EFFECTS OF BACTERIAL AGE AND METHOD OF
CULTURE ON THE INTERACTION OF *XENORHABDUS*
NEMATOPHILUS (ENTEROBACTERIACEAE) WITH
HEMOCYTES OF NONIMMUNE *GALLERIA*
MELLONELLA (INSECTA) LARVAE

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Two strains, E4 and E7 of *Xenorhabdus nematophilus* were used to examine the effect of bacterial age and culture method on the physico-chemical properties of the bacteria, the production of flagella, fimbriae, and outer membrane proteins, and subsequently, the adhesion of the bacteria to hemocytes of the insect *Galleria mellonella*. The patterns of removal of the bacteria from the hemolymph in vivo mediated by the hemocytes varied with the bacterial strain, age and culture method. The cells of both strains in the stationary phase in shaken culture tubes and flasks were more extensively removed by nodule formation than were bacteria in the early and late exponential growth phases. The level of granular cells with adhering bacteria in the hemocyte monolayers in vitro increased with increasing bacterial age for both strains. The in vivo and in vitro hemocyte responses were independent of bacterial hydrophobicity and electrostatic charge but were correlated with bacterial viability. Bacterial metabolites released into culture media did not affect the relationship between bacterial viability and hemocyte contact. Bacterial surface features influenced hemocyte responses. The glycocalyx did not mediate adhesion to hemocytes.

The insect pathogen *Xenorhabdus nematophilus* (Enterobacteriaceae) is carried into the hemolymph of insects in the gut of the entomoparasitic nematode *Steinernema carpocapsae* (2). The bacterial-nematode complex is produced commercially as a biological insecticide to control pest insects of importance to forestry and agriculture (12).

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Galleria mellonella larvae, a pest of beehives, has antibacterial systems consisting of an interactive combination of humoral and cellular responses that respond ineffectively to *X. nematophilus* (9,13). The bacteria adhere to the insect hemocytes triggering aggregations of bacteria and hemocytes called nodules. Nodule formation removes the bacteria from the hemolymph, the rate of removal varying with the bacterial strain and insect species and diet (10). The initial adhesion of *X. nematophilus* to *G. mellonella* hemocytes is mediated by bacterial surface components induced by serum enzymes but is independent of the prophenoloxidase system (15). Components of the prophenoloxidase system including prophenoloxidase are known to mediate nodule formation to some insect pathogens (10). Bacterial hydrophobicity and cationic charge also enhance bacterial-hemocyte adhesion (9).

Fimbriae, important for the colonization of vertebrate tissues by some bacterial pathogens (7,27), have been implicated in the removal of the phase one variant (the normal phase of bacteria in the nematode and insect) of *X. nematophilus* from insect hemolymph (5). Fimbriae and outer membrane proteins (OMP) of vertebrate pathogens influence bacterial interactions with the cellular defenses of the host (25), and the production of these surface features is influenced by culture age and the method of culture (5,29). The effect of culture age and method on the physicochemical properties of *X. nematophilus*, on flagella, fimbriae and on OMPs, and hence on bacterial attachment to insect hemocytes, is not known. This study addresses these aspects using two previously isolated strains of *X. nematophilus* known to differ in their virulence for *G. mellonella*.

MATERIALS AND METHODS

Cultures and media. Two strains, E4 and E7, of *Xenorhabdus nematophilus* in the phase one form (the form infecting insects) were isolated from *Galleria mellonella* parasitized by the nematode *Steinernema carpocapsae* DD136 (15). The surfaces of dead insects were disinfected by flaming in 85% (w/v) ethyl alcohol. The bacteria were isolated by streaking hemolymph (released from the larvae by amputation of a thoracic proleg) onto tergitol-7-agar (Difco, Detroit, MI, U.S.A.) supplemented with 1% (w/v) 2,3,5-triphenyltetrazolium chloride (1). The phase one form of the bacteria was confirmed by antibiotic production and dye uptake according to Boemare and Akhurst (3). The cultures were maintained on tergitol-7-agar medium at 25°C in the dark and were subcultured bi-monthly.

Unless stated otherwise, inocula were prepared by growing the bacteria in 5 ml of yeast salts broth (YSB, 3) in 20-ml culture tubes with agitation on a horizontal gyratory shaker (150 rpm) at 27°C in the dark. Bacteria were subsequently washed by centrifugation (11,000×g, 3 min, 25°C) in phosphate-buffered saline (pH 6.5, 11), adjusted to 2.1×10^7 bacteria/ml and used as inoculum for the two methods used to grow the bacteria.

Last instar larvae of *G. mellonella* were reared at 27°C on a diet of multiple grains with glycerol and sugar (16). Only nonimmune larvae were used to preclude

antibacterial cecropins and attacins influencing the results. The nonimmune state was based on the presence of phenoloxidase activity and the absence of change in the total and different types of hemocytes (8). Insect serum for the hemocyte monolayer studies was prepared according to Dunphy (9).

Culture of bacteria. Two methods of culture were used for both strains; one involved low oxygenation and the other highly oxygenated conditions. In the latter, aliquots (100 μ l) of inoculum were added to 100 ml of YSB in 500-ml Erlenmeyer flasks. The low oxygenation was used to simulate conditions found in insects and was achieved by adding 100 μ l of inoculum to 5 ml of YSB in 20-ml culture tubes. All cultures were incubated at 27°C, with agitation on a horizontal gyratory shaker (150 rpm) in the dark. Total bacterial counts, where appropriate, were determined every 3 h using a Petroff-Hausser counter and phase contrast microscopy. Early and late exponential phase, and stationary phase bacteria from the culture tube method, and stationary phase bacteria from the flask cultures were selected for study. The bacteria were washed three times by centrifugation (11,000 $\times g$, 3 min, 25°C) in phosphate-buffered saline and adjusted to 2.1×10^7 bacteria/ml (unless stated otherwise) prior to use in the subsequent experiments. Stationary phase bacteria in culture tubes (17 h) were physiologically comparable to those in flasks (23 h).

Bacterial association with the hemocytes. Ten microliters of bacterial suspension was injected into the insect hemocoel at the base of the thoracic proleg of each of five larvae in two groups. Insects were incubated at 27°C for selected time periods and bled by severing a proleg. The increase in total bacterial and hemocyte counts in the hemolymph were determined using a hemocytometer and phase contrast microscopy (9). The increase in total hemocyte counts of *G. mellonella* in the presence of *X. nematophilus* was used to assess hemocyte damage due to the bacterial endotoxins (14). Viable bacteria exhibited no growth during the first hour in vivo and subsequent increases in the bacterial numbers represent a combination of bacterial growth in the plasma and hemocytes as well as the release of bacteria from the damaged hemocytes and nodules (13–15).

Hemocyte monolayers (9) were used to determine the relationship between the physicochemical properties of the bacteria and adhesion of the bacteria to the hemocytes. The monolayers were rinsed free of larval serum and nonadhering hemocytes with three 1-ml volumes of phosphate-buffered saline. One hundred microliters of bacteria in buffer or 100 μ l of bacteria in larval serum were added to two groups (10 slides each) of hemocyte monolayers. Slides were incubated at 25°C with agitation on a horizontal gyratory shaker (25 rpm) for 20 min. Another 100 μ l of buffer was then added and incubation continued for an additional 20 min. To determine if extracellular metabolites produced by the bacteria in YSB under the different growth regimes influenced bacterial adhesion to the hemocytes, bacteria of different ages were washed and suspended as described above; 50 μ l of the bacterial suspension was added to 50 μ l of YSB that had been previously rendered free of bacteria. Spent YSB was rendered free of bacteria by centrifugation (15,000 $\times g$, 5 min, 25°C) followed by filtration (0.22 μ m pore size). Such

suspensions (100 μ l) were added to monolayers of hemocytes and incubated as described above. Controls consisted of bacteria suspended in their own spent medium and in phosphate-buffered saline. Slides were rinsed free of non-adhering bacteria and fixed with 2.5% (v/v) glutaraldehyde in buffer (9). The numbers of bacteria adhering to the two types of antibacterial hemocytes, the plasmatocytes and granulocytes (13), and the percentages of these hemocytes with attached bacteria were determined using phase-contrast microscopy and a minimum of 100 hemocytes of each type.

Bacterial surface area and viability. The surface areas of *Bacillus* species vary with bacterial age and influence the removal of the bacteria from insect hemolymph (29). This parameter was assessed for both strains of *X. nematophilus*, from the different regimens, that had been heat-fixed to slides. Surface areas were calculated as the sum of the surface area of a cylinder and a sphere according to Walters and Ratcliffe (29).

To determine bacterial viability, cultures were diluted 10^2 - to 10^5 -fold with phosphate-buffered saline and 100 μ l of the suspension were plated on yeast salts agar (13). Viability was based upon the number of colony-forming units/ml of original culture.

Hydrophobic and electrostatic interaction chromatography. Bacteria from the different growth regimens were suspended in 1 ml of a solution composed of nine salts (23) such that a 100-fold dilution of these suspensions in the salts solution yielded an optical density at 420 nm (OD_{420}) of 0.2. The hydrophobic (octyl-Sepharose CL-4B, Pharmacia, Montreal, Canada) and electrostatic (cation exchange resin, Dowex 50 WX8-200; anion exchange resin, Dowex 1 $\times$ 8-200; Aldrich Chemical Company, Toronto, Canada) resins were equilibrated in aliquots (25 μ l) of the nine-salts solution (1 mg resin/ml of solution). Twenty-five microliters of the resin suspension was then added to the bacterial suspensions, the mixtures incubated for 20 min, the resins allowed to settle from the solution and the OD_{420} values of the suspensions determined. Controls consisted of bacterial suspension diluted with 25 μ l of the nine-salts solution. The relative levels of surface hydrophobicity and type of charge exhibited were based on the levels of bacteria bound to the resins (as a percentage) using 10 replicates per treatment.

Transmission electron microscopy. Aliquots (5 μ l) of bacterial suspension from the different growth regimens were placed onto carbon-coated copper grids (200 mesh). The bacteria were negatively stained with 2% (w/v) uranyl acetate or 2% (w/v) phosphotungstic acid. After 1 min of staining, excess material was removed, the grids air-dried and the bacteria examined with a Carl Zeiss EM9 transmission electron microscope. Phosphotungstic acid stained an amorphous layer beyond the bacterial surface suggesting the existence of a glycocalyx. The presence of a glycocalyx was confirmed using the method of Brehelin et al. (5).

Outer membrane components. Lipopolysaccharides (LPS) and outer membrane proteins (OMP) have been implicated in the adhesion of *X. nematophilus* to insect hemocytes (15). To obtain LPS for electrophoresis, *X. nematophilus* strains

from selected regimes were digested with 125 μ g of proteinase K in 50 μ l of solubilizing buffer (18). The LPS electrophoretic profiles were investigated using SDS-polyacrylamide gel electrophoresis (4.5% acrylamide stacking gel, 12.5% acrylamide separating gel) and silver staining (28).

Outer membranes of the strains from selected regimens were obtained by lysing the bacteria by ultrasonication in phosphate-buffered saline that contained 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 20% (v/v) sarkosyl (22). Cell debris was removed by centrifugation (3,000 $\times g$, 10 min, 5°C), and the membranes were harvested by centrifugation (100,000 $\times g$, 1 h, 5°C). These membranes were further purified by washing with 2% (w/v) sarkosyl and then distilled water (100,000 $\times g$, 1 h, 5°C). Total OMP content was estimated using the method of Peterson (24) using bovine serum albumin as a standard.

Outer membrane preparations (15 μ g protein/lane) were subjected to electrophoresis (10% acrylamide running gel, 4.5% stacking gel) (21) and the polypeptides visualized by silver staining (Bio-Rad Inc., Richmond, CA, U.S.A.).

Statistical analysis. Except for the percentage data, all data are expressed as means $\pm$ the standard error of the mean. These data were analyzed by Tukey's test (26). Percentage data represent the decoded mean of arc sin $\sqrt{p}$ -transformed data (with the 95% confidence limits of the coded data) (26). Correlation coefficients were calculated for the relationships between bacterial physicochemical parameters and bacterial removal from the hemolymph in vivo, bacterial physicochemical parameters and adhesion to hemocytes in vitro, and bacterial viability and interaction with the hemocytes of the insects. The analyses were done using different groups of highly inbred larvae. The different insect groups and bacterial isolates were subsampled for the parameters. Because these groups of larvae responded to bacteria in a statistically similar fashion, as determined by analyses of variance, it was possible to correlate analyses between experimental groups (9).

RESULTS

Culture methods

Strains E4 and E7 grew at similar rates (average population doubling time = 1.1 ± 0.2 h) during the initial 9 h of growth in both culture tubes and flasks. Strain E4, in culture tubes, reached the stationary phase by 15 h (Fig. 1A) after inoculation whereas strain E7 continued to grow (Fig. 1B) albeit at a very slow rate (population doubling time = 6.1 ± 0.3 h). The cultures in flasks grew to higher levels than those in tubes and entered the stationary phase at 18 h postinoculation.

Bacterial association with hemocytes

The pattern of hemocyte-mediated removal of bacteria from hemolymph by the hemocytes in vivo varied with the strain, the age of the culture and the culture method. Both strains of bacteria in the early and late exponential phase from tube cultures increased in concentration throughout the 4-h study (Fig. 2A, B). How-

Fig. 1. Growth of isolates of E4 (A) and E7 (B) of *Xenorhabdus nematophilus* in yeast salts broth, in culture tubes (●) and in Erlenmeyer flasks (■).

Fig. 2. Removal of isolates E4 (open symbols) and E7 (closed symbols) of *Xenorhabdus nematophilus* at different ages and grown in tubes (A, 6 h; B, 9 h; C, 17 h) and flasks (D, 23 h).

ever, 50% of the E4 strain in the stationary phase and the E7 strain in the deceleration phase cultured in tubes had been removed by 30 min postinjection. By 1 h postinjection, both strains emerged into the hemolymph at similar rates (Fig. 2C). Stationary phase E4 and E7 from flask cultures were removed from the hemolymph at rates that differed from their counterparts grown in tubes (Fig. 2C, D). Strain E4 from a 23-h flask culture was not removed during the initial 30 min

Table 1. The effect of strains E4 and E7 of *Xenorhabdus nematophilus* of different ages and from different methods of culture on the rates of increase of the total hemocyte counts of nonimmune *Galleria mellonella* larvae.

Isolate	Culture		Rate of increase of hemocyte counts ($\times 10^4/\text{ml}/\text{min}$)
	Age (h)	Method	
E4	6	Tube	$3.3 \pm 0.2b^a$
	9	Tube	$8.3 \pm 4c$
	17	Tube	$1.7 \pm 0.4d$
	23	Flask	$5.8 \pm 0.3c$
E7	6	Tube	$6.7 \pm 0.3c$
	9	Tube	$12.0 \pm 1.0e$
	17	Tube	$13.0 \pm 5.1e$
	23	Flask	$0.8 \pm 0.1f$

^a Mean $\pm$ SE of the mean; $n \geq 10$.

Data with the same letter are not significantly different ($P > 0.05$).

postinjection but by 2 h the number of bacteria had declined by 50%; strain E7 was removed continuously throughout the study.

The numbers of damaged hemocytes increased rapidly during the infection by *X. nematophilus*. However, the rates of increase in the total hemocyte counts during infection with strains E4 and E7, although varying with the age of the bacteria, the strain and method of culture (Table 1) were not correlated with bacterial removal from ($r=0.175$, $P > 0.05$) or bacterial emergence into the hemolymph ($r=0.251$, $P > 0.05$).

Adhesion of *X. nematophilus* to hemocyte monolayers varied with the bacterial strain and age and method of culture. In the absence of larval serum, strain E4 grown in tubes for 9 or 17 h adhered to more granular cells than did the early exponential phase bacteria from tubes or bacteria from 23-h flask culture (Table 2). Bacteria from flasks adhered to more plasmatocytes than did bacteria from tubes. E7 did not exhibit these patterns; bacteria grown for 17 h in tubes adhered to more granular cells and plasmatocytes than did bacteria of other ages or from flasks. Irrespective of culture age and culture method, the numbers of bacteria per type of hemocyte for both isolates were not statistically different, averaging 1.0 ± 0.2 bacteria per hemocyte. There was no correlation between bacterial adhesion to hemocytes in vitro and removal from the hemolymph in vivo ($r=0.000$, $P > 0.05$). Insect serum increased bacterial adhesion to both hemocyte types, this effect being greatest with stationary phase bacteria (Table 2). There was a correlation between bacterial adhesion to hemocytes in vitro in the presence of larval serum and removal of the bacteria from the hemolymph ($r=0.787$, $P < 0.05$).

Physicochemical properties and bacterial viability

Bacterial hydrophobicity, with the exception of strain E4 grown in tubes for 6 h (62%), and the anionic and cationic charges of the bacteria did not vary with

Table 2. Effect of bacterial age and method of culture on the viability of strains E4 and E7 of *Xenorhabdus nematophilus* and on their adhesion to hemocytes of nonimmune *Galleria mellonella* larvae.

Isolate	Culture		Percentages of hemocytes with bacteria in phosphate-buffered salines* ***				Viable** *** counts	
	Age (h)	Method	Granular cells	Plasmotocytes	Granular cells	Plasmotocytes	(CFU $\times 10^5$ /ml)	
E4	6	Tube	12 (18.4-21.3) ^a	6 (12.5-17.7) ^c	15 (2.07-24.8) ^a	10 (17.4-21.0) ^c	10.3 $\pm$ 1.4 ^s	
	9	Tube	24 (27.3-31.3) ^b	10 (16.4-22.1) ^c	51 (44.6-47.5) ^c	26 (29.1-33.0) ^a	8.2 $\pm$ 0.8 ^s	
	17	Tube	22 (26.5-29.4) ^b	11 (17.5-22.0) ^c	41 (38.3-42.3) ^f	20 (25.4-30.2) ^a	2.3 $\pm$ 0.2 ^h	
	23	Flask	14 (19.4-24.3) ^a	24 (26.8-31.4) ^d	25 (20.7-31.4) ^a	38 (35.8-40.4) ^f	11.7 $\pm$ 1.4 ^s	
E7	6	Tube	12 (18.6-23.0) ^a	3 (6.0-17.6) ^c	18 (22.7-26.6) ^a	7 (12.2-19.7) ^c	13.2 $\pm$ 0.5 ^s	
	9	Tube	13 (19.7-23.7) ^a	7 (12.9-17.8) ^c	21 (25.5-29.7) ^a	12 (17.3-19.9) ^c	7.3 $\pm$ 1.2 ^h	
	17	Tube	24 (27.0-31.7) ^b	18 (23.0-27.4) ^d	52 (44.8-47.9) ^c	39 (36.6-41.2) ^f	6.0 $\pm$ 0.6 ^h	
	23	Flask	18 (24.0-25.9) ^a	7 (12.9-17.8) ^c	42 (39.6-42.5) ^f	12 (15.4-24.4) ^c	12.6 $\pm$ 0.4 ^s	

* Decoded mean (with 95% confidence limits of arc sin $\sqrt{p}$ transformed data); $n \geq 10$. Data are based on a minimum of 125 hemocytes/type/slide.

** Colony-forming units; mean $\pm$ SE of the mean. $n \geq 10$.

*** Values with the same letter for the same parameter are not significantly different, $P > 0.05$.

either culture age or method of culture (average range 0–8%). However, the surface areas of the strains did vary with culture age and method of culture: the areas decreased with culture age (E4 tube: 6 h = $39.47 \pm 0.38 \mu\text{m}^2$, 9 h = 37.18 ± 0.33

Fig. 3. Development of surface structures on the E4 strain of *Xenorhabdus nematophilus* at different stages of development and under different culture conditions.

(A) Six-hour culture in early exponential growth in test tubes. (B) Nine-hour culture in test tubes in late exponential growth. (C) Seventeen-hour culture in early stationary phase from test tubes. (D) Nine-hour culture in flasks in early exponential growth (same physiological age as A). (E) Fifteen-hour flask culture in late exponential growth (same physiological age as B). (F) Twenty-three hour flask culture in early stationary phase (same physiological age as C). F, fimbriae; f, flagella; g, glycocalyx.

μm^2 , 17 h = $38.36 \pm 0.86 \mu\text{m}^2$; E4 flask/23 h = $31.50 \pm 0.34 \mu\text{m}^2$; E7 tube 6 h = $43.07 \pm 0.31 \mu\text{m}^2$; 9 h = not determined, 17 h = $36.72 \pm 0.15 \mu\text{m}^2$; E7 flask/23 h = $34.25 \pm 0.05 \mu\text{m}^2$). Bacterial surface areas were correlated with neither bacterial removal from the hemolymph ($r=0.051$, $P>0.05$) nor adhesion to hemocyte monolayers ($r=0.158$, $P>0.05$).

The viability of both strains declined as the incubation time in the tubes was increased (Table 2). In flask cultures at 23 h, the numbers of viable bacteria were similar to those obtained in tubes during the early exponential phase. With bacteria from tubes, viability was correlated with the numbers of hemocytes with attached bacterial cells ($r=0.826$, $P<0.05$). It was not possible to compare adhesion of the bacteria to the hemocytes in terms of viability between the two methods of culture because the bacterial levels were significantly different (Fig. 1A, B). Mixing bacteria of different ages suspended in phosphate-buffered saline with filter-sterilized, spent YSB from cultures of unrelated ages did not alter the numbers of hemocytes with attached bacteria (data not shown), and thus of bacterial metabolites may not be an adhesion factor.

Flagella, fimbriae and outer membrane components

Both strains exhibited peritrichous flagellation at all ages and in both culture systems (Fig. 3A, B for representative strain E4). However, fimbriae were detected on stationary-phase bacteria from both agitated tube and flask cultures (Fig. 3C,

Fig. 4. Outer membrane protein patterns of strains of *Xenorhabdus nematophilus* on an SDS-10% polyacrylamide gel.

Proteins were stained with silver. Molecular mass standards (lanes 1, 4 and 7) are noted on the left. Lanes 2 and 3 contain proteins from flask and tube cultures of strain E7 and lanes 5 and 6 contain proteins from flask and tube cultures of strain E4.

F). Fimbriation was more extensive on bacteria from unshaken cultures. When isolate E4 was stained with phosphotungstic acid, a diffuse electron-opaque layer was observed around exponential-phase bacteria (Fig. 3D, E). With the advent of fimbriation, this layer became less diffuse and more electron-dense (Fig. 3F). Such a layer was also evident around bacteria fixed with glutaraldehyde and the stabilizing agent lysine establishing its identity as a glycocalyx (5).

There were no differences in the LPS electrophoretic profiles of isolates E4 and E7 grown for 17 h in tubes or for 23 h in flasks (data not shown); both isolates exhibited a rough LPS profile. OMP profiles, however, although similar for both isolates for a given incubation condition, varied with the culture method. Bacteria from flask cultures produced an OMP with an apparent molecular mass of 45 kDa that was not detected in the bacteria from tubes; the former organisms had reduced amounts of polypeptide with an apparent molecular mass of 22 kDa (Fig. 4). Both bacterial strains from tubes also contained larger amounts of OMPs with apparent molecular masses of 52, 65 and 67 kDa.

DISCUSSION

The removal of *X. nematophilus* from the hemolymph of *G. mellonella* in vivo was influenced by the age of the bacteria and method of culture which is similar to reports on bacterial pathogens in mammals (25). Bacterial removal from the hemolymph was not correlated with adhesion of the bacteria to monolayers of hemocytes in the absence of larval serum as opposed to the presence of serum. Although opsonins have not been detected, enzymes in larval serum are known to modify the outer membranes of *X. nematophilus* and the closely related organisms *Photorhabdus luminescens* (= *X. luminescens* Boemare et al. (4)), accelerating nodule formation in vivo and enhancing bacterial adhesion to the hemocytes in vitro (9).

Physicochemical properties of foreign materials influence their interaction with insect hemocytes (20). Increasing hydrophobicity and declining cationic charge of chemically mutagenized *X. nematophilus* (9) and *P. luminescens* (unpublished data) increased bacterial adhesion to hemocytes of nonimmune *G. mellonella* larvae in vivo and in vitro. The results of the present study are contrary to these and may reflect the absence of extensive surface differences as found on the chemically mutagenized bacteria.

The sizes of *Bacillus* spp., which change with bacterial age, influenced the removal of the bacteria from *G. mellonella* hemolymph by nodule formation (29). In the present study, the size of *X. nematophilus* declined with increasing age, and varied with the method of culture, but was not correlated with either bacterial removal from the hemolymph or bacterial attachment to the hemocytes. Decreasing viability of *X. nematophilus* with prolonged incubation in tubes was associated with an increase in the number of hemocytes with attached bacteria. This was not due to extracellular metabolites of exponentially growing bacteria suppressing

hemocyte activity because spent media from young cultures did not reduce the hemocyte responses to bacteria in the stationary phase, and stationary-phase bacteria did not release factors stimulating hemocyte activity towards younger organisms. Consequently, the different patterns of bacterial adhesion to the hemocytes may reflect antigenic differences at the bacterial surface as was reported for LPS mutants of *Pseudomonas aeruginosa* (11) and *Serratia marcescens* (17) in lepidopterous insects.

Flagella, whether functional or nonfunctional, facilitate the association of Gram-negative bacteria with the antibacterial defenses of vertebrate (6) and insect hosts (19). However, with both isolates of *X. nematophilus* possessing flagella at all stages of growth and in both culture systems it is unlikely that flagella mediated the attachment of the bacteria to the hemocytes. An increase in the thickness of the glycocalyx of *X. nematophilus* and *P. luminescens* may enhance hemocyte-mediated removal of the bacteria from insects (5). Herein, strain E4 possessed a glycocalyx under all growth conditions examined which brings into question the validity of the layer as the only adhesion factor. Strains E4 and E7 produced fimbriae in agitated culture but produced more fimbriae in unshaken cultures in consonance with the results of Brehelin et al. (5). Fimbriae were detected only on bacteria that adhered readily to hemocytes supporting the proposal that fimbriae function as adhesion factors (5). LPS of *X. nematophilus* is also an adhesion factor, with the O-side chains, but not the core, binding to *G. mellonella* granular cells (14) and an increase in smooth LPS was correlated with increased bacterial adhesion to hemocytes (9). LPS of isolates E4 and E7 were the rough type and identical LPS electrophoretic profiles were obtained with bacteria of different ages and from different culture systems; these results would tend to preclude O-side chain participation in bacterial adherence to hemocytes. OMPs contribute to bacterial adhesion to vertebrate and insect host tissues (7,9). The appearance of new OMPs and the increase in the amounts of existing OMPs may represent an increase in antigenic/adhesion proteins. The differences in the amounts of other polypeptides produced by bacteria that differ with respect to the hemocyte types to which they adhere may reflect differences in suppressive or masking OMPs.

In summary, the adhesion of two strains of *X. nematophilus* to the antibacterial hemocytes of nonimmune *G. mellonella* larvae was influenced by neither the physicochemical properties of the bacteria, extracellular metabolites or changes in LPS, nor the presence of flagella or glycocalyces. Attachment appeared to be mediated by fimbriae, and possibly by OMPs, and the presence of insect serum.

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