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A New Bacterial Agglutinin from Soybean

II. EVIDENCE AGAINST A ROLE IN DETERMINING PATHOGEN SPECIFICITY[1]

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ABSTRACT

The activity of a bacterial agglutinin from soybean seed [*Glycine max* (L.) Merrill cv. Clark] against two bacterial pathogens, *Pseudomonas glycinea* (causal agent of bacterial blight) and *Xanthomonas phaseoli* var. *sojensis* (causal agent of bacterial pustule) was determined. The agglutinin was active against several strains of *X. phaseoli* var. *sojensis* grown on nutrient agar, but there was no correlation between pathogenicity and agglutination. Agglutination was affected by the age of the bacterial cells and the growth medium used. None of seven strains of *P. glycinea* was agglutinated.

Bacterial agglutination was inhibited by both purified lipopolysaccharide and extracellular polysaccharide from five strains of *X. phaseoli* var. *sojensis*. The lipopolysaccharides and extracellular polysaccharides from other species of bacteria were ineffective.

Ultrastructural studies showed that an avirulent strain of *X. phaseoli* var. *sojensis* was attached to leaf mesophyll cell walls of the susceptible cultivar Clark by 34 hours after vacuum infiltration. Cells of this avirulent strain were enveloped by fibrillar and granular material at the mesophyll cell wall. In contrast, cells of a virulent strain were not attached or enveloped, and they remained free to multiply in the intercellular spaces.

Recent studies have implicated attachment of bacterial cells to plant cell walls as an initial step in a recognition process that determines host reaction. In this process, carbohydrate components of bacterial cell walls interact with plant cell surface receptors (27). For example, capsular components of the symbiotic bacteria, *Rhizobium japonicum* and *Rhizobium trifolii*, apparently interact with specific lectin receptor sites on their respective host plants (3, 4, 10). Similarly, LPS[2] components of *Pseudomonas solanacearum* appear to interact with cell wall lectins and thus induce a hypersensitive response (29). Attachment of *Agrobacterium tumefaciens* to a specific wound site on the host cell is a prerequisite for infection (20).

In the preceding article (13), the isolation, partial purification, and characterization of a new bacterial agglutinating glycoprotein obtained from seed of the soybean cultivar Clark are described. This agglutinin was active against a strain of *Xanthomonas phaseoli* var. *sojensis* (Hedges) Starr and Burk. (XPS), causal agent of bacterial pustule. The cultivar Clark is susceptible to XPS, as well as to certain strains of *Pseudomonas glycinea* Coerper, causal agent of bacterial blight. The nearly isogenic cultivar, Clark 63, is resistant to the pustule pathogen (18). It seemed important to determine whether the agglutinin was a primary determinant of specificity in the bacterial pathogen-soybean cultivar interactions.

Preliminary results indicated that crude Clark seed extracts were most active against the more virulent strains of XPS. It seemed likely, therefore, that binding of virulent cells of XPS to the Clark agglutinin at leaf mesophyll cell wall surfaces might lead to a compatible response, similar to the interaction of *A. tumefaciens* and its hosts (20). However, when a large number of strains of these pathogens were assayed against highly purified agglutinin from both Clark and Clark 63 seed, this hypothesis could not be supported, as is shown here.

MATERIALS AND METHODS

Bacterial Strains and Seed Source. The source and origin of bacterial strains used in agglutination studies are listed in Table I.

Seeds of soybean (*Glycine max* [L.] Merrill) cultivars Clark and Clark 63 were obtained from R. L. Bernard, United States Regional Soybean Laboratory, Urbana, Ill.

Pathogenicity Tests. All strains of XPS and *P. glycinea* were tested several times for pathogenicity on the soybean cultivars Clark or Clark 63, as follows.

Seeds were germinated in Vermiculite; seedlings were transferred to 10-cm pots (1-3 seedlings/pot) containing a sterilized mixture of soil-Vermiculite-sand (2:1:1) and grown in the greenhouse at an average temperature of 20 C (range, 14-30 C). In the winter months, supplemental lighting was supplied from fluorescent General Electric cool-white and Sylvania Gro-Lux tubes providing 8.5×10^3 lux on a 12-h photoperiod. No supplemental lighting was supplied during the summer months.

Inoculum was prepared from 24- to 48-h cultures grown on King's medium B agar (19) (*P. glycinea*) or nutrient agar (XPS). Cells were suspended in sterile H_2O and adjusted to $OD_{600\ nm}$ = 0.10 with a Bausch and Lomb Spectronic 20 colorimeter. This suspension was diluted with sterile H_2O to give final concentrations of approximately 10^7, 10^6, or 10^5 cells/ml as determined by reference to standard curves based on colony counts. These suspensions were sprayed on the underside of 10-day-old unifoliolate leaves, or the two uppermost trifoliolate leaves of older plants, by means of a Paasche airbrush (Paasche Airbrush Co., Chicago, Ill.) fitted with a No. 3 aircap and attached to a compressed airline at 1 kg force/cm^2, until water-soaking appeared (17). Plants inoculated with *P. glycinea* strains were maintained under the same greenhouse conditions with no supplemental lighting; plants inoculated with XPS strains were moved to a greenhouse maintained at 28 C (±2 C) with no supplemental lighting. Inoculated plants were kept in a shaded area of the greenhouse for at least 2 h after inoculation. Five to 7 days after inoculation, strains were rated as to pathogenicity on a scale of 0 (not pathogenic) to 5 (highly pathogenic) based on the amount of leaf area showing water-

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[2] Abbreviations: LPS, lipopolysaccharide; EPS, extracellular polysaccharide; XPS, *X. phaseoli* var. *sojensis*; ASF (or 50-ASF), ammonium sulfate fraction; SBL, 120,000 dalton soybean lectin; NA, nutrient agar.

Table I. *Source and Origin of Bacterial Strains Used in Agglutination Studies*

Bacterium	Strain	Source	Country or State of Origin
K. pneumoniae		R. S. Hanson	
P. glycinea	K1	B. W. Kennedy	Minnesota
	K2	B. W. Kennedy	Minnesota
	K3	B. W. Kennedy	Minnesota
	K4	B. W. Kennedy	Minnesota
	J-11-1	W. F. Fett	Wisconsin
	J-18-1	W. F. Fett	Wisconsin
	J3-17-2	W. F. Fett	Wisconsin
R. japonicum	3I1B24	L. Schrader	
	3I1B71	L. Schrader	
	3I1B84	L. Schrader	
	3I1B86	L. Schrader	
	Wisc 505	L. Schrader	
	61A118	L. Schrader	
X. campestris	42	U.W.C.C.[a]	
	43	U.W.C.C.	
	63	U.W.C.C.	
X. manihotis	6.28L	U.W.C.C.	Colombia
X. oryzae	H100	U.W.C.C.	
	TW2	U.W.C.C.	
X. phaseoli	15	A. W. Saettler	
	64	U.W.C.C.	
X. phaseoli var. *fuscans*	16	A. W. Saettler	
	27	A. W. Saettler	
X. phaseoli var. *sojensis*	1124	NCPPB[b]	Zambia
	1136	NCPPB	Rhodesia
	1716	NCPPB	Zambia
	1717	NCPPB	Rhodesia
	B83	L. Ferreira	Brazil
	B93	L. Ferreira	Brazil
	B97	L. Ferreira	Brazil
	B99	L. Ferreira	Brazil
	J3-27-1A	W. F. Fett	Wisconsin
	J3-27-1B	W. F. Fett	Wisconsin
	J3-27-1C	W. F. Fett	Wisconsin
	J3-27-1D	W. F. Fett	Wisconsin
	MINN	B. W. Kennedy	Minnesota
	R12	J. Dunleavy	Iowa
	S-9-4	W. F. Fett	Wisconsin
	S-9-8	W. F. Fett	Wisconsin
	XP3	M. P. Starr	Washington, DC
	XP21	M. P. Starr	Oklahoma
	XP29	M. P. Starr	Kentucky
	XP144	M. P. Starr	Indiana
	XP175	M. P. Starr	Sudan
	XP202	M. P. Starr	Zambia
X. pruni	P	E. Civerolo	
	SC	E. Civerolo	
	A	E. Civerolo	
X. raphani	70-5	R. E. Stall	Florida

[a] University of Wisconsin Culture Collection, Madison, Wisc.

[b] National Collection of Plant Pathogenic Bacteria, Hatching Green, England.

soaking or pustules. For 14 XPS strains, the number of pustules/cm^2 leaf area present 5 to 7 days after inoculation was determined also.

Extraction of Agglutinin. Seeds of the cultivar Clark or Clark 63 were soaked overnight in 0.15 M NaCl (1 g seed/5 ml NaCl) and then homogenized in a blender. The resultant suspension was allowed to stand overnight and then strained through two layers of cheesecloth and centrifuged at 12,100*g* for 20 min. Appropriate amounts of ammonium sulfate were added to the supernatant fluid with stirring to give stepwise saturation between 40 to 70%. At each step, the mixture was left overnight and then centrifuged at 12,100*g* for 20 min. The pellets from each fraction were dissolved in 50 mM citrate buffer containing 0.15 M NaCl (pH 5.0), and solutions were dialyzed extensively against distilled H_2O followed by the same citrate buffer (pH 5.0). All procedures were done at 4 C.

Highly purified extracts from both Clark and Clark 63 seed were obtained by single precipitation at 50% ammonium sulfate followed by chromatography of this fraction (50-ASF) on carboxymethyl cellulose as reported previously (13). Protein content was determined by the methods of Lowry *et al.* (22).

Highly purified 120,000-dalton SBL was purchased from Sigma Chemical Co.

Bacterial and Red Blood Cell Agglutination Assays. For agglutination assays, xanthomonads were grown on NA, pseudomonads on King's B agar (19), and rhizobia on yeast extract-mannitol agar (31). All bacteria were grown for 24 to 48 h at 28 C, except for *Rhizobium* strains which were grown for 5 to 7 days at 28 C. Bacteria were suspended in distilled H_2O at OD_{600nm} = 1.0 (approximately 10^9 cells/ml) measured with a Bausch and Lomb Spectronic 20 colorimeter. Serial 2-fold dilutions of agglutinin were made in citrate buffer with NaCl (pH 4.0 or 5.0), placed as drops (25 μl) on polystyrene Petri plates, and mixed with an equal volume of bacterial suspension. The plates were incubated for 2 h at 23 C. Agglutination was determined under a dissecting microscope provided with oblique lighting and rated on a scale of 0 (no agglutination) to 4 (strong agglutination). In some agglutination assays, bacterial cells previously were washed by repeatedly suspending them in sterile H_2O and centrifuging them at 4340 *g* for 10 min.

Hemagglutination assays with trypsin-treated rabbit erythrocytes were performed as described previously (13).

Purification of Bacterial Cell Surface Polysaccharides. For extraction of EPS and cell wall LPS, cultures of XPS strains 175, 1716, 1717, S-9-4, and S-9-8 were grown in nutrient dextrose broth (30) shake culture for 51 h at 23 C. Cells were harvested by centrifugation at 5860 *g* for 20 min. The supernatant was retained for extraction of EPS by the procedure of Dudman (12) as modified by Sequeira and Graham (29). The EPS fractions were scanned (1 mg/ml) for *A* between 230 and 300 nm to detect contamination with proteins and nucleic acid. To detect possible contamination with LPS, the thiobarbituric acid assay for 2-keto-3-deoxyoctonate (24), and the cysteine-H_2SO_4 assay for heptose (33) were used.

For extraction of LPS, the bacterial cells were washed three times in 0.5 M NaCl to remove EPS, (7) and then were suspended in H_2O at 68 C. The LPS was extracted by the phenol-H_2O method (32) and then lyophilized. The dry LPS was resuspended in a small volume of H_2O and further purified by repeated centrifugation at 100,000*g* for 4 h (14).

To characterize the carbohydrate constituents of LPS and EPS, samples were hydrolyzed with 2 N trifluoroacetic acid at 121 C for 1 h and alditol-acetate derivatives of the simple sugars were prepared by the method of Albersheim *et al.* (1). Sugar composition was determined quantitatively by gas chromatography.

For comparison, LPS and EPS from *P. solanacearum* E. F. Smith were obtained from T. L. Graham; LPS from *Escherichia coli* (Migula) Castellani and Chalmers and *Serratia marcescens* Bizio were purchased from Difco Laboratories. Xanthan gum [EPS from *Xanthomonas campestris* (Pammel) Dowson] was purchased from Sigma Chemical Co.

Table II. *Relationship between Pathogenicity and Agglutination of Bacterial Strains by Ammonium Sulfate Fractions from Clark Seed Extracts*

Twenty-five μl ASF were added to 25 μl bacterial cell suspension (10^9 cells/ml) in sterile H_2O as drops on polystyrene Petri plates. Agglutination was rated under a dissecting microscope with oblique lighting after 2 h incubation at approximately 23 C.

Bacterium	Strain	Agglutination by ASF					Pathogenicity[b]
		0–40%	40–50%	50–60%	60–70%	>70%[a]	
R. japonicum	3I1B86	0[c]	0	0			
	Wisc 505	0	0	0			
	61A11B	0	0	0			
X. phaseoli var. *sojensis*	21	3.0			0	0	5
	MINN	3.0	2.0	0			4
	175	2.7	3.0	0	0	0	5
	1124	2.5	2.0	0	0	0	3
	1135	1.7	2.0	0			0
	1717	1.5	1.5	0			3
	R12	1.3			0	0	2
	1716	1.0	0.9	0			0

[a] Supernatant of the 60 to 70% ammonium sulfate treatment.

[b] Trifoliolate leaves of each of two plants of the soybean cultivar Clark were inoculated with H_2O suspensions of each strain. After 7 days incubation, strains were rated on a scale of 0 (nonpathogenic) to 5 (highly pathogenic) based on leaf area showing pustules.

[c] Figures represent an average of six ratings on a scale of 0 (no agglutination) to 4 (strong agglutination).

To test for inhibitory effects on agglutination, LPS or EPS were suspended at 1 mg/ml in distilled H_2O and 25 μl were mixed separately with 25 μl of either 50-ASF from Clark seed (3 mg protein/ml) or 980 μg protein/ml purified Clark seed agglutinin. The mixtures were incubated for 1 h and then the agglutination titer against XPS strain 175 was determined.

Binding of LPS or EPS to soybean seed agglutinin was determined by precipitation. Droplets (25 μl) of serial 2-fold dilutions of LPS or EPS at 1 mg/ml in distilled H_2O were placed on polystyrene Petri plates, and to each drop was added 25 μl of 980 μg protein/ml purified Clark seed agglutinin. Precipitation was determined under a dissecting microscope after 2 to 2.5 h incubation at 23 C.

Effect of Culture Age on Agglutination. A bacterial suspension containing approximately 5×10^9 washed cells/ml was added (1 ml) to each of 12 Erlenmeyer flasks containing 100 ml nutrient broth. After 8 h incubation at 28 C and every 4 h thereafter, the OD_{600nm} values of the suspensions were determined and the cells from two flasks were harvested by centrifugation. The specific agglutinating activity of purified soybean seed agglutinin against these cells was determined as described previously (13).

Interaction of Bacteria with Host Cell Walls. To determine the interaction of XPS with soybean leaf cell walls, plants of the cultivars Clark or Clark 63 were grown in the growth room at 28 C during a 12-h photoperiod from a combination of General Electric cool-white fluorescent and tungsten incandescent lights providing 2×10^4 lux. Trifoliolate leaves were vacuum-infiltrated with a suspension containing approximately 3×10^8 cells/ml. Inoculated plants were kept at 23 C under reduced light for 3 to 5 h after infiltration and then placed back at 28 C.

Leaf samples were obtained 34 h after infiltration and fixed under vacuum with 5% glutaraldehyde in 80 mM cacodylate buffer (pH 7.4). The fixed tissues were rinsed in cacodylate buffer, postfixed in osmic acid, dehydrated, stained, embedded, sectioned, and further prepared for viewing with a JEM model 7 as described elsewhere (11).

RESULTS

Pathogenicity Tests. Typical pustular lesions were apparent on Clark leaflets 3 to 4 days after inoculation with pathogenic XPS strains. The XPS strains S-9-8 and 1716 did not cause lesions by 7 days after inoculation even at 10^7 cells/ml. At this concentration, strain 1716 caused slight browning of the laminae in the inoculated area. When cultured on tetrazolium chloride agar medium (16), NA, or nutrient-dextrose agar (30) colonies produced by XPS strains S-9-8 and 1716 were indistinguishable from those produced by pathogenic XPS strains.

The visual pathogenicity ratings of 14 XPS strains on the cultivar Clark correlated well with actual counts of pustules/cm^2 leaf area. Thereafter, visual ratings only were used as a criterion of pathogenicity for all XPS strains.

No pustules were obtained in the lesions produced by XPS strains 175, 1124, and 1717 on the resistant cultivar Clark 63.

P. glycinea strains K2, K3, K4, J-11-1, and J-18-1 produced water-soaked lesions on leaflets of Clark by 4 days after inoculation, as is characteristic of a susceptible response. Inoculation with strains K1 and J3-17-2 resulted in a typical resistant response, characterized by browning of the laminae and the absence of water-soaked lesions (17).

Agglutinating Properties of Soybean Seed Extracts. Only the 0 to 40 and 40 to 50% ammonium sulfate fractions from Clark seed extracts had agglutinating activity against XPS strains (Table II). In general, the most pathogenic XPS strains were the most strongly agglutinated by these two fractions. The 0 to 40 and 40 to 50% fractions contained only low hemagglutinating activity (titer = 32) against trypsin-treated rabbit erythrocytes, but the 50 to 60% fraction had a titer of 4096; the 60 to 70%, and >70% fractions both had titers of 8192. Similar results were obtained with fractions from seed extracts of the cultivar Clark 63. No agglutinating activity was present in similar extracts from trifoliolate leaves of these two cultivars, however.

The apparent correlation between agglutination and pathogenicity of XPS strains obtained with the 0 to 40 and 40 to 50% ammonium sulfate fractions (Table II) was not confirmed when a more highly purified agglutinin preparation was used (Table III). Out of 21 XPS strains, only the pathogenic strains B97 and 175 (unwashed cells) were strongly agglutinated (average agglutination rating of 3.0 or higher). The pathogenic strains B99, J3-27-1B, S-9-4, XP 21, XP 29, and XP 202 also were agglutinated, but to a lesser degree. Several other pathogenic strains (*eg.* 1124, 1717, and B93) were not agglutinated, however. Only two avirulent

Table III. *Relationship between Pathogenicity and Agglutination of X. phaseoli var. sojensis Strains by Soybean Agglutinins*

Purified Clark seed agglutinin or commercial SBL was placed as drops (25 μl) on polystyrene Petri plates. An equal volume of bacterial cell suspension (10^9 cells/ml) was added to each drop. Plates were incubated for 2 h at approximately 23 C and then agglutination was determined under a dissecting microscope provided with oblique lighting.

Strain	Location	Agglutination index ± SE		Pathogenicity rating[c]
		Clark Seed Agglutinin[a]	SBL[b]	
1124	Zambia	0[d]	0	4
1716	Zambia	0	0	0
1717	Rhodesia	0	0	5
B83	Brazil	0	0	3
B93	Brazil	0	0	4
B97	Brazil	3.0 ± 0.0	0	4
B99	Brazil	0.8 ± 0.2	0	4
J3-27-1A	Wisconsin	0	0	1
J3-27-1B	Wisconsin	0.5 ± 0.2	0	1
J3-27-1C	Wisconsin	0	0	3
J3-27-1D	Wisconsin	0	0	3
Minn	Minnesota	0	0	5
R12	Iowa	0	0	4
S-9-4	Wisconsin	2.0 ± 0.0	0	4
S-9-8	Wisconsin	0.5 ± 0.2	0	0
XP3	Washington, DC	0	0	3
XP21	Oklahoma	1.0 ± 0.0	0	3
XP29	Kentucky	0.5 ± 0.2	0	1
XP144	India	0	0	3
XP175	Sudan	4.0 ± 0.0	0	1
XP202	Zambia	0.5 ± 0.2	0	3

[a] Clark seed bacterial agglutinin (purified by chromatography on carboxymethyl cellulose) at 30 to 38 μg protein/ml.

[b] SBL = 120,000 mol wt soybean lectin at 125 μg/ml.

[c] Unifoliolate leaves of each of three plants of the soybean cultivar Clark were inoculated with H_2O suspensions of each strain. After 7 days incubation, strains were rated as to pathogenicity on a scale of 0 (nonpathogenic) to 5 (highly pathogenic) based on leaf area showing pustules.

[d] Figures represent average rating of at least six drops; rated on a scale of 0 (nonagglutination) to 4 (strong agglutination).

strains of XPS (1716 and S-9-8) was tested and neither was strongly agglutinated. (When the cells were washed, only those of XPS strain 175 were agglutinated.) Commercial SBL at 125 or 62.5 μg/ml did not agglutinate any of the 21 XPS strains tested (Table III).

Out of 14 strains of other xanthomonads tested, only *X. phaseoli* strain 64 was strongly agglutinated by the purified Clark seed agglutinin (Table IV). None of the *P. glycinea* or the *R. japonicum* strains were agglutinated by this preparation.

Cells of XPS strains 21, B97, S-9-8, and J3-27-1B and of *X. phaseoli* strain 64, all of which showed agglutination in plate assays (Tables III and IV), totally removed the agglutinin from solution, as shown by SDS-gel electrophoresis. Cells of XPS strains 1717, Minn, and R12, which did not agglutinate in plate assays (Table III), did not remove or lessen the intensity of agglutinin bands.

Effect of Bacterial Cell Wall Fractions on Agglutination. Yields of LPS and EPS ranged from 28 to 55 mg and from 230 to 330 mg/l, respectively. Contamination of EPS fractions with 2-keto-3-deoxyoctonate, heptose, protein, and nucleic acid was below detection levels by the methods that were used.

Both LPS and EPS from XPS strains 175, 1716, 1717, S-9-4, and S-9-8 (1 mg/ml) completely inhibited agglutination of XPS

Table IV. *Agglutination of Xanthomonads by Clark Seed Bacterial Agglutinin*

See Table III for details.

Bacterium	Strain	Agglutination Index ± SE[a]	
		Unwashed	Washed
X. campestris	42	0[b]	0
	43	0	1.0 ± 0.0
	63	0	0
X. manihotis	6.28L	0	0
X. oryzae	H100	0	0
	TW2	0	0
X. phaseoli	15	1.0 ± 0.0	0
	64	3.0 ± 0.0	2.0 ± 0.0
X. phaseoli var. *fuscans*	16	0	1.0 ± 0.0
	27	0	0
X. pruni	P	0.2 ± 0.2	0
	SC	0	0
	A	0	0
X. raphani	70-5	0	0

[a] Clark seed bacterial agglutinin after chromatography on carboxymethylcellulose at 30 to 38 μg protein/ml. Agglutination assays and washing procedure were performed as stated under "Materials and Methods."

[b] Figures represent average rating of at least six drops; rated on a scale of 0 (no agglutination) to 4 (strong agglutination).

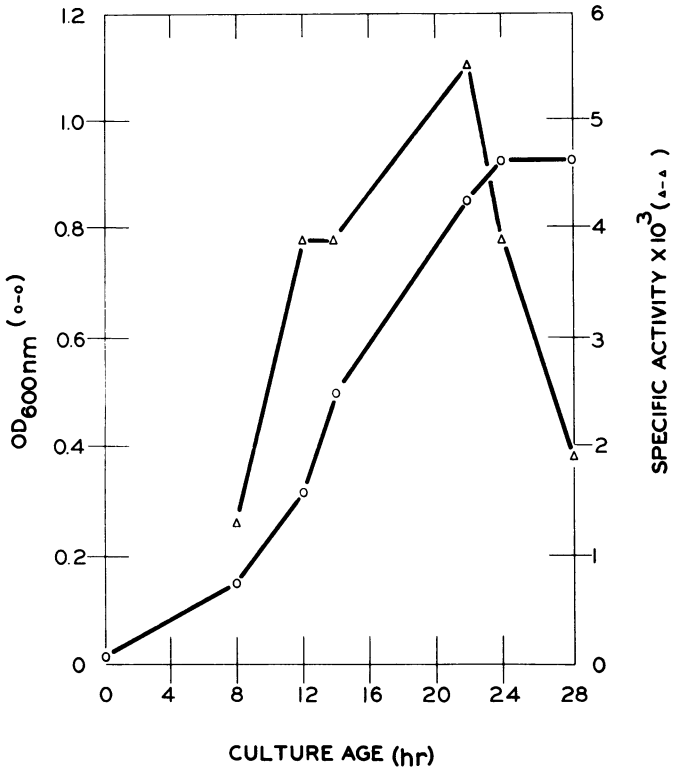

FIG. 1. Effect of culture age on agglutinability of cells of *X. phaseoli* var. *sojensis* strain 175 by purified Clark seed agglutinin.

strain 175 by the purified Clark seed agglutinin. EPS from XPS strains 175 and S-9-8 (15.5 and 31.0 μg/ml) totally inhibited agglutination. The LPS from these two strains also totally inhibited agglutination at 15.5 μg/ml. Agglutinated bacterial cells could not be resuspended by adding excess LPS (1 mg/ml) or EPS (1 and 5 mg/ml) of XPS strains 175 and 1717. Xanthan gum (EPS from *X. campestris*), EPS and LPS from *P. solanacearum* strains K60 and S210, and LPS from *E. coli* and *S. marcescens* did not

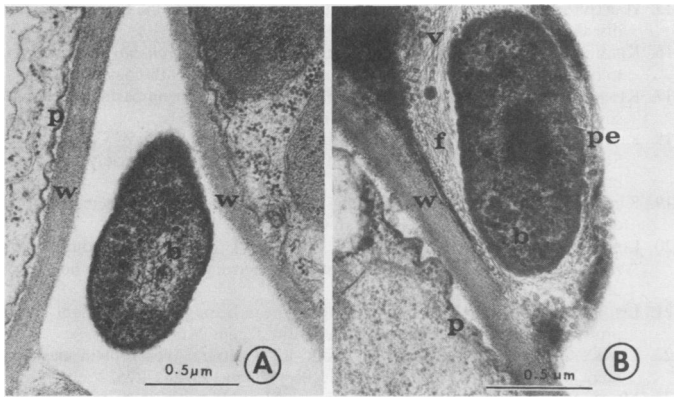

FIG. 2. Electron micrographs of Clark leaves infiltrated with a virulent strain (175) and an avirulent strain (S-9-8) of *X. phaseoli* var. *sojensis* and incubated for 34 h at 28 C. A, cell of virulent strain 175 in intercellular space. B, cell of avirulent strain S-9-8 immobilized at the mesophyll cell wall: w, mesophyll cell wall; pe, pellicle; p, plasmalemma; b, bacterium; v, vesicles; f, fibrils.

inhibit agglutination of XPS by the Clark seed agglutinin.

Although LPS from XPS strains 175, 1717, and S-9-8 inhibited agglutination, it was not precipitated when mixed with Clark seed agglutinin and incubated for 2.5 h. The LPS from 1716 and S-9-4 did show slight precipitation at concentrations as low as 83 μg/ml, however. The EPS from all five XPS strains, at concentrations as low as 4 to 8 μg/ml, formed a visible precipitate when incubated under similar conditions.

Gas chromatographic analysis of the alditol acetate derivatives of hydrolyzed LPS and EPS from XPS strains 175, 1716, 1717, S-9-4, and S-9-8 showed that they contained fucose, glucose, glucosamine, mannose, and rhamnose. These simple sugars, however, did not inhibit agglutination of XPS strain 175 by the Clark seed agglutinin (13).

Effect of Culture Age on Binding. Cells of XPS strain 175 either did not agglutinate or agglutinated only weakly when obtained from NA cultures grown for less than 15 h or for more than 72 h. Agglutinability of XPS strain 175 generally increased as the cells went from lag phase (8 h) to late log phase (22 h), but dropped sharply as cells entered stationary phase (28 h) (Fig. 1). Strains 1717 and S-9-8 showed similar patterns, but these two strains did not agglutinate or agglutinated weakly when grown for 24 h on NA (Table III).

Cells of XPS strains 175 and B97 grown on NA for 24 h at 28 C or strains 175, 1717, and S-9-8 grown in nutrient broth for 20 h at 23 C did not appear to have a capsule, as indicated by the India ink test (8). When grown under identical conditions, cells of *Klebsiella pneumoniae* (Schroeter) Trevisan were shown to contain large capsules by the India ink test.

Ultrastructural Studies. Where trifoliolate leaves of the susceptible cultivar Clark were vacuum-infiltrated with XPS strains 175 (virulent) and S-9-8 (avirulent) and incubated for 34 h at 28 C, cells of strain 175 remained free in the intercellular spaces, but those of S-9-8 were attached at the leaf mesophyll cell wall by a layer of electron-dense material and fibrous strands (Fig. 2). Vesicles accumulated near the site of attachment. Cells of avirulent strain S-9-8 often appeared dead (Fig. 2) and bacterial cell wall lysis was seen occasionally.

DISCUSSION

The hemagglutinating activity in the 50 to 60% ammonium sulfate fraction from Clark seed extracts was expected because SBL precipitates out of the solution in this fraction (21). Although this fraction did not agglutinate strains of *R. japonicum* in the tests reported here, there are reports that *R. japonicum* strains 3I1B 24 and 3I1B 71, when grown on synthetic salts media, bind to SBL strongly and weakly, respectively (3, 4). Others have been unable to demonstrate actual agglutination of *R. japonicum* by SBL (5).

The Clark seed agglutinin may bind to a specific sugar or sugars present in the surface polysaccharide layer of XPS cells. First, removal of part of the surface polysaccharides by repeated washing led to decreased agglutination of XPS cells. Second, when grown in broth culture, the ability of XPS cells to agglutinate increased as the viscosity of the culture increased (presumably due to slime formation by the bacterial cells) up to 20 h, as noted for agglutination of *R. japonicum* by SBL (3). Attachment of the clover lectin trifoliin and of SBL to *R. trifolii* and *R. japonicum*, respectively, also has been found to be influenced by the stage of growth of the cells used for assay (3, 9). This has been attributed to the presence of capsular polysaccharide (2, 6, 9). It was not possible to demonstrate the presence of capsules on cells of XPS grown on agar medium or in broth culture.

Third, agglutination of XPS strain 175 by the Clark seed agglutinin was inhibited by both LPS and EPS from five strains of XPS. Also, all the EPS, and two out of five LPS, preparations precipitated when mixed with the agglutinin. The agglutinin may be specific for a sugar or a specific glycosidic linkage common to both EPS and LPS of XPS strains. Alternatively, it is possible that the EPS preparations contained the *O*-polysaccharide portion of LPS as a contaminant. The tests reported here for LPS contamination were based on the presence of 2-keto-3-deoxyoctonate or heptose, sugars which are usually not present in the *O*-polysaccharide. Release of LPS into culture fluid by growing bacterial cells is a common phenomenon (26).

The decreased agglutination of XPS cells during stationary phase may result from changes in the composition of the cell surface polysaccharides. For example, the lowered galactose content in the EPS of *R. japonicum* as cells reach stationary phase may lead to reduced binding of the galactose-specific SBL (23).

We conclude that the Clark soybean seed agglutinin does not play a role in determining specificity in the interaction of bacterial pathogens with the soybean plant. Strains of *P. glycinea* differing in pathogenicity to soybean were not agglutinated by the Clark seed agglutinin. Initial results with crude fractions from Clark seed extracts showed that cells of the most virulent XPS strains were strongly agglutinated, but those from avirulent strains were not. However, when agglutination tests were run with a large number of XPS strains and highly purified Clark seed agglutinin, there was no correlation between virulence and agglutination. Also, the agglutinin was not specific for XPS, strongly agglutinating one strain of *X. phaseoli*.

Our initial hypothesis was that the presence of the recessive gene pair for resistance to XPS (15) in Clark 63 might be associated with the absence of the agglutinin and, thus, to the absence of a specific attachment site for XPS strains. However, the agglutinin is present in the seed of Clark 63.

Although only crude extracts were used, we failed to detect the agglutinin in leaves of Clark soybean. The presence of the agglutin in leaves would be essential for it to play a role in determining specificity of bacterial leaf infection.

Ultrastructural studies failed to show any attachment of cells of virulent XPS strain 175 to Clark leaf mesophyll cell walls, as would be expected because of the strong agglutinating properties of this strain. In contrast, cells of the avirulent XPS strain S-9-8 were immobilized by a layer of electron-dense material similar to that described for avirulent strains of *P. solanacearum* in tobacco leaves (28). We are presently examining the interaction of other bacterial strains with soybean leaf mesophyll cell walls in an effort to determine if attachment of bacterial cells is a defense mechanism in soybean.

In addition, no firm conclusions can be made concerning a possible role for SBL in determining specificity of XPS to soybean.

However, several XPS strains were not agglutinated by SBL and they did not remove SBL from solution. In addition, as happened in other experiments (25), SBL in soybean leaves on plants more than 2 to 3 weeks old was not detected here. This casts serious doubts on a possible role of SBL in determining specificity of soybean infection by bacterial leaf pathogens.

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