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Surface Interactions between *Escherichia coli* and Hemocytes of the Mediterranean Mussel *Mytilus galloprovincialis* Lam. Leading to Efficient Bacterial Clearance

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The role of type 1 fimbriae in the interactions between *Escherichia coli* and *Mytilus galloprovincialis* Lam. hemocytes was evaluated. The association of fimbriated strain MG155 with hemocyte monolayers at 18°C was 1.5- and 3- to 4-fold greater than the association of unfimbriated mutant AAEC072 in artificial seawater and in hemolymph serum, respectively. Such differences were apparently due to different adhesive properties since MG155 adhered more efficiently than AAEC072 when hemocytes were incubated at 4°C to inhibit the internalization process. Hemolymph serum increased both association and adherence of MG155 two- to threefold but did not affect association and adherence of AAEC072. MG155 was also 1.5- to 1.7-fold more sensitive to killing by hemocytes than AAEC072, as evaluated by the number of culturable bacteria after 60 and 120 min of incubation. The role of type 1 fimbriae in MG155 interactions with hemocytes was confirmed by the inhibitory effect of D-mannose. In in vivo experiments MG155 cells were cleared from circulating hemolymph more rapidly than AAEC072 cells were cleared. These results confirm that surface properties are crucial in influencing bacterial persistence and survival within mussel hemolymph.

Marine bivalves (such as mussels, clams, and oysters) are filter feeders and use ciliated gill epithelia and mucous membranes to sieve suspended food particles from the aquatic environment (2, 5, 23, 29). Bivalves are also able to trap and accumulate bacteria and viruses present in the harvesting waters and may act as passive carriers of human pathogens. Two general groups of pathogenic bacteria may be transmitted by bivalves: bacteria indigenous to the marine environment, predominantly members of the family *Vibrionaceae*, which may be pathogenic for humans; and nonindigenous bacterial pathogens that are shed into the water from infected animals and humans. Consumption of raw or inadequately cooked bivalves has been implicated in numerous food-poisoning outbreaks; thus, the microbial flora of these animals is of great concern to public health.

Shellfish depuration in controlled waters is used extensively worldwide to decrease the number of unwanted microorganisms to levels acceptable for human consumption. Bacteria have different sensitivities to this purification procedure (15, 20, 21, 26); for instance, some *Vibrio* species have been reported to be particularly resistant to the process and are able to persist and multiply within shellfish tissues (12, 16, 29).

A relationship between bacterial resistance to depuration and sensitivity to hemolymph killing activity has been suggested (11). In fact, shellfish hemolymph contains both hemocytes, which are responsible for cellular defense mechanisms (i.e., phagocytosis, production of reactive oxygen intermediates, and release of lysosomal enzymes), and humoral defense factors, such as opsonizing lectins and hydrolytic enzymes (1, 7, 9, 14, 22, 24, 31, 33). The capacities of different bacteria to survive hemolymph microbicidal activity depend on their sensitivities to combinations of these factors.

At present, why certain bacteria are more sensitive than others to hemolymph killing is not fully understood. The role of bacterial cell wall ligands in the mechanisms of recognition of microorganisms by host cells has been extensively analyzed in humans (17–19). For instance, it has been shown that type 1 fimbriae expressed by *Escherichia coli* strains enable these organisms to adhere to several types of epithelial cells, mediate attachment to human polymorphonuclear leukocytes, and trigger intracellular killing (17, 18, 28).

Although several studies have described the bactericidal activity of marine bivalve hemolymph (2, 12, 16), few authors have examined in detail the bacterial surface properties that may influence the interactions with hemocytes (8, 11). Since type 1 fimbriae can be expressed by several enteropathogenic bacteria (28) that are introduced into the aquatic environment through fecal contamination and can be concentrated by bivalve molluscs (5), a study was designed to investigate the role of these ligands in the fate of bacteria within mussel hemolymph. In this paper we describe interactions of fimbriated and unfimbriated *E. coli* strains with hemocytes of *Mytilus galloprovincialis* Lam. Mussels were chosen as representatives of important and appreciated seafood in the Mediterranean area, where cultivation of these animals is extensive.

E. coli MG1655 (=CGSC6300) (10), a wild-type strain carrying type 1 fimbriae, and an unfimbriated derivative of this strain, AAEC072 Δ*fim* (3), were used in this study. All cultures were grown in Luria-Bertani (LB) broth (27) at 37°C for 18 h under static conditions. To radiolabeled bacteria, the strains were grown overnight in LB broth containing 10 μCi of [*meth-*

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yl-^{3}H]thymidine (25 Ci/mmol) ml^{-1}. Cells were then harvested by centrifugation (3,000 × *g* for 15 min at 4°C), washed three times with phosphate-buffered saline (PBS) (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, 0.15 M NaCl; pH 7.2 to 7.4), and resuspended in PBS at an A_{650} of 1 (2×10^9 to 4×10^9 CFU ml^{-1}). The number of counts per minute per milliliter and the number of CFU per milliliter were evaluated to calculate the efficiency of cell labeling (number of CFU per count per minute), which varied in the different bacterial preparations from 120 to 330 CFU/cpm. Mussels (*M. galloprovincialis* Lam.) were obtained from the Casa del Pescatore depuration plant (Cattolica, Italy) during spring 1999. The average monthly temperature and average salinity at the collection site were 15°C and 35‰, respectively. Mussels were transferred to the laboratory, the epibiota was removed, and the mussels were kept in an aquarium at 16°C in static tanks containing artificial seawater (ASW) (1 liter/animal) for 1 to 3 days before they were used; the seawater was changed daily. Hemolymph was extracted from the posterior adductor muscle of at least 30 mussels for each experiment by using a sterile 1-ml syringe with an 18-gauge, 0.5-in.-long needle (4). After the needle was removed, the hemolymph was filtered through sterile gauze and pooled in 50-ml Falcon tubes at 4°C. To obtain hemolymph serum (i.e., hemolymph free of cells), whole hemolymph was centrifuged at 200 × *g* for 10 min, and the supernatant was passed through a filter (pore size, 0.22 μm) to sterilize the hemolymph serum.

To study bacterium-hemocyte interactions, the number of hemocytes per milliliter of hemolymph was determined before each trial to obtain the desired experimental ratio of hemocytes to bacteria (1:10). A separate aliquot of hemolymph was stained with 1% (vol/vol) Gram's crystal violet in ASW, and the cells were counted by microscopic examination with a Thoma chamber. An approximately 0.3-ml portion of hemolymph (corresponding to about 2×10^6 to 3×10^6 cells) was seeded onto glass coverslips (20 by 22 mm) placed in plastic culture dishes. The coverslips were incubated at 18°C for 30 min. After nonadherent hemocytes were removed by gently washing the preparations three times with 3 ml of ASW, 1.5 ml of either ASW or hemolymph serum containing radiolabeled bacteria at a final concentration of 2×10^7 to 3×10^7 CFU ml^{-1} was added, and the dishes were incubated with gentle shaking at either 18°C (to evaluate all associated bacteria [i.e., attached plus internalized]) or 4°C (to evaluate attached bacteria only). Triplicate preparations were made for each sample. After 60 and 120 min of incubation the coverslips were rinsed three times with 3 ml of cold ASW to remove nonadherent bacteria and transferred to PICO-FLUOR 15 scintillation fluid (Packard Instrument Company Inc., Meriden, Conn.). The number of counts per minute per coverslip was evaluated with a Beckman L5 1801 scintillation counter. For each sample, the number of bacteria per monolayer was calculated by multiplying the counts per minute by the efficiency of cell labeling. The values obtained by this method included both live bacteria and bacteria killed by the hemolymph. To evaluate background counts due to bacterial attachment to coverslips, triplicate samples for each treatment were added to coverslips without hemocytes; the radioactivity of these controls (typically 50 to 250 cpm) was subtracted from the sample values. Bacterium-hemocyte interactions were also evaluated in the presence of D-mannose and D-galactose at a final concentration of 2 mg ml^{-1}.

To evaluate bacterial sensitivity to killing by hemocytes, *E. coli* suspensions (about 3×10^7 CFU ml^{-1} each) were added to hemocyte monolayers at 18°C in the presence of hemolymph serum as described above. Triplicate preparations were made for each sampling time. Immediately after inoculation (zero time) and after 60 and 120 min of incubation, supernatants were collected from the monolayers and hemocytes were lysed by adding cold distilled water and agitating the preparations for 10 min. The supernatants and lysates were pooled, 10-fold serially diluted, and plated onto LB agar to enumerate the culturable bacteria. Percentages of killing were determined in comparison to values obtained at zero time. To evaluate the presence of endogenous bacteria in hemocytes, we included controls consisting of hemocyte monolayers without additional bacteria. The number of CFU in controls never exceeded 0.1% of the number in experimental samples. To detect and correct for bacterial growth in hemolymph serum, separate samples were seeded with bacteria and 1.5 ml of sterile hemolymph serum. No appreciable bacterial growth was observed at the same time intervals used in the killing experiments. All the experiments were also performed in the presence of D-mannose and D-galactose at a final concentration of 2 mg ml^{-1}. Filter-sterilized ASW was used in all experiments.

When bacterial clearance was studied, 50 μl of each bacterial suspension (1×10^9 CFU ml^{-1}) was injected into the anterior adductor muscle of 15 mussels. The mussels were placed in a plastic tank containing filter-sterilized ASW at 18°C (0.5 liter/mussel). Before the first hemolymph sample was removed, the injected bacterial suspension was allowed to equilibrate for 30 min in the whole hemolymph volume, as previously described for other bivalves (1). At this time (arbitrarily considered zero time) and after another 60 and 120 min, 0.1-ml hemolymph samples were withdrawn from the anterior adductor muscles of five animals; each sample was placed in a tube containing 9.5 ml of sterile distilled water and mixed to osmotically lyse the hemocytes. Tenfold serial dilutions in PBS of this lysate were plated onto LB agar. We also included controls in which hemolymph samples withdrawn from a parallel set of 15 noninjected animals were plated and incubated under the conditions described above. The hemolymph samples from control mussels were virtually free of bacteria.

Hemolymph serum agglutination assays were performed as previously described (32) by challenging bacteria with serial twofold dilutions of filter-sterilized hemolymph serum in round-bottom microtiter plates. A positive reaction consisted of an even layer of bacteria spread over the surface of a U-shaped well. Agglutination was also checked by microscopic examination (magnification, ×1,000). The agglutination titer (AT) was the reciprocal of the highest dilution that showed agglutination activity. Adsorbed hemolymph serum was obtained by adding 0.1 ml of a bacterial suspension (2×10^9 bacteria ml^{-1}) to 2 ml of hemolymph serum. After 2 h of incubation at 4°C, the bacteria were removed by centrifugation, the supernatant was treated as described above, and the procedure was repeated three times.

Data, representing means based on at least three separate trials, were analyzed for significance by the Mann-Whitney U test. Differences were considered significant at $P \leq 0.05$.

TABLE 1. Association of fimbriated strain MG155 and unfimbriated strain AAEC072 of *E. coli* with *M. galloprovincialis* hemocytes at 18°C

Strain and exptl conditions	No. of bacteria/monolayer (10^5)			
	ASW		Hemolymph serum	
	60 min	120 min	60 min	120 min
MG155	15 ± 0.8[a]	31 ± 2.5	31 ± 2.1	83 ± 6.5
AAEC072	10 ± 0.5	20 ± 1.2	11 ± 0.9	19 ± 1.1
MG155 + D-mannose	11 ± 0.7	23 ± 1.1	14 ± 0.6	28 ± 1.9
MG155 + D-galactose	16 ± 0.9	33 ± 1.9	35 ± 2.5	88 ± 7.9
AAEC072 + D-mannose	11 ± 0.9	25 ± 2.2	13 ± 0.6	21 ± 1.9
AAEC072 + D-galactose	11 ± 0.8	19 ± 0.8	11 ± 0.8	24 ± 2.1

[a] Mean ± standard deviation.

TABLE 2. Adherence of fimbriated strain MG155 and unfimbriated strain AAEC072 of *E. coli* to *M. galloprovincialis* hemocytes at 4°C

Strain and exptl conditions	No. of bacteria/monolayer (10^5)			
	ASW		Hemolymph serum	
	60 min	120 min	60 min	120 min
MG155	9 ± 0.6[a]	19 ± 1.2	20 ± 1.7	55 ± 4.9
AAEC072	5 ± 0.4	9 ± 0.7	5 ± 0.3	9 ± 0.9
MG155 + D-mannose	6 ± 0.5	10 ± 0.9	8 ± 0.7	14 ± 1.3
MG155 + D-galactose	10 ± 0.8	21 ± 1.9	21 ± 1.8	58 ± 4.9
AAEC072 + D-mannose	5 ± 0.4	11 ± 1.0	6 ± 0.5	10 ± 0.9
AAEC072 + D-galactose	7 ± 0.6	9 ± 0.8	5 ± 0.4	11 ± 0.9

[a] Mean ± standard deviation.

To study the role of type 1 fimbriae in *E. coli* interactions with *Mytilus* hemocytes, a wild-type strain expressing type 1 fimbriae (MG155) and an unfimbriated mutant (AAEC072) were used to infect hemocyte monolayers in both ASW and hemolymph serum at 18°C. Table 1 shows that in both ASW and hemolymph serum, the association of the fimbriated strain with hemocytes was greater than the association of the mutant lacking fimbriae after both 60 and 120 min of incubation ($P \leq 0.05$). Moreover, the presence of hemolymph serum greatly increased the association of the fimbriated strain ($P \leq 0.05$), but it did not affect the association of the unfimbriated mutant. The observed differences between the two strains were more evident in hemolymph serum than in ASW; under the former conditions the association of the fimbriated strain was three- to fourfold greater than the association of AAEC072, whereas under the latter conditions the number of associated fimbriated bacteria was only about 1.5-fold greater than the number of unfimbriated cells.

To verify that the observed differences between the two strains were due to the presence of type 1 fimbriae, we took advantage of the fact that these ligands bind D-mannose, methyl-α-D-mannoside, and other D-mannose derivatives (18). Therefore, the same experiment was performed in the presence of D-mannose. As shown in Table 1, D-mannose reduced the association of the fimbriated strain ($P \leq 0.05$), whereas it did not affect the association of the mutant. Interestingly, the reduction in association due to D-mannose was greater in hemolymph serum (55 to 66%) than in ASW (26 to 27%). D-Galactose did not affect the association of either strain.

Adhesion is one of the key factors that affect bacterial and mammalian cell interactions (19). To clarify to what extent the observed differences in the association of the two strains with mussel hemocytes were due to different adherence capabilities, the same experiment was performed at 4°C. At this temperature the internalization process is almost completely inhibited both in mussel hemocytes (Canesi, personal observations) and in clam hemocytes (30). As shown in Table 2, both in the presence and in the absence of hemolymph serum, more MG155 cells than AAEC092 cells adhered to hemocytes ($P \leq 0.05$) after both 60 and 120 min of incubation. This difference was particularly evident in the presence of hemolymph after 120 min of incubation. Moreover, hemolymph serum significantly increased the adherence of the fimbriated strain ($P \leq 0.05$) but did not affect the adherence of the unfimbriated mutant. The adherence of the fimbriated strain in hemolymph serum was four to six times greater than that of AAEC072, whereas in ASW the number of adherent fimbriated bacteria was only about twice the number of adherent unfimbriated cells. In the presence of D-mannose, adhesion of fimbriated bacteria was significantly reduced both in ASW and in hemolymph serum ($P \leq 0.05$), whereas adherence of the unfimbriated strain was not affected (Table 2). No effect was observed with D-galactose.

These data demonstrate that type 1 fimbriae play a role in the surface interactions between *E. coli* and mussel hemocytes and indicate that the difference in association between the two strains studied could be largely due to differences in adhesion both in ASW and in hemolymph serum. The results obtained in ASW indicate that mussel hemocytes express receptors for type 1 fimbriae; on the other hand, the results obtained in the presence of hemolymph serum suggest that humoral factors may specifically opsonize fimbriated bacteria, enabling them to more efficiently interact with hemocytes. Therefore, the presence in hemolymph serum of agglutinating molecules was tested by challenging the strains with mussel hemolymph serum and evaluating the AT. Since we observed no difference between the two strains (AT = 16), a similar experiment was performed after hemolymph serum adsorption with the unfimbriated mutant to remove common agglutinins. Although AAEC072-adsorbed hemolymph serum was no longer able to agglutinate the unfimbriated bacteria, it still caused agglutination of the fimbriated cells (AT = 4); this agglutination was inhibited by D-mannose. These data support the hypothesis that hemolymph serum contains agglutinins specific for type 1 fimbriae and that the agglutinins may play a role in mediating interactions with hemocytes.

The possibility that the observed differences between the two strains could lead to differences in sensitivity to hemocyte bactericidal activity was investigated. In vitro experiments showed that fimbriated strain MG155 was more sensitive to killing by hemocyte monolayers than mutant AAEC072 was. In fact, after 60 and 120 min of incubation, the percentages of culturable bacteria compared to the zero-time values were 49 and 30%, respectively, for the fimbriated strain and 67 and 52%, respectively, for the unfimbriated strain. All differences between the two strains were statistically significant ($P \leq 0.05$). In the presence of D-mannose (but not D-galactose), MG155 sensitivity to killing did not differ significantly from AAEC072 sensitivity to killing. Neither sugar affected the sensitivity of the

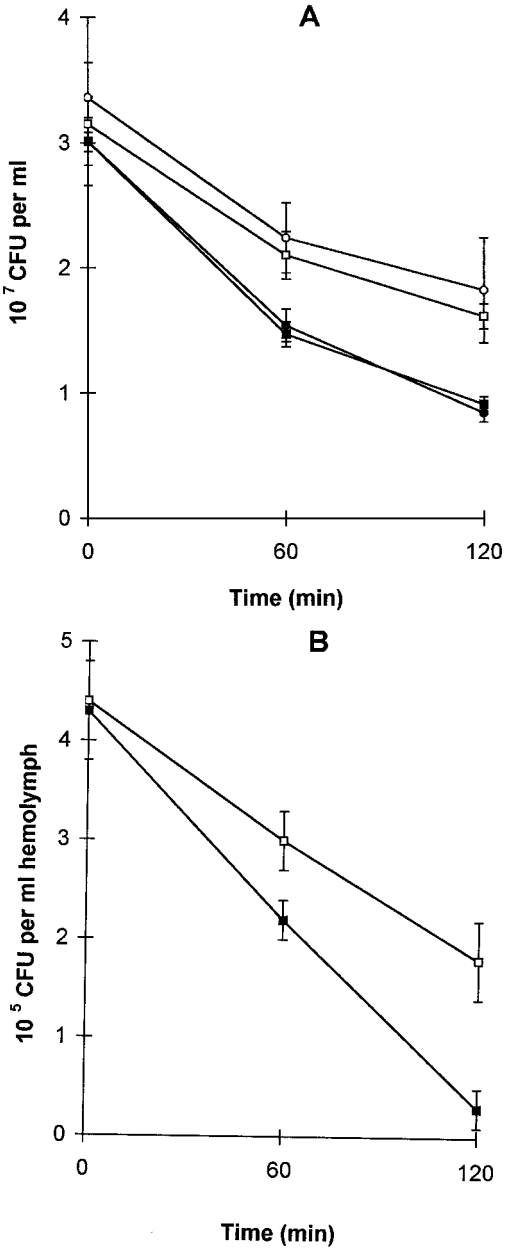

FIG. 1. Survival of fimbriated strain MG1655 and unfimbriated strain AAEC072 of *E. coli* in hemocyte monolayers in vitro (A) and in mussel hemolymph in vivo (B). (A) Number of culturable bacteria per milliliter in the hemocyte monolayer. Symbols: ■, MG1655; □, AAEC072; ●, MG1655 in the presence of D-galactose; ○, MG1655 in the presence of D-mannose. (B) Number of culturable bacteria per milliliter of hemolymph. In this experiment, zero time corresponded to 30 postinjection (that is, the time interval required for hemolymph to reach equilibrium after bacterial injection), as described in the text. Symbols: ■, MG1655; □, AAEC072. Bars indicate standard deviations.

unfimbriated strain to hemocyte bactericidal activity (data not shown). These data indicate that type 1 fimbriae increased the adherence of *E. coli* to hemocytes, which led to increased sensitivity to hemolymph killing in vitro. Experiments are in progress to identify the main mechanism responsible for the observed higher bactericidal activity of mussel hemolymph towards *E. coli* strains expressing type 1 fimbriae.

In vivo experiments with bacterium-injected mussels showed that a fimbriated *E. coli* strain was more efficiently cleared from circulating hemolymph than its unfimbriated derivative. In fact, the number of culturable MG155 cells was significantly lower than the number of AAEC072 cells ($P \leq 0.05$) at both 60 and 120 min (Fig. 1B). In particular, at 120 min the number of CFU per milliliter of hemolymph was about 14-fold lower than the number present at zero time for MG155, whereas only a 2.5-fold decrease was observed with AAEC072. These data suggest that surface properties of bacteria could also influence the interactions with mussel host cells in vivo. The faster clearance of the fimbriated strain may have been due to its greater sensitivity to hemolymph killing; however, the possibility that some fimbriated bacteria could have adhered to tissues along the sinus wall cannot be ruled out.

These data, although preliminary, are of interest considering that type 1 fimbriae are also expressed by human pathogens (e.g., *Salmonella* spp.) that are transmitted to humans by shellfish cultured in unsafe waters (5). Since type 1 fimbria oligosaccharide receptors are different in different genera (6, 13), the role of these structures in the interactions of pathogenic species other than *E. coli* with hemocytes and other mussel cell types is currently under investigation.

Overall, our results show the role of surface properties in the interactions between bacteria and mussel hemocytes and suggest that these properties may affect the fate of bacteria in bivalve tissues. Understanding the molecular basis of such interactions may elucidate the mechanisms that influence enteric bacterial ecology in the marine environment.

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