Origin of the Polysaccharide Component of Ooze from Plants Infected with *Erwinia amylovora*

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(Received 21 May 1979)

The composition of extracellular polysaccharides produced by *Erwinia amylovora* in defined culture media was compared with that of the polysaccharide present in the ooze produced during fireblight infection. The results strongly suggested that the ooze polysaccharide was of bacterial origin.

Two distinct polysaccharides were produced *in vitro*. One contained galactose (61 to 69%), glucose (8 to 11%), mannose (2 to 6%) and uronic acid (16 to 23%) and the other contained only fructose. Both the quantity and composition of the polysaccharide produced was determined by the nature and concentration of the sugar or sugar alcohol supplied and by the nature of the nitrogen source.

Evidence is also presented which suggests that production of the ooze-like polysaccharide is a virulence determinant and that this polysaccharide is similar in composition to the capsule. Preliminary studies indicate the presence of capsule and polysaccharide-hydrolysing enzymes in lysates of phage-infected bacteria.

INTRODUCTION

One of the commonest early symptoms of fireblight disease is the exudation of a gummy bacterial mass or ooze which appears as globules, or a spreading film or fine strands on the surface of infected plant tissues. Apart from bacteria, the ooze consists mainly of a high molecular weight polysaccharide composed of galactose (74%), uronide (16%) and small quantities of glucose and possibly mannose (Eden-Green & Knee, 1974; Goodman *et al.*, 1974). The origin and function of the polysaccharide constituent of the ooze are not clear. Eden-Green & Knee (1974) extracted similar polysaccharides from bacteria grown on yeast extract peptone agar with added galactose, glucose or sorbitol. Beer *et al.* (1977) have also reported finding a similar polysaccharide in nutrient broth shake cultures of *Erwinia amylovora*. Goodman *et al.* (1974), however, failed to detect this polysaccharide in cultures of *E. amylovora* after 48 h growth in a defined liquid medium and suggested that the ooze polysaccharide was produced as a result of an interaction between the pathogen and a susceptible host.

The initial object of this study was to determine whether the ooze polysaccharide was of bacterial origin. Evidence is also presented which suggests that polysaccharide production is a virulence determinant, that the free extracellular polysaccharide is similar in composition to the capsule and that the nature and concentration of the carbon source affects the formation of both.

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Table 1. *Origin and yields of polysaccharides analysed*

Source of polysaccharide	Strain*	Polysaccharide yield†
<i>In vivo</i>		
Apple, pear or hawthorn shoots	T	++
Pear fruit slices	T, AT, E9	++
<i>In vitro</i> ‡		
ASP		
+ 1 % sorbitol	T, AT, E9, P66	++
+ 1 % sorbitol	P70, 4S1, 4S2	
	4L1, 3A2B, E8	—
+ 1 % glucose	T	++
+ 1, 5 or 10 % sucrose	T	++
+ 5 or 10 % glucose	T	—
+ 5 or 10 % sorbitol	T	—
ARJ		
+ 1, 5 or 10 % glucose	T	—
+ 1, 5 or 10 % sorbitol	T	—
+ 1, 5 or 10 % sucrose	T	+

* Virulent strains: T, AT, E9. Avirulent strains: P70, 4S1, 4S2, P66, 4L1, 3A2B, E8.

† Polysaccharide yield: high ++, low +, not detected —.

‡ ASP, Asparagine salts medium; ARJ, ammonium salts medium.

METHODS

Cultures. Most of the cultures were from a collection at East Malling Research Station; these were virulent strains T and AT (a variant colony from T) and avirulent strains 4S1, 4S2, 4L1 (phage-resistant mutants from T), P66, P70 and 3A2B. Two further cultures (one avirulent, E8, and one virulent, E9) were from Professor R. N. Goodman (Columbia University, Mo., U.S.A.).

Sources of polysaccharides. These were from three sources: infected shoots, infected immature pear fruit slices and supernatants from shake cultures grown in defined liquid media. With two hosts, two defined media, three carbon sources and several different strains of the organism it was impossible to examine the polysaccharides from all the possible combinations. Those selected were chosen to compare the polysaccharides produced by (1) a single strain in several hosts, (2) several strains in a single host, (3) a single strain in several media and (4) different strains in a single medium. Details of the hosts, media and strains used are shown in Table 1.

(i) *Production in vivo.* Ooze was collected from apple, pear and hawthorn shoots which had been inoculated with *E. amylovora* strain T and from immature 'Conference' pear fruits which had been inoculated with strains T, AT or E9. Bacteria were removed from aqueous solutions of the ooze (about 1 %, w/v) by centrifuging at 3750 g for 15 min followed by membrane filtration (Millipore filter, type HA, 0.45 µm pore size).

(ii) *Production in vitro.* Eden-Green & Knee (1974) extracted polysaccharides from bacterial masses grown on the surface of agar plates containing yeast extract and peptone with added galactose, glucose or sorbitol. Use of this method raised several problems as the yeast extract contained polysaccharides and the agar contained D-galactose and derivatives such as 6-methyl-D-galactose and galactose 6-sulphate. Although the risk of contamination of the bacterial polysaccharide may have been negligible it could not be ruled out. A suitable defined liquid medium was therefore sought for polysaccharide production.

The first medium chosen was that of Ayres *et al.* (1919), a mineral salts medium (ARJ) which contained (g l⁻¹): NH₄H₂PO₄, 1.0; KCl, 0.2; MgSO₄·7H₂O, 0.2; nicotinic acid, 0.2; thiamin.HCl, 0.2; Oxoid agar, 12. The second medium was an asparagine-based mineral medium (ASP) which contained (g l⁻¹): asparagine, 4.0; K₂HPO₄, 2.0; MgSO₄·7H₂O, 0.2; NaCl, 3.0; nicotinic acid, 0.2; thiamin.HCl, 0.2; Oxoid agar, 12. Filter-sterilized solutions of glucose, sucrose or sorbitol were added to a final concentration of 1, 5 or 10 % (w/v).

Polysaccharide was produced in 250 ml conical flasks containing 50 ml broth inoculated with about 5 × 10⁷ cells of selected strains of *E. amylovora* grown overnight on yeast peptone agar slopes. Cultures were incubated at 25 °C for 48 h in a Mk 6 rotary shaker incubator (L.H. Engineering, Stoke Poges, Bucks.) at 100 cycles min⁻¹. A 2 cm diameter stainless steel coil spring was positioned around the inside of the flask to aid aeration during incubation. Bacteria were removed from the culture fluids as described above.

(iii) *Extraction.* Before extraction of the polysaccharides, the bacteria-free samples were concentrated to a

final volume of about 10 to 15 ml on a rotary evaporator at 30 °C and then dialysed (Visking tubing 3/32) against 2 × 1 l distilled water at 4 °C for 48 h. Ethanol was then added (final concn 80 %, v/v) and the polysaccharide was precipitated by adding a few drops of saturated CaCl₂ solution. After centrifuging (1500 g, 10 min), the polysaccharide pellet was washed three times in absolute ethanol, dried and stored *in vacuo* over P₂O₅. No attempt was made to purify the polysaccharides further at this stage.

Comparison of polysaccharides.

(i) *Infrared spectroscopy.* Samples were dispersed in KBr discs at a concentration of about 2 mg per 200 mg KBr and spectra were obtained using a Unicam SP1200 infrared spectrophotometer.

(ii) *Descending paper chromatography.* Polysaccharide (2 mg) was heated in a boiling water bath for 40 min with 0.2 ml 2 M-HCl. The solution was evaporated to dryness on a rotary evaporator at 40 °C; the dried sample was redissolved in 1 ml distilled water and re-evaporated. This process was repeated twice more. The dried solid was finally redissolved in 10 % (v/v) aqueous propan-2-ol for chromatography. Samples (3 or 6 µl) of the hydrolysates were spotted on to Whatman no. 1 paper (48 × 21 cm) and descending chromatograms were developed for 24 h using, as solvent, either methyl ethyl ketone/saturated aqueous boric acid/glacial acetic acid (MEK, 9:1:1, by vol.) or butan-1-ol/pyridine/water (BPW, 6:4:3, by vol.). The chromatograms were then dried and the spots were visualized either by dipping the chromatograms in 4-aminobenzoic acid reagent (Saini, 1966) and heating at 105 °C for 10 to 15 min or by dipping them in AgNO₃ solution followed by NaOH and Na₂S₂O₃. The latter is a modification of the method of Trevelyan *et al.* (1950) in which excess Ag₂O was removed using 6 M-NH₄OH.

(iii) *Paper electrophoresis.* Polysaccharide hydrolysate solutions were also subjected to paper electrophoresis. Electrophoretograms were developed on Whatman no. 1 paper in sodium phosphate buffer (0.1 M, pH 8.5) for 90 min at about 30 V cm⁻¹. After drying, the spots were visualized as described above.

(iv) *Gas-liquid chromatography.* Hydrolysis and preparation of volatile sugar acetates was done by the method of Holligan & Drew (1971). The acetate derivatives were separated using a Pye series 104 chromatograph with a flame ionization detector. The oven temperature was 225 °C and 1 µl samples were injected at 300 °C into a 1.7 m × 4 mm column of 5 % silicone OV-275 on Chromosorb W (100 to 120 mesh) fed with N₂ carrier gas at 40 ml min⁻¹. Peaks were eluted and detected isothermally at 275 °C.

(v) *Estimation of uronide content.* This was done using the method of McCreedy & McComb (1952).

(vi) *Gel-filtration on Sepharose 2B.* Eden-Green & Knee (1974) reported that the polysaccharide found in ooze was eluted from a Sephadex G-200 column in the void volume, suggesting a molecular weight in excess of 200000. To obtain a more accurate idea of the molecular weight of this and the other polysaccharides, Sepharose 2B was used, as this has a separation range for globular proteins of between 500000 and 2500000. Fractionation was done at room temperature on a column (37 × 1.8 cm, void volume 30 ml, bed volume 90 ml) equilibrated with 0.4 % (v/v) aqueous pyridine buffer adjusted to pH 5.4 with glacial acetic acid. The height of the column reservoir was adjusted to give a working pressure head of 30 cm and a flow rate of about 25 ml h⁻¹. Samples (1 ml) containing 2.5 mg polysaccharide were loaded on to the column and fractions were collected automatically every 5 min over a 5 h period. Eluate samples were analysed for the presence of carbohydrate by adding 0.5 ml 5 % (w/v) aqueous phenol solution to 1 ml sample, then quickly adding 2.5 ml conc. H₂SO₄ so that the mixture boiled. After cooling, the mixture was incubated at 25 °C for 10 min to allow for colour development and the A₄₈₅ of the samples was measured on a Unicam SP800 u.v. spectrophotometer.

(vii) *Electrophoresis on cellulose acetate.* Unhydrolysed polysaccharides were subjected to cellulose acetate electrophoresis in 0.1 M-sodium acetate buffer pH 6.7 for 10 min at 15 V cm⁻¹. The polysaccharides were visualized by dipping the cellulose strips in toluidine blue for 3 min and then washing in 80 % (v/v) aqueous ethanol until most of the background stain was removed.

(viii) *Polysaccharide-hydrolysing enzyme in crude phage L3H preparations.* Three phages (4S, 4L, L3H) produced haloes of reduced turbidity around their plaques on capsulated cultures. Billing (1960) suggested that these haloes might be caused by a capsule-hydrolysing enzyme. Some evidence supporting this idea has been described (Bennett, 1978*a*). If the loosely bound extracellular polysaccharides produced by *E. amylovora* are the same or very similar to the capsular material, such enzymes could be useful tools in helping to characterize these compounds. An attempt was made to determine whether extracellular polysaccharide-hydrolysing enzymes were present in crude phage L3H preparations (CPP).

A 10 ml portion of L3H phage suspension containing about 1 × 10⁸ plaque-forming units ml⁻¹ was added to 10 ml of a broth shake culture [which contained (g l⁻¹): yeast extract, 3; peptone, 5; glycerol, 10] of strain T containing about 1 × 10⁸ cells ml⁻¹. After 6 h incubation at 25 °C in a Mk 6 rotary shaker incubator (L.H. Engineering), the mixture was centrifuged at 3750 g for 15 min followed by membrane filtration to remove any remaining bacteria. The 20 ml CPP was then concentrated to 5 ml on a rotary evaporator at 30 °C.

Polysaccharides from broth shake culture (ASP+1 % sorbitol) of strain T and from pear slice ooze (strain T) were used in these experiments. To determine whether degradative activity was present, the CPP

Fig. 1. Infrared spectra of polysaccharides produced by *Erwinia amylovora* strain T: (a) polysaccharide from pear slice ooze; (b) polysaccharide from ASP+1% sorbitol; (c) polysaccharide from ASP+10% sucrose.

was mixed in equal proportions with aqueous polysaccharide solutions (10 mg ml^{-1}) and incubated in a water bath at 25°C for 5 h. The incubated solutions were analysed for changes in viscosity, behaviour on gel-filtration with Sepharose 2B and composition determined by descending paper chromatography and paper electrophoresis. Viscosity was measured using an Ostwald viscometer (capacity 1.3 ml), immersed in water at 20°C . Measurements were taken at 5 min intervals for the first 30 min and then at 30 min intervals for the next 4.5 h. Controls were polysaccharide alone and polysaccharide plus heat-treated (80°C for 10 min) CPP.

RESULTS

Comparison of polysaccharides

Infrared spectroscopy. Examples of the spectra from different polysaccharides are shown in Fig. 1. The spectra of polysaccharides produced *in vivo* were the same irrespective of their origin and have been labelled as type 1. The polysaccharides produced by strains T, AT, E9 and P66 in the ASP+1% sorbitol or by strain T in ASP+1% glucose or sucrose, also gave the type 1 spectrum. However, the polysaccharides produced by strain T in ASP+5 or 10% sucrose or in ARJ+1, 5 or 10% sucrose showed a different spectrum (type 2). Neither of the spectra produced by the *E. amylovora* polysaccharides appeared to be the same as those produced by the known polysaccharides inulin, starch or dextran.

Paper chromatography. The results are summarized in Table 2 and show the components of each polysaccharide. All the polysaccharides produced *in vivo* and those produced by strains T, AT, E9 and P66 in ASP+1% sorbitol and by strain T in ASP+1% glucose or sucrose were similar and contained mainly galactose with smaller quantities of glucose and two unidentified compounds. The unidentified compounds had similar R_F values to galacturonic and glucuronic acid when developed in the BPW solvent, but in the MEK solvent the unknowns remained close to the origin whereas the uronic acids had the same R_F values as glucose and galactose. The two unknown compounds also stained differently from the uronic acids with the 4-aminobenzoic acid reagent: the neutral hexose sugars and the unknowns stained orange/brown whereas the uronic acids stained pink. As the uronic acid standards had the same R_F value in BPW as the unknowns and the same R_F value in MEK

Table 2. *Paper chromatographic analysis of acid hydrolysates of polysaccharides produced by Erwinia amylovora*

Source of polysaccharide	Strain	Components in acid hydrolysates (in order of spot density)
<i>In vivo</i>		
Apple, pear and hawthorn shoots	T	Galactose Unknown 1 Unknown 2 Glucose
Pear fruit slices	T, AT, E9	
<i>In vitro</i> *		
ASP		
+ 1 % sorbitol	T, AT, E9, P66	Fructose
+ 1 % glucose	T	
+ 1 % sucrose	T	
ASP		
+ 5 or 10 % sucrose	T	Fructose
ARJ		
+ 1, 5 or 10 % sucrose	T	

* ASP, Asparagine salts medium; ARJ, ammonium salts medium.

as the neutral sugars, it was not possible to say whether or not free galacturonic or glucuronic acids were present in the hydrolysates.

The polysaccharides produced by strain T in ASP + 5 or 10 % sucrose and ARJ + 1, 5 or 10 % sucrose contained only fructose and might possibly be levan.

Paper electrophoresis. The polysaccharides produced *in vivo* and those produced by strains T, AT, E9 and P66 in ASP + 1 % sorbitol and by strain T in ASP + 1 % glucose or sucrose, all contained traces of glucuronic acid and two unidentified compounds both of which had fairly high electrophoretic mobilities (though not as high as the uronic acids) and migrated towards the positive electrode which showed them to be acidic. The levan-like polysaccharide from the other sucrose media remained at the origin of the electrophoretogram.

Gas-liquid chromatography and uronide content. The results of the tests described above show that *E. amylovora* is capable of producing at least two polysaccharides *in vitro* and suggest that one of these is the same as that found in ooze. To test this further, the polysaccharides from pear slice ooze (strain T), ASP + 1 % sucrose or glucose (strain T) and ASP + 1 % sorbitol (strains T, AT and P66) were analysed by gas-liquid chromatography. All six polysaccharides contained galactose, glucose and small quantities of mannose and some minor components of uncertain identity. Details of the results, expressed as a percentage of the total hexose positively identified, are shown in Table 3. These six polysaccharides also contained between 16 and 23 % uronide (Table 3).

Column chromatography on Sepharose 2B. Gel-filtration was done on the polysaccharides from pear slice ooze (strain T) and ASP + 1 % sorbitol (strains T, AT, P66). All four polysaccharides were just retained by the gel and appeared as a single peak 5 ml after the void volume. This suggests they have a very high molecular weight. Although these results do not give an accurate molecular weight determination, they do show that the polysaccharides produced *in vivo* and *in vitro* are comparable in size.

Cellulose acetate electrophoresis. The polysaccharides listed above all migrated as a single peak the same distance towards the positive electrode, showing that their mass/charge ratios are similar and that they are all acidic.

Degradative activity in crude phage L3H preparations. The polysaccharides from both the pear slice ooze and the broth shake culture gave the same results in the following experiments.

Viscosity. After incubation with CPP, the polysaccharides showed a rapid and marked drop in viscosity (Fig. 2). However, no such change was observed when the polysaccharides

Table 3. *Composition of polysaccharides produced by Erwinia amylovora*

Source of polysaccharide	Strain	Percentage composition*			
		Galactose	Glucose	Mannose	Uronide
<i>In vivo</i>					
Pear slice ooze	T	73.3	7.0	3.4	16.3
<i>In vitro</i>					
ASP†					
+1 % glucose	T	61.6	10.8	4.9	22.7
+1 % sucrose	T	69.3	9.4	4.6	16.7
+1 % sorbitol	T	67.7	8.5	2.3	21.5
+1 % sorbitol	AT	65.0	10.8	1.5	22.7
+1 % sorbitol	P66	64.3	8.1	6.0	21.6

* Hexoses were determined by gas-liquid chromatographic analysis of acid hydrolysates; uronides were estimated by a colorimetric method (McCready & McComb, 1952).

† ASP, Asparagine salts medium.

Fig. 2. Viscosity measurements of the ooze polysaccharide before (●) and after (○) the addition of phage L3H preparation.

Fig. 3. Behaviour of polysaccharide from ooze on Sepharose 2B gel-filtration before (●) and after (○) incubation with phage L3H preparation. The elution volumes of Blue dextran (BD) and NaCl are shown.

were incubated with heat-treated CPP or alone. This shows that CPP contains heat-labile polysaccharide-degrading activity.

Behaviour on gel-filtration. Prior to incubation with CPP and after incubation with heat-treated CPP, the polysaccharides were eluted as a single peak just after the void volume. However, after incubation with CPP, a mixed population of lower molecular weight oligosaccharides were eluted as a broad peak near the bed volume for the column (Fig. 3).

Descending paper chromatography and paper electrophoresis. Irrespective of treatment, both polysaccharides remained at the origin of the chromatograms and electrophoretograms showing that simple sugar compounds had not been released.

DISCUSSION

The results strongly suggest that in certain defined culture media, *E. amylovora* produces the same polysaccharide as that found in ooze. The polysaccharides produced by a single strain of the organism in different hosts and by different strains in the same host all appeared to be similar. These findings support and extend those of Eden-Green (1972), Eden-Green & Knee (1974) and Beer *et al.* (1977).

Both the quantity and composition of the polysaccharides produced *in vitro* were affected by the nature and concentration of the sugar or sugar alcohol supplied and by the nature of the nitrogen source; capsulation is also affected in the same way (Bennett & Billing, 1978*a*). Large quantities of ooze-like polysaccharide were produced by strain T in the asparagine-based medium (ASP) supplemented with 1% glucose, sucrose or sorbitol, but if the concentration of glucose or sorbitol was increased to 5 or 10%, polysaccharide production was completely suppressed. When the concentration of sucrose was increased to 5 or 10%, however, polysaccharide production was still high but the polysaccharide produced contained only fructose.

In the ammonium-based medium (ARJ), no polysaccharide was produced when glucose or sorbitol were supplied as the carbon source, though in the presence of sucrose, small quantities of the fructose polysaccharide were produced. This agrees with the findings of Goodman *et al.* (1974) who were unable to isolate ooze-like polysaccharide from *E. amylovora* cultures grown in defined inorganic media for 48 h.

All three virulent strains and the capsulated avirulent strain P66 produced the ooze-like polysaccharide in ASP + 1% sorbitol. In contrast, no polysaccharide was produced in this medium by any of the non-capsulated avirulent strains. This suggests that production of the ooze-like polysaccharide, as well as capsulation (Bennett & Billing, 1978*a*), is associated with virulence. The fact that strain P66 is capsulated and produces the ooze-like polysaccharide *in vitro*, but is avirulent, shows that at least one other factor is involved in virulence. Evidence supporting this idea is reported elsewhere (Bennett, 1978*a, b*; Bennett & Billing, 1978*b*; Bennett, 1980).

In histological studies, several workers (Nixon, 1927; Haber, 1928; Miller, 1929) observed that the bacteria in host tissue were embedded in a gelatinous matrix within 10 h of inoculation. This suggests that extracellular polysaccharide is present during the early stages of infection but its role (if any) has not yet been determined.

It has been postulated, on the basis of cut shoot and petiole wilt induction tests, that the ooze polysaccharide (amylovorin) is a host-specific toxin (Goodman *et al.*, 1974, 1978; Hsu & Goodman, 1978). These authors suggested that the polysaccharide might be used to evaluate varietal resistance of apple and pear to the fireblight bacterium, but Beer & Aldwinckle (1976) were unable to confirm this with shoots of apple cultivars and so far success has not been reported from other laboratories. Furthermore, Sjulín & Beer (1978) in experiments with *Cotoneaster* concluded that the polysaccharide induced wilt of cut shoots by restriction of water movement rather than by the direct toxic effect suggested by Huang & Goodman (1976); they observed no detrimental effect of the polysaccharide on

host cells that could account for the wilting observed by various workers during the early stages of infection.

So far, capsulation has always been associated with ooze-like polysaccharide production. How closely the bound capsular material resembles the free polysaccharide is uncertain, but there is now evidence which suggests that they are very similar in composition. Lysates from bacteria infected with phages 4S, 4L and L3H appear to contain capsule-hydrolysing enzymes while those from bacteria infected with phage L3H (and possibly 4S and 4L) possess heat-labile polysaccharide-degrading ability. Further work using purified enzymes from phage lysates could help to characterize these compounds.

There are conflicting views about the importance of the sugar concentration in nectar in relation to the susceptibility of pear and apple blossom to fireblight (Schroth *et al.*, 1974). Apart from the effect of sugar concentration on the growth of the pathogen, Thomas & Ark (1934) reported that cultures of *E. amylovora* grown in the presence of high concentrations of sugar were weakened in virulence. Furthermore, virulence could be restored by growth in media containing 1 to 2% sugar. Ivanoff & Keitt (1941) found, during experiments using contaminated bees to transmit the disease from one blossom to another, that there were many cases in which the nectar was at a favourable concentration for bacterial growth but no infection occurred. The work reported here suggests one possible explanation for these observations which merits further study, i.e. that capsule and polysaccharide production are suppressed by sugar concentrations which are not sufficient to inhibit growth and that this results in a reduction in the virulence of the organism.

Note added in proof. After completion of this work, Slade & Tiffin (1978) showed, using serological techniques, that *E. amylovora* produced the same exopolysaccharide *in vivo* and *in vitro*, both as a capsule and an extracellular slime.

We are grateful to R. C. Hignett for help and advice. One of us (R. A. Bennett) was supported by an Agricultural Research Council Grant.

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