Agglutinating Activity in Apple Cell Suspension Cultures Inoculated with a Virulent Strain of Erwinia amylovora

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ABSTRACT

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Filtrates from apple cell suspension cultures (ACSC) inoculated with *Erwinia amylovora* contained a factor(s) which agglutinated cells of *E. amylovora* and red blood cells (RBC) of several animals. The agglutinating factor was produced in ACSC by a virulent strain but not by an avirulent strain of *E. amylovora*, and the factor preferentially agglutinated cells of the avirulent strain of the pathogen. Nine partially purified preparations of the toxin amylovorin also possessed agglutinating activity. Virulent cells of *E. amylovora*, however, agglutinated following pre-treatment with either heat or papain. Cells from older cultures of the virulent strain also were mildly agglutinable.

Additional key words: bacterial capsule, toxin, amylovorin, extra-polysaccharidal slime.

Agglutinating factors in plants inoculated with incompatible bacterial pathogens have been reported by several investigators (6, 8, 10, 13, 15). Huang et al. (10) showed that cells of an avirulent strain of the fireblight bacterium, *Erwinia amylovora* (Burr.) Winsl. et al., were agglutinated in the xylem vessels when apple petioles were inoculated with the bacteria. A virulent strain similarly inoculated into the petioles was not agglutinated but appeared to be protected from clumping by a layer consisting of an electron-lucent zone and fine filaments emanating from the walls of the bacteria. The agglutination reaction in situ was believed to be a host defense mechanism which restricted the movement of the avirulent bacteria in host tissue (10). Although the nature of the agglutinating factor is unknown, a lectin-like substance was suggested (6, 17).

We have reported that a host-specific wilt inducing toxin is produced in apple cell culture (ACSC) systems (9), as well as in intact host tissues (5) by *E. amylovora* (E9). In the present study, we describe the production of an agglutinin by the virulent E9 strain in ACSC that is able to agglutinate in vitro the avirulent E8 strain as well as certain animal red blood cells (RBC).

MATERIALS AND METHODS

The procedures for preparation of apple (cultivar Antonovka) cell suspension cultures (ACSC), their inoculation with *E. amylovora*, and collection of filtrates from the inoculated ACSC were as described previously (9). Agglutination tests were performed with filtrates from ACSC 7 days after they were inoculated with the bacteria unless otherwise indicated.

Agglutination of bacteria by ACSC filtrates was performed by pipetting 0.05 ml of 10^9 bacterial cells/ml suspended in 0.85% saline NaCl solution, prepared from 2-day-old nutrient yeast glucose agar slant (NYGA) cultures (9) into a test tube containing 0.2 ml of the filtrate. The mixture was then examined as a hanging drop at 1 hr with a phase-contrast microscope.

Agglutination activity also was determined against animal red blood cells (RBC). Animal RBC were obtained from the Department of Veterinary Pathology, School of Veterinary Medicine, University of Missouri. The RBC were separated from serum by centrifugation, then washed three times with saline by centrifugation, and finally suspended in saline. The ACSC filtrate was diluted 1:1 with 1.7% NaCl solution. Subsequent serial twofold dilutions of the filtrate were made with saline. Then 0.2 ml of each dilution was mixed with 0.05 ml of a 5% RBC suspension. Immediately after mixing, a drop of the mixture was placed on a glass slide and covered with a cover slip. The edge of the slip was sealed with mineral oil to slow evaporation, and observations were made at 1 hr with a phase-contrast microscope.

RESULTS

Agglutination of Erwinia amylovora.—Sterile culture filtrates from ACSC that had been inoculated with either a virulent (E9) or an avirulent (E8) strain of *E. amylovora* were assayed for agglutination activity on both E8 and E9 bacterial cells. A factor(s) which agglutinated avirulent bacterial cells was produced only in the suspension cultures inoculated with the virulent strain. The "agglutinin(s)" was not detected in filtrates from either noninoculated ACSC or ACSC inoculated with the avirulent strain (Table 1). Aggregation of bacteria started within a few minutes after mixing with the filtrate, and became more intense after 1 hr. The intensity of bacterial agglutination is characterized in Fig. 1. Nine preparations

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TABLE 1. Agglutination[a] of *Erwinia amylovora* by filtrates[b] from apple cell suspension cultures[c] or culture medium[d] 7 days after inoculation with *E. amylovora* (strain E8 or E9)

Filtrate from:	Agglutination of *E. amylovora*	
	Virulent strain (E9)	Avirulent strain (E8)
Suspension culture inoculated with strain E9	−	+
Suspension culture inoculated with strain E8	−	−
Control suspension culture	−	−
Culture medium inoculated with strain E9	−	−
Culture medium inoculated with strain E8	−	−
Control culture medium	−	−

[a]Agglutination aggregates equivalent to those in Fig. 1.
[b]Filtrates from two identical experiments were tested with precisely the same results.
[c]The apple cell suspension cultures were grown in SH medium for 14 days prior to inoculation with *E. amylovora* strains E8 and E9 (10^8 cells/50 ml culture medium).
[d]Culture medium is Schenk and Hildebrandt's SH medium (Can. J. Bot. 50:199-204).

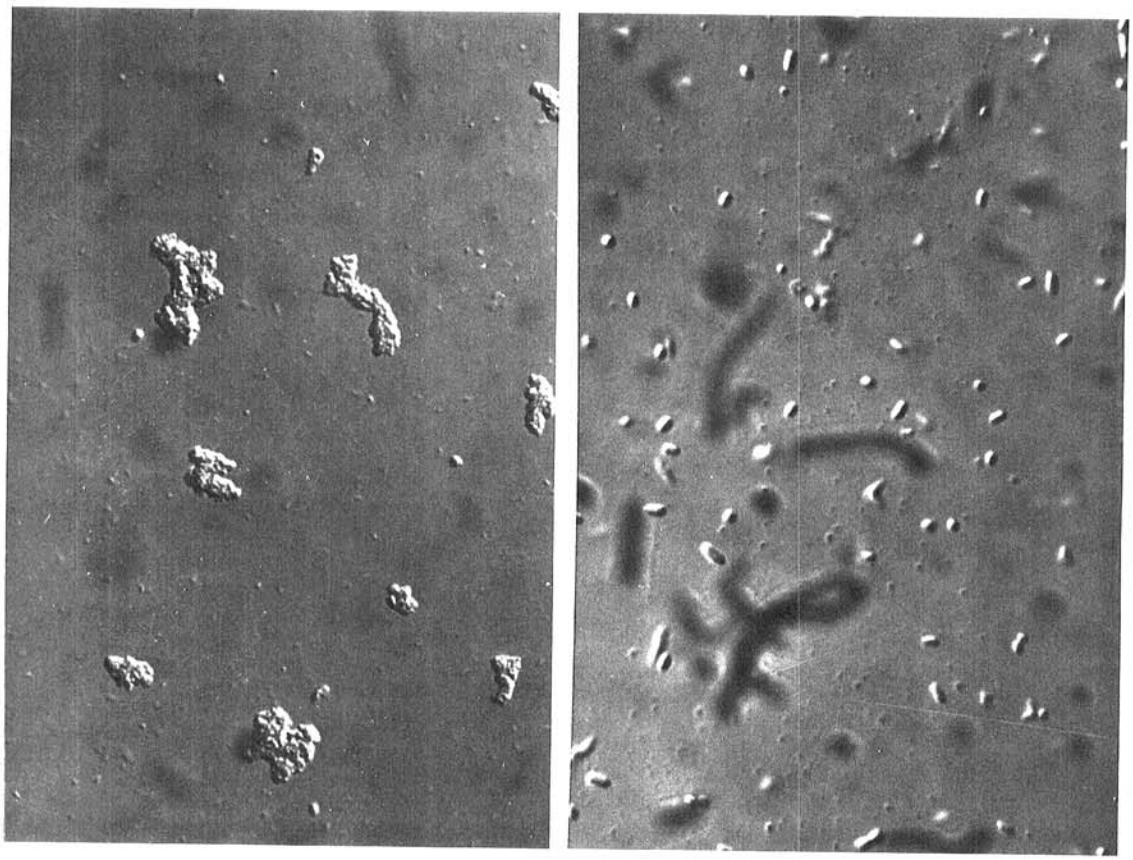

Fig. 1. Agglutination of E8 *Erwinia amylovora* cells by filtrates prepared from apple suspension cultures 7 days after inoculation with the virulent E9 strain (left) and the avirulent E8 strain (right).

of amylovorin also agglutinated E8 cells.

The agglutinating factor was nondiayzable against distilled water at 4 C for 24 hr. It also was active after boiling for 30 min. The pH of the filtrate prior to boiling was 6.2 and it was not altered by boiling. Attempts to precipitate the agglutinin from ACSC filtrates and from nine partially purified preparations of amylovorin with $(NH_4)_2SO_4$ at 20-80% saturation were unsuccessful.

Accumulation of the agglutinating factor in the suspension cultures.—The time course of agglutinin accumulation was monitored by sampling duplicate ACSC filtrates at 0, 1, 2, and 3 days and then at 2-day intervals following inoculation with the E9 bacterium. Assay for agglutinating activity in the ACSC filtrates was performed with avirulent E8 bacteria.

The agglutinating factor was detectable on three occasions at 5 days and on two others 3 days after inoculation of the ACSC with E9 bacteria and increased gradually in titer during the course of the experiments (Fig. 2). Similarly, when we used the highly sensitive RBC of chinchilla, we noted that 11, 13, and 15 days after inoculation the ACSC filtrates could be diluted 1:8 and still give strong evidence of agglutination.

Effect of pH on agglutination.—Filtrates from virulent E9-inoculated ACSC were adjusted to different pH values with 1 N NaOH or 1 N HCl, and were used for agglutination tests. The avirulent E8 bacteria were agglutinated by the filtrates at all pH values tested (4.0 - 9.0) whereas the virulent E9 cells were not agglutinable at any pH value except pH 4.0 at which slight clumping of bacterial cells was observed.

Effect of heat or papain treatment of bacterial cells on agglutination.—A suspension of virulent E9 bacteria (10^9 cells/ml) was prepared from 2-day-old NYGA slants. A portion of the suspension was boiled in water for 10 min and a second portion of the suspension was treated with papain (crude powder, Type II, Sigma Chemical Co., St. Louis, MO 63178). In the latter, 9 ml of bacterial suspension was incubated with 1 ml of 1% papain at 28 C for 1 hr. Heated and papain-treated cells were centrifuged and washed three times with sterile distilled water and finally resuspended in 9 ml of sterile distilled water. A similarly washed and centrifuged nontreated suspension served as a control. These treated and nontreated bacterial cells were tested for agglutination by the filtrates from E9-inoculated ACSC or from noninoculated ACSC. We observed that filtrates from E9-inoculated ACSC were capable of agglutinating heat- or papain-treated E9 cells, but were unable to agglutinate untreated bacterial cells. Filtrates from noninoculated ACSC had no agglutinating effect on either treated or untreated bacterial cells.

Effect of bacterial culture age on agglutinability.—Agglutinability of the avirulent E8 strain increased with culture age over a 7-day period, however, only the oldest E9 (virulent) cultures were agglutinated by the filtrates and then only weakly so (Table 2). In addition, it was noted that heat-treating a 1-day-old E8 culture fostered an agglutination intensity approximating that detected in 4- to 7-day-old cultures.

Agglutination of animal red blood cells (RBC).—The culture filtrates from ACSC inoculated with E9 cells (and amylovorin preparations) were able to agglutinate chicken, chinchilla, dog, horse, pig, rabbit, and sheep RBC (Fig. 3) but not cow, guinea pig, hamster, or mule RBC. Chinchilla RBC were the most sensitive to the agglutinating factor (Table 3).

Attempts to block the agglutination of chinchilla RBC by ACSC filtrates with 1,000 μg/ml of glucose, galactose,

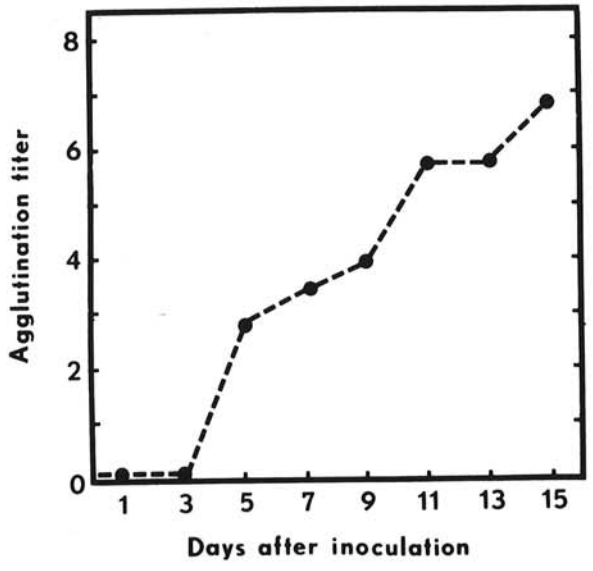

Fig. 2. Accumulation of the agglutinating factor in the apple suspension cultures following inoculation with *Erwinia amylovora* (strain E9). Agglutinating factor was assayed by the procedure described for agglutinating the E8 strain of *E. amylovora* in the Materials and Methods. Apple cell suspension cultures were grown in SH medium for 14 days prior to inoculation with *E. amylovora* strain E9 (10^8 cells/50 ml of culture medium). This experiment was performed five times and the agglutinating factor was detected three times after 5 days and twice after 3 days.

TABLE 2. Agglutination[a] of *Erwinia amylovora* strains E8 and E9 with filtrates[b] of apple cell suspension cultures (ACSC)[c] obtained at time intervals after inoculation of ACSC with *E. amylovora* strain E9

Inoculation of ACSC with E9 bacteria (days)	Agglutination titer with: E8	E9
1	1:2	–
2	1:4	–
3	1:4	–
4	1:8	–
5	1:8	1:2
7	1:8	1:4

[a]Agglutination aggregates equivalent to those in Fig. 1.
[b]Filtrates from three identical experiments were tested and each provided essentially the same results recorded above.
[c]Apple cell suspension cultures were grown in SH medium (14) for 14 days prior to inoculation with *E. amylovora* strain E9 (10^8 cells/50 ml of culture medium).

raffinose, melibiose, stachyose, lactose, ribose, arabinose, xylose, mannose, maltose, sorbitol, mannitol, glucosamine-HCl, N-acetyl-glucosamine, N-acetyl-galactosamine, or N-acetyl-muramic acid were unsuccessful (similar results were obtained in attempts to block E8 *E. amylovora* cell agglutination).

TABLE 3. Sensitivity of various animal red blood cells[a] (RBC) to the filtrate[b] from apple cell suspension cultures[c] (ACSC) 9 days after inoculation with *Erwinia amylovora* (strain E9)

Red blood cell (source)	Haemagglutination of RBC by ACSC filtrates at Reciprocals of: dilution end-point						
	1	2	4	8	16	32	64
Chicken	±[d]	−	−	−	−	−	−
Chinchilla	+	+	+	+	+	±	−
Dog	+	+	−	−	−	−	−
Horse	+	+	±	−	−	−	−
Pig	+	+	+	−	−	−	−
Rabbit	+	+	−	−	−	−	−
Sheep	+	+	±	−	−	−	−

[a]Red blood cells from hamster, guinea pig, cow, and mule were not agglutinable in these experiments.
[b]Filtrates from two identical experiments gave essentially the same results.
[c]Apple cell suspension cultures were grown for 14 days in SH medium (14) prior to inoculation with *E. amylovora* strain E9 (10^8 cells/50 ml of culture medium).
[d]Symbols indicate the degree of haemagglutination: − no aggregates of RBC, ± a few small aggregates, + aggregates of RBC analogous in size to Fig. 3, left side. The haemagglutination procedure is presented in Materials and Methods.

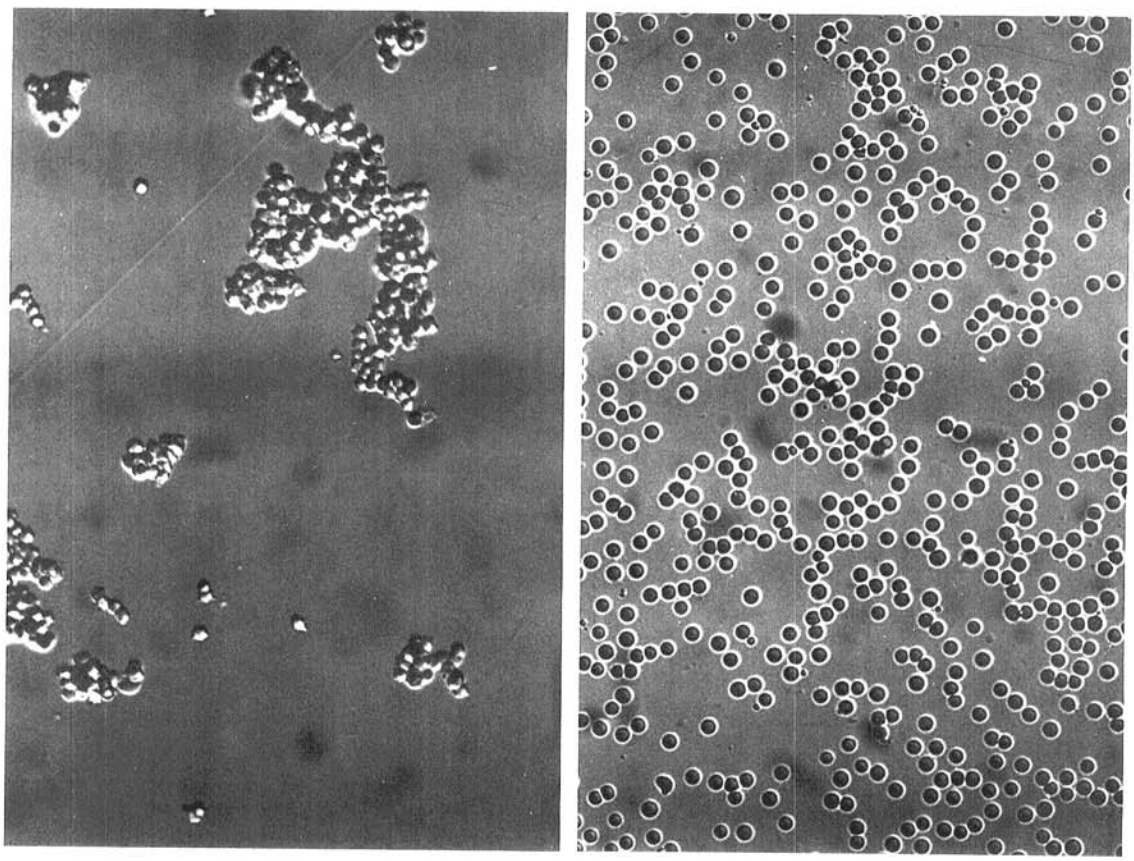

Fig. 3. Haemagglutination of sheep erythrocytes by the filtrate from apple suspension cultures 9 days after inoculation with a virulent strain of *Erwinia amylovora* (left) or by the filtrate from control suspension cultures (right).

DISCUSSION

Prior to this study, agglutination of plant pathogenic bacteria in vitro or in vivo reflected the activity of either pathogen-induced or preformed plant substances on avirulent or incompatible strains of a pathogen. For example Huang et al. (10) reported that cells of an avirulent strain of *E. amylovora* lacking extrapolysaccharidal slime (EPS) were agglutinated and localized in xylem vessels of apple leaf petioles near the site of inoculation whereas virulent cells were not affected. Their electron micrographs suggested that the agglutinins detected in xylem vessels were of xylem parenchyma origin. At about the same time, and at the ultrastructural level, Horino (8) observed envelopment and localization of an incompatible strain of *Xanthomonas oryzae* in rice xylem vessels by an induced fibrillar material. This was not observed in rice inoculated with a compatible strain of the pathogen. An earlier report by Main (13), indicated that an avirulent strain of *Pseudomonas solanacearum* injected into tobacco leaves induced the production of an extractable substance capable of agglutinating the avirulent strain in vitro. More recently, Sequeira and Graham (15) revealed that 34 avirulent mutants (lacking EPS) of *P. solanacearum* could be agglutinated by a highly purified potato lectin. Conversely, 55 virulent isolates were insensitive to the lectin. Their failure to bind to the lectin was clearly correlated to the presence of an EPS envelope.

The agglutination phenomenon that we report here is different at least in part, in that it reflects the production of an agglutinin by a *virulent* isolate of *E. amylovora* in ACSC that agglutinates an avirulent mutant in vitro. It is unlikely that this agglutinin is identical to the one responsible for in vivo agglutination of an avirulent strain of *E. amylovora* in susceptible host xylem cells. The latter has been judged to be of host origin (5).

We suggest that the agglutinin produced in ACSC is largely, if not totally, extracellular polysaccharidal slime (EPS) which is produced as a fibrillar mass that extends from the outer envelope of the bacterial cell. Our electron micrographs (Goodman, *unpublished*) have revealed EPS fibrils of $> 1\mu m$ in length on the surface of virulent cells in pear fruit tissue. On the other hand, EPS has been observed in barely perceptible traces on the surfaces of occasional avirulent cells. The facts that wilt-inducing and agglutinating activities are induced only by the virulent strain, that they appear only in cultures containing ACSC, and that the active component in each instance is a polysaccharide (5, 9, 18) leads us to suspect that the wilt-inducing toxin amylovorin and the agglutinin produced in ACSC are very similar if not identical. In addition, partially purified fireblight toxin obtained from nine different lots of immature apple fruits each possessed agglutinating activity. Partial purification of crude toxin activity from either ooze from inoculated green fruit or filtrates of ACSC was accomplished by precipitating it in 70% ethanol and successively passing the lyophilized ethanol precipitate over Dowex 1-X8 and Bio-Glas 500 columns (18).

Our failure thus far to detect either wilt-inducing or agglutinating activity in culture filtrates of *E. amylovora* grown in vitro may be explained in two ways. First, EPS may be produced in smaller quantities in vitro than in vivo. Second, it is possible that EPS is modified in association with host cells in a way that potentiates its biological activity. It is well known that EPS is produced in copious amounts in green apple fruit (5), apple stems (12), and in ACSC (9). Analogously, *Escherichia coli* B and *Salmonella typhimurium* cells grown in rabbit peritoneum develop a significantly thicker EPS envelope than they do in vitro (16). Ostensibly, the function of EPS is, to protect the pathogen from adverse environmental conditions, for example, host-produced agglutinins (10). The other biologically important function of EPS is its host-specific toxigenicity. Growth of *E. amylovora* in host tissue is accompanied by toxin production in a linear fashion (9).

The mechanism that accounts for the agglutination of avirulent *E. amylovora* cells by either the ACSC filtrate or a partially purified preparation of the fireblight toxin is not fully understood. Preferential agglutination of avirulent cells suggests that the surface structure of the avirulent cells differs significantly from that of the virulent cells (1). Enhanced agglutination of virulent cells by heat or papain treatment would tend to support this contention. It would seem that modification of the surface of the virulent E9 bacterial cells is affected by papain and heat, thus becoming more accessible to the agglutinating factor(s). In order to visualize this we might assume that the schematic of Costerton et al. (2) is a fairly accurate representation for the spatial arrangement of the components of the Gram-negative cell envelope. Hence, we must add to the LPS protruding from the outer membrane the EPS which is prominent, for example, on E9 but not on E8 cell surfaces. Heating bacterial suspensions has long been known to strip amorphous and weakly bound EPS away from the bacterial cells (4). This would provide sites to which amylovorin might bind and thus agglutinate adjacent E9 cells. Papain, on the other hand, could actually remove segments of the outer membrane of the cell envelope (2, 11); i.e., lipoprotein as well as LPS and EPS. Amylovorin could then bind to the newly exposed sites and thus agglutinate adjacent E9 cells (7).

Agglutination of a variety of animal RBC by ACSC culture filtrates and partially purified amylovorin suggests that the agglutinin is not blood-type specific. Actually, haemagglutinins associated with bacteria and bacterial components have been known since the turn of the century and may be of three kinds (4). The haemagglutination that we have observed is interpreted by us to conform to the Gold and Balding (4) classification of "direct agglutination" in that it involves the bacterial cells per se or an agglutinin produced by the bacteria. Our data suggest that the agglutinin production also requires some participation by host cells (Table 1).

Direct agglutination of the RBC of a number of animal species by a haemagglutinin in *Escherichia coli* was reported to take place over a pH range of 3-10. Furthermore, these agglutinins were associated most abundantly with cells in host tissue rather than in filtrates or supernatants of axenic cultures. Agglutinins also were present in filtrates of saline suspensions of *E. coli* (4). Agglutinins produced by *Pseudomonas aeruginosa* exhibit no loss of activity when heated to 70 C for 30 min and retain significant activity following exposure to 100 C

for 30 min. It also was reported that this haemagglutinin was resistant to pronase treatment. However, its agglutinating activity was inhibited by D-galactose and its derivatives (3). Even though some similarities exist between the mode of action of the haemagglutinin produced by *E. amylovora* and those produced by other bacteria, the literature also contains data that indicate inactivation of agglutinating activity by heat, protein-degrading enzymes, and low pH. Though the literature on bacterial haemagglutination is extensive, it is conflicting; nothing is known of combining sites, and little about the receptors. At the moment eight genera of the *Enterobacteriaceae* (to which *E. amylovora* belongs) produce haemagglutinins (4). The nature of the interaction between the agglutinin produced by *E. amylovora* and RBC remains as obscure as those reported for the other genera in the *Enterobacteriaceae*.

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