

Extracellular Polysaccharide of *Erwinia amylovora*: a Correlation with Virulence†

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The extracellular polysaccharides produced as slime or capsule layers by bacterial pathogens of animals and plants have been often implicated as factors essential to pathogenesis. In the present study, virulence of the plant pathogen *Erwinia amylovora* was correlated with the ability to produce extracellular polysaccharide (EPS). EPS production by a series of field isolates and bacteriophage-resistant mutants differing in the extent to which they cause symptoms in host tissues was examined by quantitation with a modified Laurell rocket immunoelectrophoresis assay. The amount of EPS produced as an easily removed capsular layer or slime on solid nutrient agar approximated the capacity to exhibit symptoms in host inoculation tests. Features common to the virulent isolates are mucoid colony morphology, sensitivity to EPS-specific bacteriophages [$S\phi 3$ and PEal(h)], and ability to produce a characteristic EPS. Mutants selected for resistance to $S\phi 3$ or nonmucoid colony morphology are deficient in EPS production and have lost the ability to multiply in host tissue and cause symptoms. We conclude that EPS may be directly involved in symptom expression and provide a function essential to the growth of the pathogen in host tissues.

The surface of gram-negative bacterial cells is covered with a variety of carbohydrates. These carbohydrates, including most prominently the extracellular polysaccharide (EPS) and the lipopolysaccharide, have been implicated as determinants in the pathogenesis of animals and humans (24). The production of EPS in the form of a capsule or a free slime or both has been also associated with virulence in several plant pathogenic bacteria (4, 8, 10, 12, 15, 19). These polysaccharides are thought to be involved in symptom expression, e.g., wilting due to decreased vascular flow (6, 8, 10, 26), and it has been suggested that the production of EPS may interfere with plant defenses which involve recognition of other bacterial cell wall components such as lipopolysaccharide (22).

Virulence in *Erwinia amylovora*, the fire-blight pathogen of rosaceous plants, is also characterized by the copious production of EPS in the form of a capsule and slime (2, 3, 8). Mutants lacking EPS are avirulent (9, 10, 21). EPS-deficient mutants of *E. amylovora* fail to multiply in host tissue (S. Ouchi, personal communication) and are observed by electron microscopy to be agglutinated in xylem vessels by minute

granules apparently of host origin (13). In the present study, virulence of *E. amylovora* is correlated with the amount of EPS produced by a series of field isolates and mutants differing in the extent to which they cause symptoms in host tissues.

(A preliminary account of the production of the EPS amylovorin in vitro was presented at the annual meeting of the American Phytopathological Society, 1977, East Lansing, Mich. [Proc. Am. Phytopathol. Soc. 4:107, abstr. no. 116, 1977].)

MATERIALS AND METHODS

Cells. *E. amylovora* (Burrill) isolates E9, 101b, 60b, and 121a were obtained from infected apple trees, cultivar Jonathan. Identification as *E. amylovora* was based on characteristic cratered colony morphology on Crosse-Goodman medium (6) and by assay for virulence using petiole inoculation of apple shoots (13). A spontaneous acapsular mutant E8 of the capsulated strain E9 was isolated from agar culture plates by using a shift from fluidal-mucoid to butyrous colony morphology as a guide for selection. Cultures were stored in lyophilized form or in glycerol (40%) at -20°C . Working cultures were initiated from single colonies of correct colony morphology and were periodically checked for bacteriophage sensitivity. The characteristics of the isolates and mutant strains are presented in Table 1.

Media. Nutrient broth supplemented with yeast extract and glucose, prepared as previously described

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TABLE 1. Bacteriophage sensitivity, virulence, and colony morphology of strains of *E. amylovora*^a

Isolate	Bacteriophage sensitivity				Virulence	Colony morphology ^b
	Sφ2	Sφ3	Sφ4	Sφ5		
E9	+	+	+	+	13	M
101b	-	+	-	-	6	M ^c
60b	-	+	+	+	2	M ^d
121a	-	-	+	+	0	S
AA2 ^e	-	+	+	+	13	M
AA3,E8 ^e	+	-	+	+	0	S
AA4 ^e	+	+	-	+	13	M
AA5 ^e	-	-	+	+	0	S
AA6 ^e	-	-	-	+	0	S

^a Four field isolates of *E. amylovora* (E9, 101b, 60b, 121a) and six mutants of E9 were compared for their ability to support the growth of four bacteriophages, virulence as judged by symptom expression in apple shoot inoculations (centimeters of tissue involvement), and colony morphology.

^b Colony morphology was found to be of two basic types: mucoid (M); and nonmucoid, smooth (S).

^c Isolate 101b exhibited a convoluted central region of high cell density surrounded by a mucoid matrix giving an opalescent appearance.

^d The mucoid matrix surrounding cells of isolate 60b was very sticky in comparison to the more fluidal matrix of other isolates.

^e Mutant of E9; these were selected by their resistance to the bacteriophages indicated; e.g., AA3 was selected for resistance to Sφ3. E8, which had properties identical to AA3, was selected by altered colony morphology.

(23), was the basic liquid (NYGB) and solid medium (NYGA) used. TTC medium, composed of NYGA supplemented with 0.5% triphenyltetrazolium chloride (16), was used for the determination of colony morphology. The soft-agar medium used in overlay assays was composed of 10 g of tryptone (Difco Laboratories), 1 g of yeast extract, 8 g of NaCl, 1 g of glucose, and 8 g of agar per liter.

Bacteriophages and bacteriophage-resistant mutants. Phages were obtained from D. F. Ritchie, Michigan State University, East Lansing [PEal(h)], and locally from raw, filter-sterilized sewage after enrichment (1). All bacteriophages were purified by repeated single-plaque isolation as described by Adams (1). Phages Sφ2 and Sφ3 were isolated directly on E9. Sφ4 was isolated on a mutant of E9 (AA5), which was selected for resistance to phages Sφ2 and Sφ3. Similarly, Sφ5 was isolated on a mutant (AA6) resistant to phages Sφ2, Sφ3, and Sφ4.

Plaque morphology was determined at 24 and 48 h of incubation with E9 at 27°C. Absorption of bacteriophages to cells of *E. amylovora* was measured by the reduction in the titer of bacteriophage suspensions (3×10^8 plaque-forming units/ml) after various periods of incubation (27°C) with cells (10^9 colony-forming units/ml) subsequently killed by chloroform (1). A one-step growth experiment with PEal(h) was performed as described by Ritchie and Klos (21), with the

exception that NYGB was used at a temperature of 27°C.

Sensitivity to phages was assayed by the spot assay and soft-agar overlay methods (1). The soft agar used was composed of 10 g of tryptone (Difco), 1 g of yeast extract (Difco), 8 g of NaCl, 1 g of glucose, and 8 g of agar per liter.

Spontaneous mutants resistant to particular bacteriophages were obtained as single colonies growing in soft-agar overlays containing a sufficient number of the phage of interest to yield confluent lysis of the parent bacterium (1).

Quantitation of free EPS. NYGA slants were streaked with bacteria from single colonies confirmed to be of the correct colony morphology. The slants were incubated horizontally for 24 h at 27°C. The surface of the slants was washed thoroughly by blending in a Vortex mixer with two 5-ml volumes of phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.0, 0.15 M NaCl). This and all subsequent steps were performed at 0 to 4°C. The two washes were combined to form the original suspension of bacteria and associated polysaccharide. Two samples were removed from the original suspension for determinations of cell density. The first sample was diluted 1:100 into PBS containing 0.01 M sodium azide. This sample was used, after appropriate dilutions, for measurements of cell densities by turbidity at 550 nm and for direct cell counting, using interference contrast optics with a Petroff-Hausser counting chamber. Turbidity (one unit of absorbance at 550 nm is equivalent to 8×10^8 colony-forming units) was found to be the most reliable of the cell counting methods and was used in subsequent calculations involving cell numbers. The second sample was diluted into NYGB and plated by spreading on NYGA plates, which were scored for viable cell numbers after 30 h at 27°C.

Bacteria in the original suspension were separated from soluble polysaccharide by centrifugation ($27,000 \times g$, 10 min). Two further washings in 5 ml of PBS with Vortex mixing to suspend the bacteria were performed. The EPS content was determined with a modified Laurell rocket assay developed for EPS (see below). The EPS production per cell was determined from the amount of EPS released in each of three washes compared with the total number of bacteria present in the original suspension. A minimum of six determinations was performed on each strain.

Isolation and fractionation of EPS. NYGA plates were spread with approximately 10^6 bacteria per plate and incubated at 27°C for 48 h. The bacteria and the associated extracellular polysaccharide were washed from the surface of the agar by two applications of 5 ml of PBS. The concentration of bacteria in the suspension was determined by dilution plating, turbidity at 550 nm, and direct cell counts using a Petroff-Hausser counting chamber. Suspended bacteria were removed by centrifugation ($27,000 \times g$, 10 min). The supernatant was then passed through a nitrocellulose filter (0.65 μm; Millipore Corp.) and rotary evaporated under reduced pressure to achieve a fivefold increase in solute concentration. Ethanol was added to a final concentration of 70%. The resulting precipitate was collected by centrifugation, dissolved in 0.1 M sodium ethylenediaminetetraacetate,

pH 7.0, and exhaustively dialyzed against deionized water. This material was then lyophilized to yield crude EPS. The yield of crude EPS per bacterium was based on the total number of cells extracted.

EPS was fractionated by chromatography on a column (2.2 by 90 cm) of Bio-Gel A-50m (Bio-Rad Laboratories), equilibrated and eluted with E buffer (0.1 M potassium phosphate buffer, pH 7.0, 0.005 M EDTA, 0.10 M NaCl) at a flow rate of 8 ml/h. The void volume (120 ml) was determined by using glutaraldehyde-fixed bacteria, and the included volume (291 ml) was determined with glucose. Fractions of 6.3 ml were collected and analyzed for polysaccharide by the anthrone method (7). Polysaccharide content is expressed in equivalents of galactose. Molecular weights were estimated from the nominal exclusion limit of Bio-Gel A-50m (5×10^7 daltons).

Degradation of EPS with phage-bound enzyme. Bacteriophage PEal(h) was purified by differential centrifugation. Phage particles (10^{10} plaque-forming units) were incubated with E9 EPS (20 mg of galactose equivalents by anthrone) for 15 h at 27°C in 3 ml of PBS. The control sample of EPS was incubated under the same conditions, but without bacteriophages. Bacteriophages were removed by centrifugation ($27,000 \times g$, 1 h).

The polysaccharides in the control and after treatment with bacteriophage particles were fractionated by chromatography on a column (2.2 by 80 cm) of Bio-Gel A-15m (Bio-Rad), using E buffer at 8 ml/h. The void volume (127 ml) was determined, using glutaraldehyde-fixed bacteria, and the included volume (320 ml) was determined with glucose. Fractions of 6.5 ml

were collected and analyzed for polysaccharide by the anthrone method (7).

Immunological techniques. Suspensions containing approximately 5×10^8 cells of E9 per ml were treated with Formalin (0.3%) for 1 h at 20°C and were washed twice by centrifugation in PBS. Two 0.5-ml subcutaneous injections of the Formalin-inactivated bacteria emulsified with equal volumes of Freund incomplete adjuvant were made at weekly intervals in white New Zealand rabbits (2 kg). A week after the fifth injection, blood was withdrawn by heart puncture and stored at 20°C for 4 h, and serum was collected. The immunoglobulins were partially purified by ammonium sulfate precipitation (14). Typical agglutination titers for the partially purified antisera were 5,000 to 10,000 with washed cells of E9.

A modified form of the Laurell rocket assay (17, 28) was developed for determinations of EPS content in bacterial extracts. This assay also permits detection of monovalent antigenic determinants corresponding to those present in EPS. Two adjacent sections of agarose were formed between two glass slides (57 by 85 mm) separated by 0.8 mm with glass spacers at the corners (Fig. 1). The first section (15 by 85 mm) was formed from 0.6% ME agarose (Miles Laboratories) in 0.025 M sodium barbital buffer, pH 8.2 (B buffer). The second section was formed from 0.5% ME agarose in B buffer, containing 7.5 μ l of partially purified antiserum raised to E9 per ml. A 2-mm-wide trough was cut in the first section of agarose, 3 mm from the junction of the two sections. The trough was filled with 100 μ l of melted 0.3% ME agarose in B buffer, containing 0.1 μ g of purified E9 EPS per ml. Wells 2.5 mm in diameter

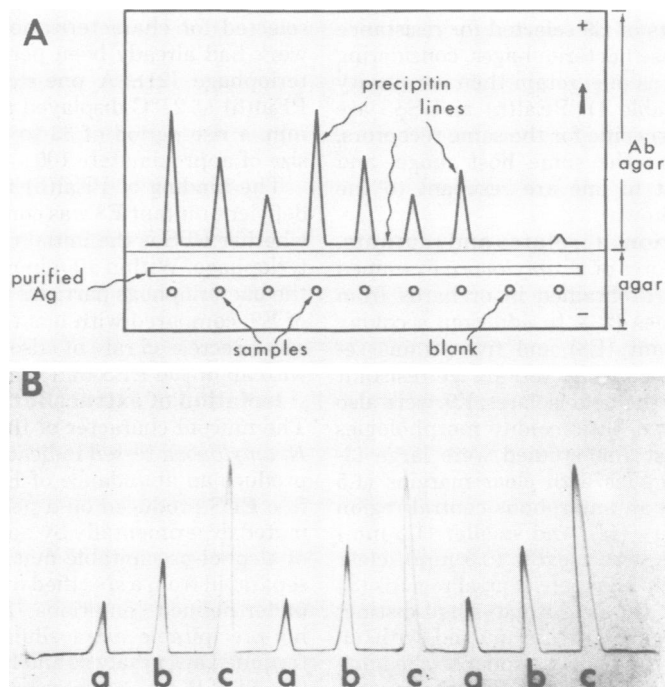


FIG. 1. (A) Diagram of the line-rocket assay; (B) precipitin lines with replicates of three samples (a, 5 ng; b, 10 ng; c, 15 ng) of E9 EPS. Precipitin lines were stained with Coomassie brilliant blue.

were punched in the first agarose section, and these were filled with 3.0 μ l of purified E9 EPS or a sample to be tested for EPS antigen. An electric potential of 10 V/cm with a current of approximately 2 mA per slide was applied for 10 h, using a water-cooled electrophoresis cell (Bio-Rad model 1400). The developed slides were stained by the procedure of Weeke (27). The EPS concentration is proportional to the height and area of the precipitin rockets and is calibrated by a series of standards of purified EPS from E9 included on each slide. Monovalent antigens were detectable by the solubilization of the precipitin base line.

Wilt assay. The ability of EPS to wilt shoots of apple, pear, and quince cultivars representing a broad spectrum of sensitivity to *E. amylovora* was tested with the standard wilt assay and the cultivars previously described (11). EPS from E9 was used in these tests at a concentration of 100 μ g of galactose equivalents per ml, as measured by the anthrone procedure (7).

RESULTS

Bacteriophage characteristics. The plaque morphologies of the bacteriophages examined in this study differed significantly. PEal(h), obtained from blighted Jonathan apple terminals (21), produced a clear plaque 4 to 5 mm in diameter with a spreading halo. Bacteriophage S ϕ 3 produced the same large plaques with spreading halos as PEal(h). S ϕ 2 and S ϕ 4 produced small (0.2-mm), clear plaques. The plaques of S ϕ 2 are surrounded by a cloudy margin. S ϕ 5 produces a clear plaque 2 to 3 mm in diameter. Mutants of E9 selected for resistance to each of the these bacteriophages, considering S ϕ 3 and PEal(h) as one, retain their sensitivity to the others (Table 1). PEal(h) and S ϕ 3 are considered to be specific for the same receptors, since they exhibit the same host range, and mutants resistant to one are resistant to the other (data not shown).

Characterization of isolates and mutants. The four field isolates of *E. amylovora* examined in this study were obtained in orchards from infected apple trees (23). In addition, a colony morphology mutant (E8) and five strains selected as spontaneous bacteriophage-resistant mutants of one of the field isolates, E9, were also examined. The two basic colony morphologies observed in the strains studied were large (3-mm), mucoid colonies with clear margins (1.5 mm) surrounding an amorphous central region of high cell density (M), and smaller (1.5 mm) butyrous colonies with narrow (0.5 mm), clear margins surrounding discrete central regions (S) (Table 1). Two of the field isolates had distinct colony morphologies; 101b is mucoid with an opalescent center, and 60b is similar to the large colony type, with the exception that the mucoid matrix of the colonies is very sticky.

The sensitivity of the various strains to a

series of five bacteriophages isolated from sewage or an infected apple tree was determined by spot assays and the ability to form plaques (Table 1). All of the bacteriophages obtained from sewage were isolated on E9 or mutants of E9. The field isolates each differ in their bacteriophage sensitivity, with the single exception that all virulent isolates are sensitive to S ϕ 3. Bacteriophage PEal(h) was identical to S ϕ 3 in its host range. The mucoid colony morphology characteristic of EPS production was observed in all strains sensitive to phage S ϕ 3.

Virulence of the strains was investigated by using a standard petiole inoculation of apple shoots (Table 1). Four different levels of virulence could be distinguished, ranging from avirulence (0 cm of tissue involvement) with minimal symptoms only at the wound site, e.g., 121a and AA3, to involvement of the entire apical region of the inoculated test shoot (13 cm), e.g., E9. Virulence, regardless of the magnitude of symptom expression, is associated with sensitivity to S ϕ 3 [or PEal(h)]. Moreover, mutants selected for resistance to S ϕ 3 or PEal(h), e.g., AA3, have a smooth colony morphology indicative of a deficiency in EPS production and have lost their virulence. Mutants selected for resistance to phages other than S ϕ 3 retain their virulence.

The observation that sensitivity to S ϕ 3 and PEal(h) was correlated with virulence prompted further study of this phage type. PEal(h) was selected for characterization, since some basic work had already been performed on this bacteriophage (21). A one-step growth curve of PEal(h) at 27°C displayed a latent period of 33 min, a rise period of 33 to 43 min, and a burst size of approximately 100.

The binding of PEal(h) to E9 and the EPS-deficient mutant E8 was compared to determine whether EPS is the initial receptor for this bacteriophage. Within 10 min of incubation, 98% of the bacteriophage particles are adsorbed to cells of E9, compared with less than 10% for E8. The same decreased rate of adsorption was observed with all of the EPS-deficient strains tested.

Isolation of extracellular polysaccharide. The mucoid character of the virulent strains of *E. amylovora* tested indicates that these strains produce an abundance of EPS. The amount of free EPS produced on a per cell basis was estimated experimentally by measuring the amount of alcohol-precipitable material which could be separated from a specified number of cells grown under defined conditions. The amount of alcohol-precipitable extracellular material was determined after dialysis and lyophilization (Table 2). The three isolates produced comparable quantities of extracellular material, regardless of their virulence. The EPS-deficient mutants, rep-

TABLE 2. Production of alcohol-precipitable material and EPS by strains of *E. amylovora*

Isolate	Virulence	Extracellular precipitate (mg/10 ¹² cells) ^a	Free EPS content (mg/10 ¹² cells) ^b
E9	13	20.5	14.5 ± 1.1
101b	6	23.1	9.3 ± 3.8
60b	2	21.4	7.8 ± 2.9
AA3	0	0.6	0.0

^a Extracellular alcohol-precipitable material produced by *E. amylovora* isolates E9, 101b, and 60b and an EPS-deficient mutant of E9, AA3, was measured by weighing the material obtained by precipitation of the supernatant of a 48-h culture grown on NYGA after precipitation in 70% ethanol.

^b Determined directly on diluted supernatant using the line-rocket assay. Free EPS is expressed in milligrams (\pm standard error of the mean) of galactose equivalents of EPS required to give the same peak height in the line-rocket assay. Cell numbers were determined by turbidity at 550 nm.

represented by AA3, produced very little extracellular material, and as will be demonstrated subsequently, no EPS.

The term EPS is reserved for material which reacts immunochemically in the line-rocket assay to give a line of identity with the EPS from E9. The amount of EPS removed from cells of E9 by three successive washings was determined to estimate the ease with which EPS present as a loosely bound layer or soluble slime can be separated from cells. The first wash routinely yields 95% of this free material, and the second wash yields 4 to 5%. Subsequent washes yield less than 1% of the total free material.

The amounts of free EPS obtained by a single wash from four field isolates and two mutants selected for decreased EPS production were compared by using the line-rocket assay to quantify EPS (Table 2). E9, the most virulent isolate tested, produced the largest quantity of free EPS per cell (14.5 \pm 1.1 mg/10¹² cells). Isolates 101b and 60b, which exhibit attenuated virulence, show a decreased ability to produce EPS relative to E9. AA3, representative of the avirulent strains, such as 121a, E8, and AA5, which have smooth colony morphology, was found to produce less than 0.01 mg of EPS per 10¹² cells, the detection limit of the assay. These EPS-deficient strains fail to produce even fragments of free EPS, since no monovalent antigen homologous to E9 EPS is detectable.

Fractionation of EPS. Cells from suspensions of several strains of *E. amylovora* grown on solid media were removed by centrifugation to yield preparations of soluble extracellular ma-

terial. The soluble polysaccharide was precipitated in 70% ethanol, treated with ethylenediaminetetraacetic acid to remove divalent cations, dialyzed extensively against water, and lyophilized. The crude EPS from each of these strains was fractionated on a gel permeation column (Bio-Gel A-50m, nominal exclusion limit 50 \times 10⁶ daltons), and the effluent was analyzed for hexose content (Fig. 2). Two of the strains tested (E9 and 101b) had similar elution profiles (estimated mean molecular weight of the heterogeneous EPS, 40 \times 10⁶), whereas 60b appeared to contain in addition a component with a lower molecular weight than the E9 EPS. The alcohol-precipitable polysaccharide found in the supernatant of AA3 suspensions was much lower in molecular weight (ca. 2.5 \times 10⁶) than the E9 EPS and was present in a much lower amount (Table 2).

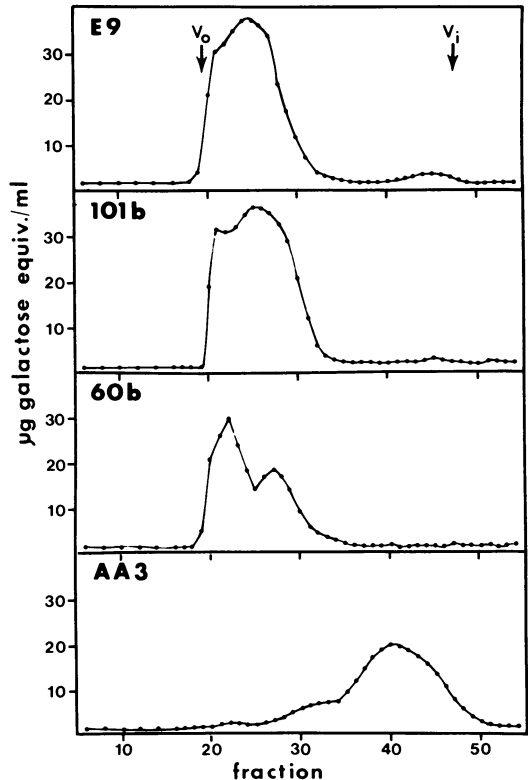


FIG. 2. Gel permeation chromatograph of the ethanol-precipitable material from isolates E9, 101b, 60b, and an EPS-deficient mutant, AA3, of *E. amylovora*. Samples (10 mg each) of material precipitated from the supernatant of cultures grown for 48 h on nutrient agar plates were fractionated by chromatography on Bio-Gel A-50m. Polysaccharide content is expressed in microgram equivalents of galactose as measured by the anthrone procedure.

The antigenic characteristics of each of the peak fractions of the sizing column effluent (Fig. 2) were examined by using the line-rocket assay. Equal hexose equivalents (10 ng) of each of the peak fractions were applied in the assay, and the resultant precipitin lines formed with purified E9 EPS as a standard were developed. E9, 101b, and 60b each produce a predominant EPS which gives the same response in the line-rocket assay. The lower-molecular-weight component of 60b EPS gives only 30% of the response observed with EPS from the other strains, indicating that the predominant high-molecular-weight component extending into this region may be solely responsible for the activity of this fraction. Hence, the lower-molecular-weight component of 60b is likely to be antigenically distinct from E9 EPS. The alcohol-precipitable material obtained from the EPS-deficient mutant, AA3, and the avirulent isolate 121a gave no response in the line-rocket assay for EPS.

EPS degradation with phage-bound enzyme. The spreading halos observed around plaques of S ϕ 3 and PEal(h) are characteristic of bacteriophages which bind to and depolymerize the capsular polysaccharides of their hosts (18). The depolymerizing enzymes are found both as structural components of phage particles or in a free, soluble form in lysates of capsule-specific bacteriophages or both (18). PEal(h) was examined for a phage-bound, depolymerizing enzyme by incubating phage particles with EPS

from E9. The size distribution of EPS after phage treatment was compared with that of untreated EPS by gel permeation chromatography (Fig. 3); it had decreased dramatically to a molecular weight of approximately 10^5 .

Wilting activity of EPS. EPS induced wilting in apple, pear, and quince shoots with the same pattern as previously observed with the closely related, if not identical, polysaccharide termed amylovorin obtained from apple tissue inoculated with *E. amylovora* (13). Cultivars resistant to *E. amylovora* exhibited only minimal wilting in a few leaves throughout the course of the test (3 h), whereas cultivars susceptible to infection by *E. amylovora* showed severe wilting induced by EPS.

DISCUSSION

The field isolates of *E. amylovora* examined in this study differ in colony morphology, bacteriophage sensitivity, and virulence. These differences underscore the wide natural variation in the surface characteristics of this plant pathogen. The features common to the virulent isolates, mucoid colony morphology and sensitivity to bacteriophage S ϕ 3, can be attributed to the production of a characteristic EPS.

The observed differences, with the exception of mucoid character, in the colony morphologies of *E. amylovora* field isolates may result from surface components other than EPS. The distinctive opalescent colony morphology of isolate

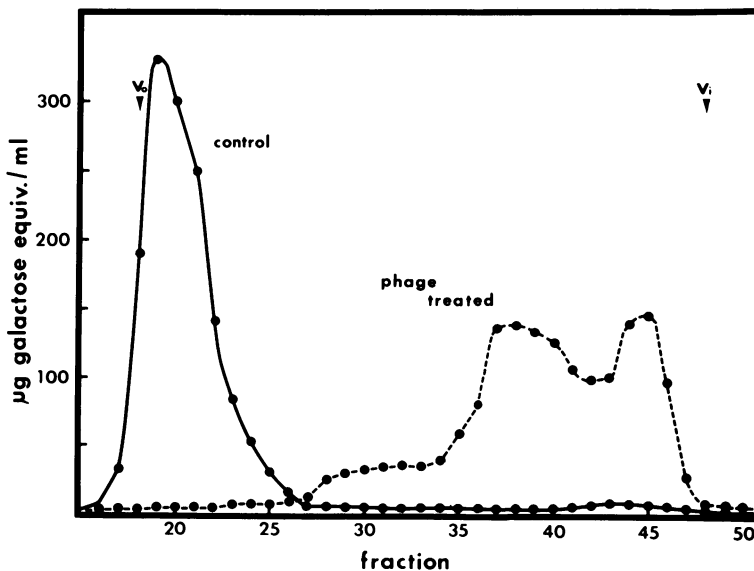


FIG. 3. Bacteriophage treatment of EPS. EPS from E9 was treated with particles of bacteriophage PEal(h). The treated EPS was fractionated by chromatography on Bio-Gel A-15m (---). Untreated EPS was similarly fractionated by size for comparison (—). Polysaccharide content is expressed in microgram equivalents of galactose as measured by the anthrone procedure.

101b, for example, is not likely to result from its EPS, since the EPS of 101b has the same size distribution and antigenic determinants as that of E9, although it is produced in lower quantity. Moreover, the observation that 101b is insensitive to three bacteriophages (S ϕ 2, S ϕ 4, and S ϕ 5) to which E9 is sensitive is consistent with significant differences in the surface components of E9 and 101b. The opalescent morphology may, in fact, result from the absence of surface components which serve as receptors for bacteriophages S ϕ 2, S ϕ 4, and S ϕ 5.

The variation observed in this study with a series of bacteriophages isolated from sewage is in contrast to that observed with bacteriophages isolated only from apple trees infected with *E. amylovora* (3, 9, 21). In the previous studies with bacteriophages from orchards, virulent isolates could not be distinguished by differences in bacteriophage sensitivity.

The bacteriophages isolated from sewage appear to have a much broader range of receptor specificities than those obtained from sources in which *E. amylovora* is prevalent. The increased use of a large standard set of bacteriophages with a broad spectrum of receptor specificities should permit the unequivocal identification of the strains of *E. amylovora* now being studied in various laboratories and be very useful in investigating the spread of strains during epiphytotic.

Our assertion that EPS is the initial receptor for the binding of bacteriophages S ϕ 3 and PEal(h) to *E. amylovora* is based on two lines of evidence. First, a comparison of the rate of binding to cells of *E. amylovora* possessing either normal capsules of EPS or deficient in EPS demonstrates a much decreased rate for EPS-deficient cells. These results are consistent with those of Ritchie and Klos (21; personal communication). Presumably, the decreased binding efficiency is responsible for the inability of EPS-specific bacteriophages to produce plaques on EPS-deficient cells. Also, the apparent molecular weight of EPS is reduced by an enzyme bound to virions of PEal(h). This enzyme may in fact provide the basis for binding of EPS-specific phages to capsulated cells. Thus, the initial interaction of the phage with the cell may be through the binding of the phage-bound enzyme to its substrate, the EPS in the capsule. Subsequently, the phage will come into contact with deeper layers of the cell wall of the bacterium as the enzyme degrades more of the capsule. These deeper layers might then provide the receptor sites which trigger irreversible binding and the transfer of nucleic acids from the phage to the bacterium (18).

Four field isolates representing a gradation in

the ability to exhibit symptoms in host tissues were selected from a collection of several hundred isolates of *E. amylovora* obtained from all of the areas of the world reporting fireblight (Second International Congress of Plant Pathology, Minneapolis, Minn., abstract no. 934, 1973). It was hoped that the decreased virulence expressed by other than the most virulent isolate would reflect modifications in factors essential to virulence. The present study indicates that the role of pathogen surface components in pathogenesis is more complex than anticipated. Several variations exist in the components of the isolates tested, as reflected in the diverse patterns of bacteriophage sensitivity. Many components are apparently nonessential for virulence. The single component found to be essential for virulence was EPS.

EPS production in vitro roughly correlates with the extent of symptom expression observed in host tissue (Table 2). The isolate producing the largest amount of EPS in culture, E9, exhibited the most extensive symptoms in host tissue, whereas isolates producing less EPS exhibited correspondingly decreased symptoms. Moreover, mutants selected for nonmucoid colony morphology (E8) or resistance to EPS-specific bacteriophages (AA3) are deficient in EPS production and lack the ability to multiply and cause symptoms in host tissue. These data confirm and extend the correlation of virulence with capsulation in *E. amylovora* (3, 9, 10), and with mucoid colony characteristics in *Pseudomonas solanacearum* (15, 16) and *Xanthomonas phaseoli* (4).

The fact that EPS production is related to symptom expression presents the possibility that EPS may be directly involved in the production of symptoms. A polysaccharide obtained from apple fruit inoculated with *E. amylovora* was previously demonstrated to duplicate the wilt symptom of fireblight disease when introduced into the vascular system of susceptible tissue (10). This polysaccharide was given the trivial name of amylovorin. Amylovorin, a galactan with branches consisting of glucuronic acid and glucose (P. R. Stoffl, M. S. thesis, University of Missouri-Columbia, 1976) has the unique property of producing this symptom with cultivar specificity (11). Thus, apple, pear, and quince cultivars which are resistant to inoculations of *E. amylovora* are resistant to the wilting effects of amylovorin solutions. The EPS obtained from a virulent isolate of *E. amylovora* grown in vitro shows this same pattern of cultivar-specific wilting in the series of cultivars used in tests on the activity of amylovorin. In addition, preliminary experiments indicate that the size distribution, composition, and antigenic determinants of EPS

and amylovorin are identical (data not shown). EPS is, therefore, apparently in abundance in inoculated susceptible tissue and can be obtained as the polysaccharide called amylovorin.

We conclude that the extracellular polysaccharide produced as a capsule and slime by virulent isolates of *E. amylovora* is an essential factor in virulence. It is probably directly involved in the wilt symptom exhibited by infected tissue and may be necessary for the growth of the bacteria in host tissue. Other surface components which may be visualized in modifications in colony morphology or bacteriophage sensitivity may, however, contribute to the level of symptom expression observed in response to various isolates of *E. amylovora*.

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