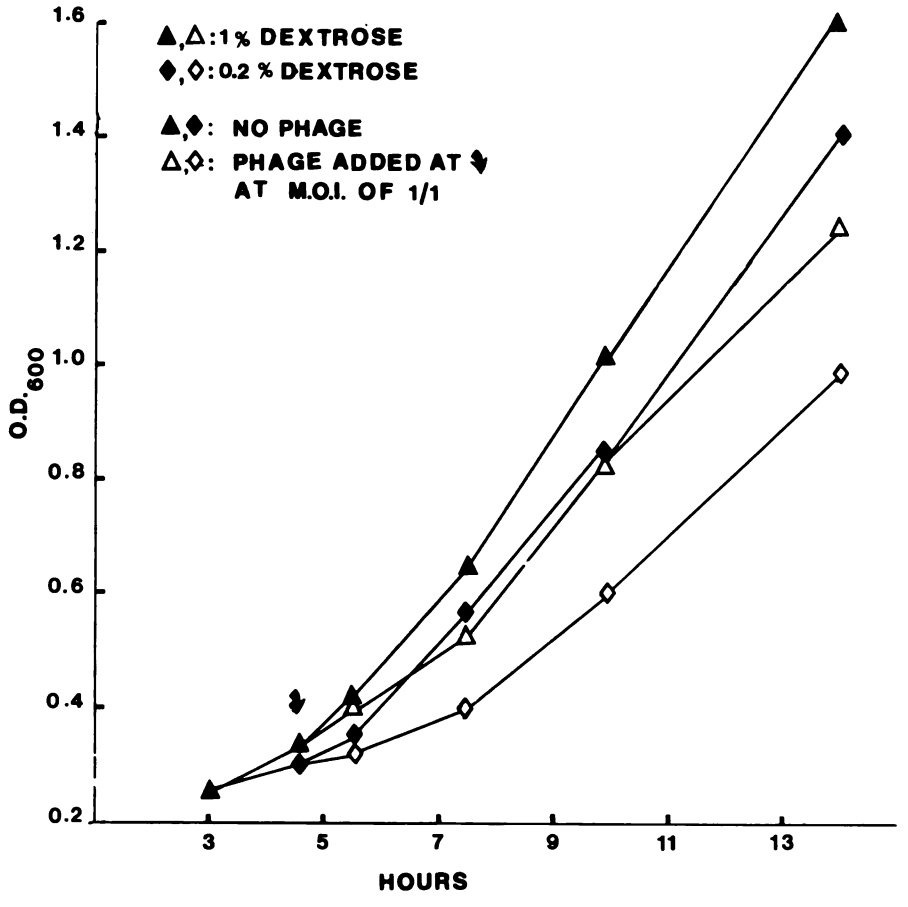

Figure 18. Interaction of Ea110R and phage PEa1(h) at different concentrations of glucose in Yurewicz broth.

like those from similar experiments carried out in nutrient broth (Figure 16 and (107)).

Phage PEA1(h) was better able to lyse Ea110R when grown in DM-17 containing 0.2% or 1% glucose at all M.O.I. tested. Figure 19 shows the results of the experiment with a M.O.I. of 1/1 with DM-17 containing 0.2 % and 1 % dextrose. Phage PEA1(h) was able to lyse Ea110R in DM-17 liquid medium but not in NBGYE or Yurewicz broth.

PEa1(h) was able to completely lyse Ea110R grown in DM-17 broth when added to cultures at several points during exponential growth. The ability of PEA1(h) to lyse Ea110R in DM-17 was not influenced by the growth stage of the culture at the time the phage were added, within the range tested (Figure 20).

Effect of Divalent Cations on Growth of PEa1(h) - $CaCl_2$ added to Davis broth at concentrations of 0.1 - 2.5 mM had no effect on the lysis of Ea110R by PEa1(h) (Figure 21). The results are indistinguishable from the results of the same experiment carried out in medium lacking $CaCl_2$ (Figure 20).

When $MgSO_4$ was added at concentrations greater than 0.4 mM, the concentration used in Davis broth, no effect on lysis of Ea110R by PEa1(h) was observed. When no $MgSO_4$ was included in Davis broth the growth of Ea110R was very restricted although the culture was apparently lysed by PEa1(h).

The growth of Ea110R and its lysis by PEa1(h) was

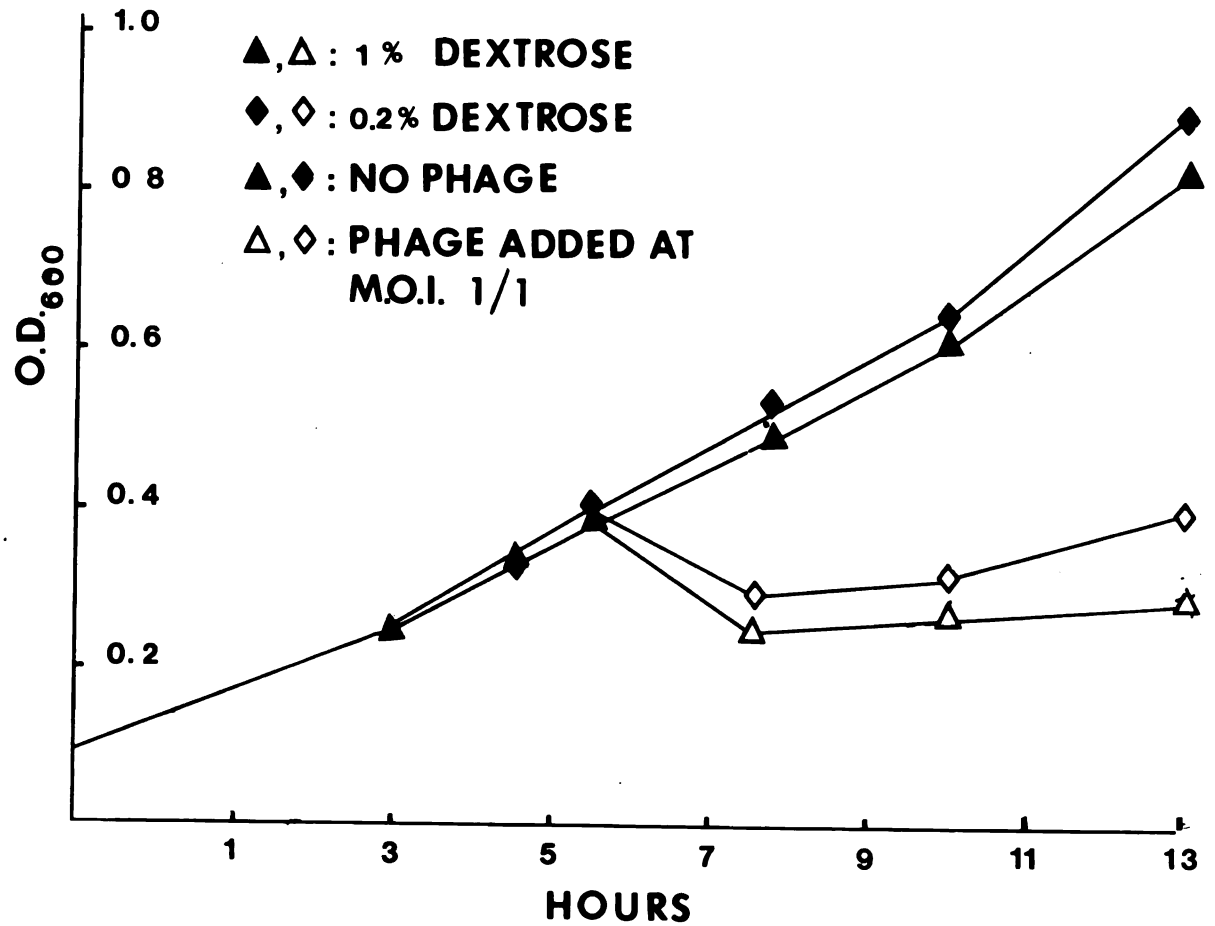

Figure 19. Interaction of Ea110R and phage PEa1(h) at different concentrations of glucose in DM-17 broth.

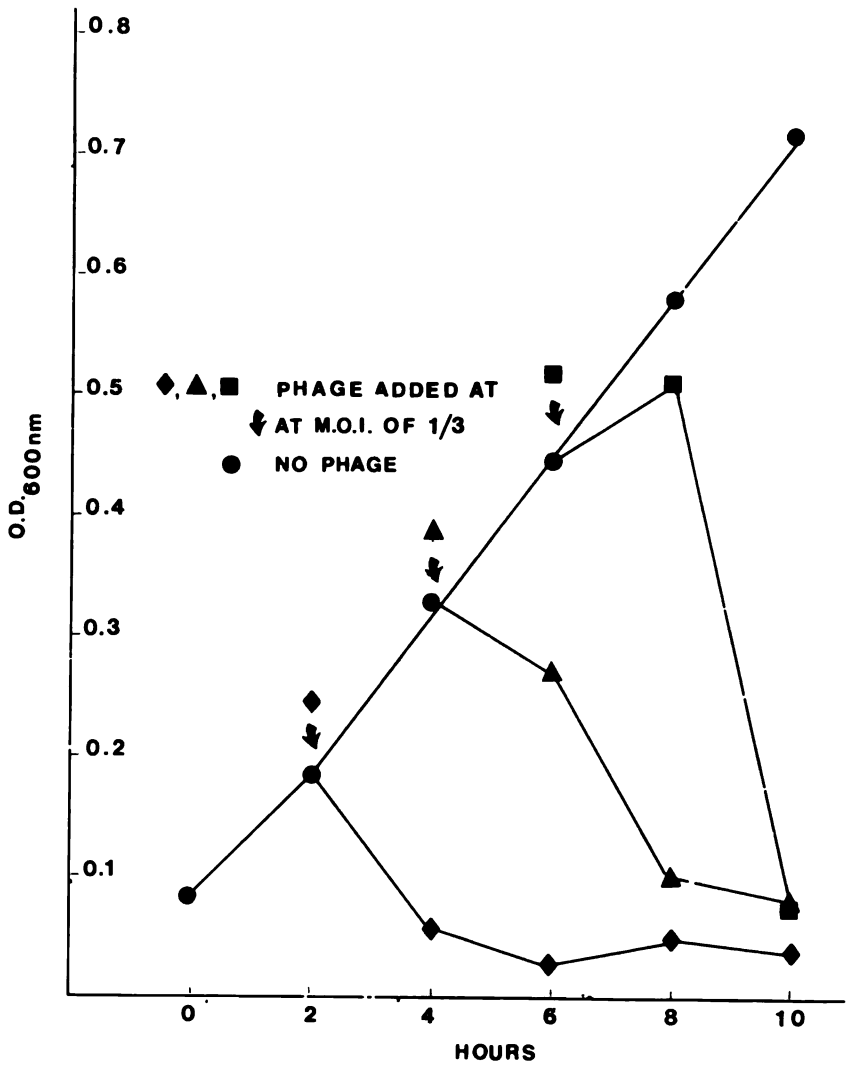

Figure 20. Effect of growth stage on lysis of Ea110R by PEa1(h) in DM-17 medium.

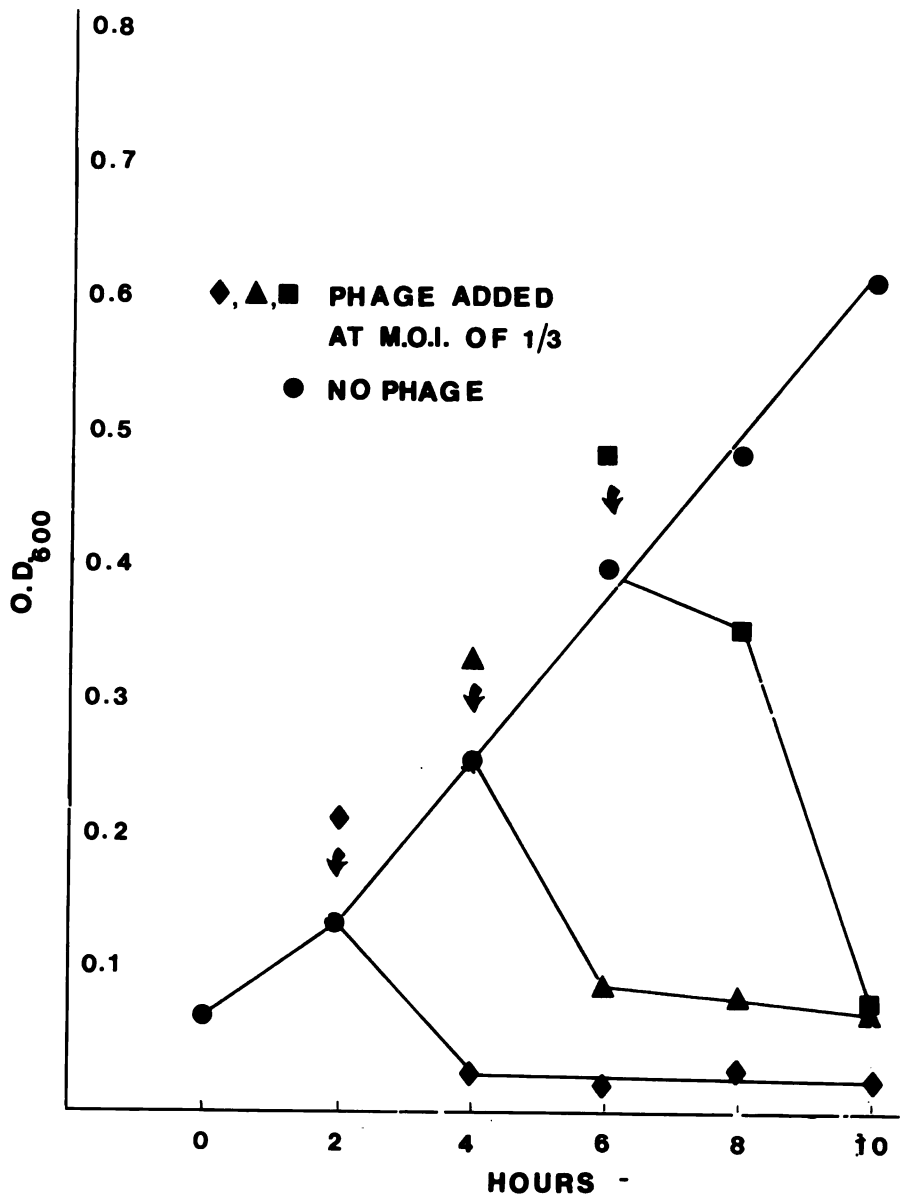

Figure 21. Effect of growth stage on lysis of Ea110R by PEa1(h) in DM-17 medium supplemented with 1mM $CaCl_2$

unaffected by the addition of $ZnSO_4$ to concentrations ranging from 2-100 uM (Figure 22, A&B). Although neither $CaCl_2$ nor $ZnSO_4$ had any effect on lysis of Ea110R by PEa1(h) when added to Davis broth separately, when present together in Davis broth they seemed to greatly enhance lysis. In this experiment the culture grown in Davis broth without added $CaCl_2$ or $ZnSO_4$ only lysed partially and then recovered as was the case for cultures grown in Yurewicz broth or in rich media (Figure 23). The culture grown in the presence of 1 mM $CaCl_2$ and 100 uM $ZnSO_4$ lysed completely.

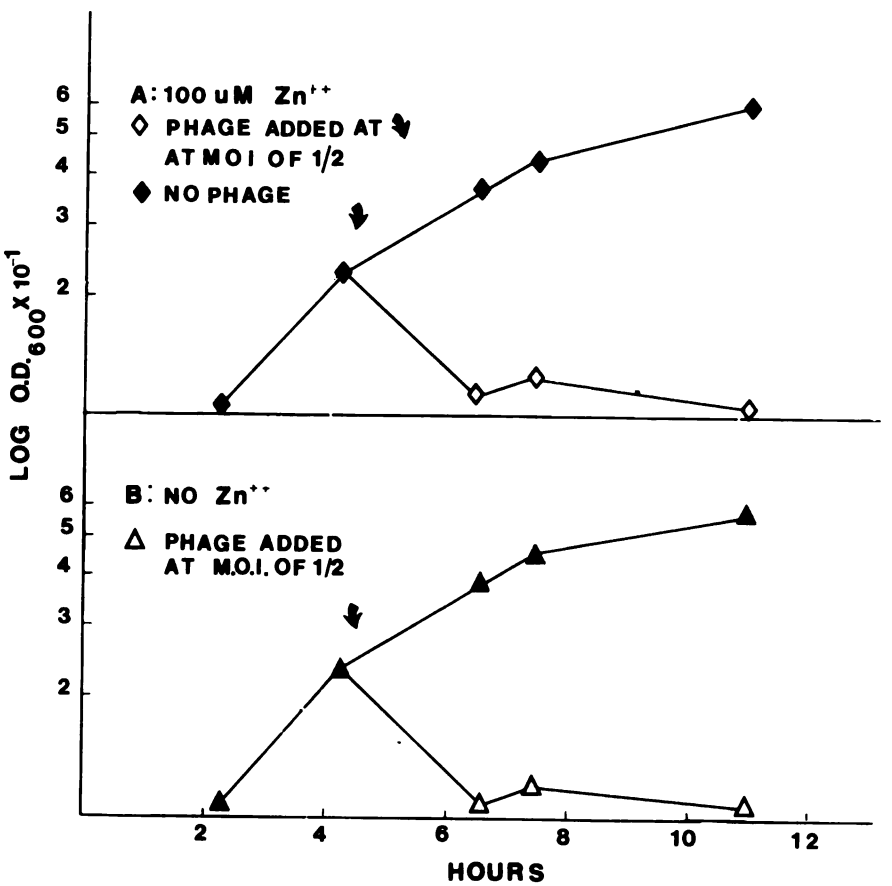

Figure 22. Effect of $ZnSO_4$ on the lysis of Ea110R by PEa1(h) in DM-17 medium. (A). DM-17 supplemented with 100 uM $ZnSO_4$. (B). DM-17 not supplemented with $ZnSO_4$.

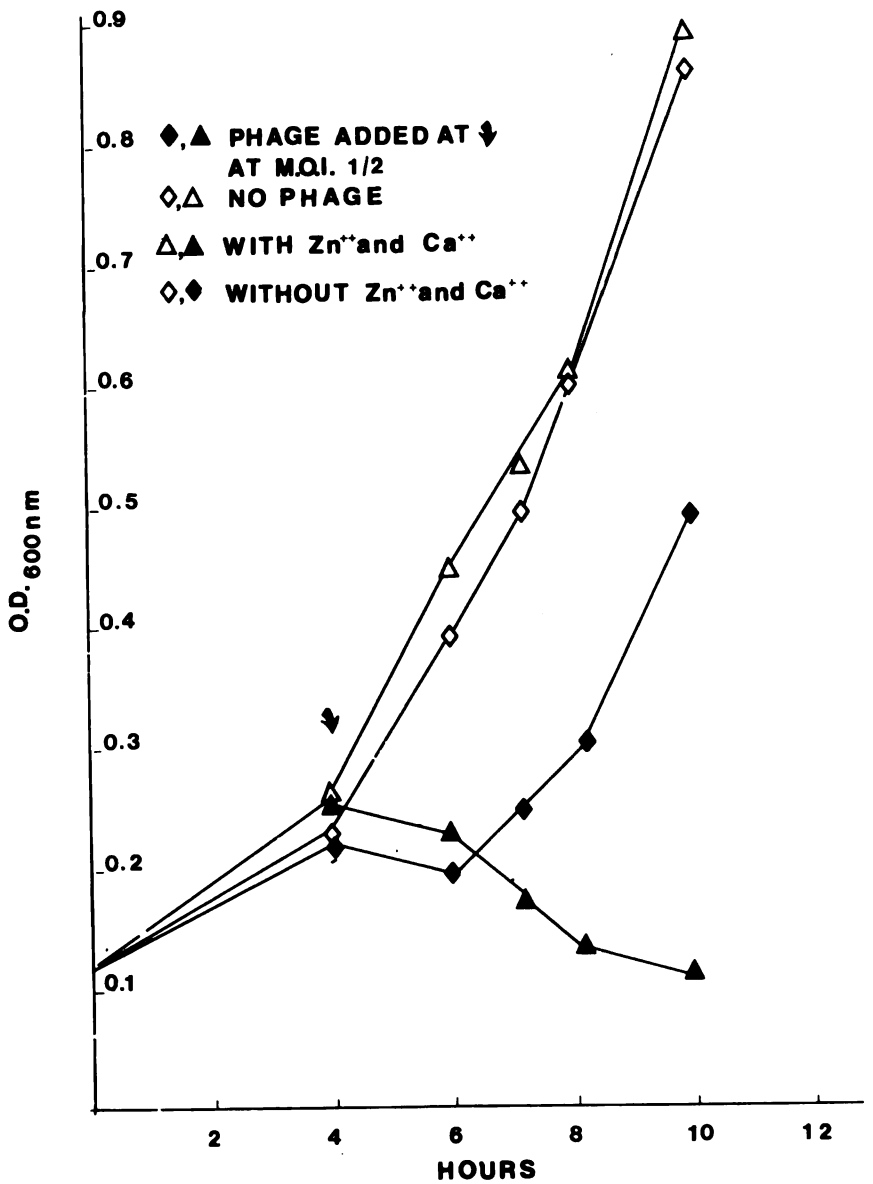

Figure 23. Apparent synergistic effect of $CaCl_2$ and $ZnSO_4$ on lysis of Ea110R by PEa1(h) in DM-17 medium.

DISCUSSION

The results confirm Ritchie's observation that PEA1(h) does not completely lyse Ea110R when grown in NBGYE (Figure 15 and 105).

Yurewicz et. al. (137) purified a polysaccharide depolymerase from bacteriophage infected Aerobacter aerogenes grown in a liquid mineral salts medium supplemented with casamino acids and glucose . Their phage completely lysed the bacterial culture within three hours after inoculation with the phage at a M.O.I. of 1/25. When Ea110R was grown in Yurewicz broth and inoculated with PEa1(h), the phage failed to lyse the bacterial culture (Figure 16), even at a M.O.I. of 1/1, just as was the case when NBGYE was the growth medium.

In contrast to the situation observed with other liquid media, phage PEa1(h) was able to completely lyse a culture of Ea110R growing in D-17 medium (Figure 18). Lysis was not influenced by either Ca^{++} or Zn^{++} ions when they were added singly to D-17 broth (Figures 20 and 21). However the presence of Ca^{++} and Zn^{++} ions together in D-17 did promote lysis in one experiment (Figure 22). The reason for

this apparent synergistic interaction of the ions without any effect when the ions were present singly is not understood.

As can be seen in Table 1, the composition of Yurewicz broth and DM-17 are quite similar, yet PEA1(h) can lyse Ea110R in DM-17 but not in Yurewicz's broth. The difference in glucose concentration was shown not to be significant since more complete lysis occured in DM-17 containing either 0.2% or 1.0% glucose than in Yurewicz's broth at either glucose concentration (Figures 17-19). The very slight difference in pH between the two media appears insignificant, but the difference in total ionic strength of the two media may be significant (3). Since phage PEa1(h) lyses Ea110R much better in DM-17 broth than in other liquid broths, this broth should be useful in producing PD for purification and characterization.

The conclusion of Ritchie (107), that PEa1(h) requires intact capsular polysaccharides for infection of E. amylovora, is similar to that of Stirm (121) and Bayer (16). This model is supported by the observation reported in chapter 2 that a strain Ea110R which contained and expressed the cloned polysaccharide depolymerase gene of PEa1(h) produced turbid, not clear plaques on DM-17 agar. This result mimics in a solid medium the rapid growth of phage resistant bacteria observed in NBGYE or Yurewicz broth which resulted in very turbid cultures after phage infection, presumably due

to the enzymatic removal of capsular polysaccharides required for phage binding in each case.

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