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Interaction of the Fish Pathogen *Aeromonas salmonicida* with Rainbow Trout Macrophages

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A procedure was developed to culture rainbow trout macrophages (Mϕ) on supported glass coverslips. Using this method and a variety of well-characterized *Aeromonas salmonicida* strains with normal or altered cell surfaces, we investigated the role of this unusual bacterial surface in the bacterium-Mϕ interaction. An intact crystalline protein array, the A-layer, mediated adherence of *A. salmonicida* cells to Mϕ even in the absence of opsonins. In contrast, unopsonized cells of an A-layer-negative (A^-) mutant with a smooth lipopolysaccharide (LPS) layer were unable to interact with Mϕ. However, this ability was recovered when the A-layer was reconstituted onto the smooth LPS surface of these A^- LPS^+ cells. Two *A. salmonicida* mutants possessing the A-layer in different disorganized states had a reduced ability to interact with Mϕ. A^+ cells grown under calcium limitation produced A-layers locked into an alternative conformation which mediated the highest levels of Mϕ association in the absence of opsonins or any other surface coating. Coating A^+ cells with hemin greatly increased their levels of Mϕ association, and bacterial cells grown on trout blood agar plates also had a dramatic increase in their ability to interact with Mϕ. Only A^+ *A. salmonicida* cells were highly cytotoxic to trout Mϕ, especially after being coated with hemin, presumably due to a more focused targeting of the bacterial cell onto the Mϕ surface and/or into the intracellular regions of the Mϕ.

A large number of bacterial surfaces have now been shown to be defined by highly organized protein monolayers known as S-layers (1, 22, 37). Such layers are actually giant two-dimensional protein multimers, the single-protein subunits of which are arranged in one of a variety of possible ways (34). Very few S-layers have been described for pathogenic bacteria, the archetype being the A-layer of the fish pathogen *Aeromonas salmonicida* (43), which has been amply demonstrated to be an important virulence factor (17, 18; reviewed in references 19 and 21).

The A-layer covers the entire surface of all virulent strains of *A. salmonicida* (32). It is composed of subunits of a hydrophobic protein species, the A protein (18, 32), disposed as a paracrystalline tetragonal array (39) of defined three-dimensional structure (9). The gene encoding A protein, *vapA* (for virulence array protein), has been cloned and sequenced (2, 6), and the regions defining the major protein domains have been identified.

A protein is capable, in the absence of any other bacterial cell component, of self-assembling in vitro to form arrays similar to those observed in sloughs dislodged from wild-type strains of *A. salmonicida* (13). This self-assembly process requires the presence of divalent cations. Furthermore, in the absence of calcium, the A-layer can adopt unusual, regularly arrayed patterns, recently observed by electron microscopy (13). Although assembly into regular arrays may occur in the absence of other bacterial cell components, the layer requires lipopolysaccharide (LPS) O-chains to remain anchored to the cell surface (3). Some of the O-polysaccharide chains penetrate the A-layer and remain exposed on the cell surface (5). O-chain-deficient mutants produce assembled A-layer sheets (9) or tetrameric structures (15) that are released into the culture medium.

We recently demonstrated that the A-layer is capable of mediating attachment to and penetration of murine macrophages (Mϕ), apparently through a specific mechanism (12). Furthermore, only the assembled layer was effective, yet different preparations of assembled A-layer varied in efficacy, suggesting that specific properties of the A-layer may modulate its adhesin-like activity. These properties included the number of LPS O-chains associated with the assembled layer, the proportion and type of regular arrays present in the layer, and the layer's orientation.

Recognizing the importance of surface macromolecular structures of bacterial pathogens in determining specific interactions with their host (38, 45), we proposed to study the effect that some structural surface modifications, especially those effecting the A-layer, have on the ability of *A. salmonicida* to associate with trout Mϕ.

MATERIALS AND METHODS

Bacterial strains. *A. salmonicida* A450, a virulent A^+ LPS^+ strain isolated from a brown trout with furunculosis in France, was used as the wild type (18). Different derivatives of A450, isolated in this laboratory, were also used: A450-3, an avirulent A^- LPS^+ strain (17); A450-1, an avirulent A^+ LPS^- (O-chain-deficient), A-layer-excretor strain (18); A450-10S, an A^+ LPS^+, slow-growing, attenuated mutant (40); and A450-10SR, an A^+ LPS^+, fast-growing pseudorevertant of A450-10S (40).

Mϕ. Mϕ were routinely isolated from the head kidney of rainbow trout (*Oncorhynchus mykiss*) by a modification of the method described by Braun-Nesje et al. (4). Briefly, the head kidney tissue, dissected from a rainbow trout (150 to 300 g) under aseptic conditions, was forced through a nylon mesh of 20-μm pores to produce a cell suspension in incomplete L-15 tissue culture medium. Mϕ were separated from other cell populations in a continuous-density gradient of Percoll (29). The continuous-density gradient was self-generated in situ in 10 ml of Nalgene polycarbonate tubes

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(containing 4.5 ml of stock isotonic Percoll solution and 4.0 ml of head kidney cell suspension in incomplete L-15) by centrifugation at 20,000 × *g* for 20 min in an angle-head rotor (Beckman JA-20). After centrifugation, five main bands and a pellet of melanin granules had formed. The band enriched in Mϕ (third from the top, with a density distribution of 1.069 to 1.075) was transferred to a separate tube and washed three times with incomplete L-15. A suspension of 2×10^6 cells per ml was then prepared and dispensed in 0.5-ml aliquots onto supported coverslips. Supported coverslips consisted of glass coverslips (22 by 22 mm) raised from the bottom of 35-mm tissue culture dishes by means of a glass piece (10 by 10 by 3 mm). Coverslips were centered so that no contacts occurred between their edges and the culture dish. Melted Vaseline was used to attach the supporting glass piece to the bottom of the 35-mm tissue culture dish and to attach the coverslip to the supporting glass piece. Alternatively, Mϕ were plated and kept in 24-well tissue culture plates (Falcon Laboratories). In this case, Mϕ were originally plated at 0.5×10^6 per well.

Two hours after plating, nonadherent cells were washed off, and the adherent Mϕ were covered with complete L-15 medium. The yield of adherent Mϕ varied between 10 and 15% of the plated cells. The purity of our Mϕ preparations varied from batch to batch, but the minimal final purity, after nonadherent cells were removed, was ≥95%.

Buffers, media, and cultivation conditions. Hanks balanced salt solution (HBSS, pH 7.6) was used as the main buffer throughout. Occasionally, it was substituted by phosphate-buffered saline (PBS, pH 7.4), especially for the washing of bacterial cells. Bacteria were grown in tryptic soy broth (TSB), in fish peptone medium (FPM), on tryptic soy agar plates (TSA), or on supplemented TSA plates, as needed. For FPM, peptone powder p-0100 (Marine Biochemicals A.S.) was dissolved at a final concentration of 0.2% in modified minimal Davis salts, containing (in grams per liter): K_2HPO_4, 5.23; KH_2PO_4, 2.25; Na_2HPO_4, 4.26; NaH_2PO_4, 1.98; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1. After the medium was autoclaved, glucose was added to a final concentration of 0.4%. TSA-Congo red (CR) (30 μg/ml) was prepared as described previously (16). TSA-hemin plates were supplemented with hemin at a final concentration of 10 μg/ml. Hemin was first dissolved in a 1:1 mixture of dimethyl sulfoxide-Tween 20, further diluted in deionized water, filter sterilized, and used as needed. TSA-blood plates contained 10% (vol/vol) aseptically collected, heparinized fresh trout blood. Hemin and blood supplements were added to the TSA base at 50°C before the medium was poured into plates. The dilution medium used for counting viable cells was nutrient broth containing 0.5% (vol/vol) Tween 20.

The bacteria used in association assays with Mϕ were routinely grown on TSA or TSA-blood plates at 20°C for 48 to 72 h. Alternatively, bacteria were grown in TSB or FPM at 20°C, with agitation, for 14 to 16 h. Bacteria grown on plates were scraped off the surface, suspended in PBS, and pelleted by centrifugation (1,500 × *g* for 5 min). Bacteria grown in liquid media were pelleted under the same conditions. Pellets were washed twice in PBS, and bacterial suspensions in HBSS or incomplete L-15 were prepared.

Mϕ were kept in culture at 15°C for up to 1 week but preferably were used after 1 or 2 days in culture. The tissue culture medium used was L-15 with glutamine (GIBCO) supplemented with 0.33% glucose (incomplete L-15). Complete medium was prepared by adding bovine calf serum (Flow Laboratories) to the incomplete medium at a final concentration of 10% (vol/vol) and also adding a mixture of antibiotics and an antimycotic agent (streptomycin, penicillin, and amphotericin B), following the manufacturer's recommendations (14).

Staining and counting methods. Monolayers of Mϕ were air dried, fixed for 5 min in methanol, and stained with Wright or Giemsa stain (Sigma). Bacteria were stained dark purple by these stains.

Mϕ in suspension were counted in a Neubauer hemacytometer after 1:2 dilution in a 0.4% solution of trypan blue in PBS. The number of attached Mϕ or attached bacteria per coverslip, the percentage of infected Mϕ, and the number of bacteria per infected Mϕ were determined by direct microscopy (95% confidence level) by the recommendations of Mallette (24). Usually, 250 Mϕ per coverslip were analyzed. The average number of bacteria per infected Mϕ was multiplied by the percentage of infected Mϕ to obtain the number of bacteria per 100 Mϕ.

Several counts of viable cells in suspensions of the different bacterial strains used, at an optical density at 650 nm (OD_{650}) of 1, were made by the standard dilution plate method, and the average number of CFU per milliliter was determined (7.5×10^8 CFU/ml of a bacterial cell suspension at an OD_{650} of 1). Dilutions of suspensions (OD_{650} of 1) were made routinely to adjust the bacterial cell density as needed.

SEM. Scanning electron microscopy (SEM) of Mϕ was done by conventional methods. Briefly, Mϕ attached to round (10-mm-diameter) glass coverslips were fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical-point dried, and mounted on aluminum stubs for gold coating. Specimens were examined with a JEOL JSM 35 scanning electron microscope at 10 kV. Negative staining of A-layers was done on Formvar-coated copper grids with ammonium molybdate as described previously (40).

Purification of A-layer and A protein. Outer membrane (OM) preparations of A450, extracted with sodium lauryl sarcosinate, were prepared as reported previously (18, 31). OM preparations were subjected to serial extractions with sodium deoxycholate (DOC) as follows. An OM preparation (4.5 ml), 4.0 ml of 10-mg/ml lysozyme, and 31.5 ml of 20 mM Tris (pH 8.0) with 5 mM EDTA were mixed and incubated for 30 min at room temperature with agitation. Then, 40 ml of 4% DOC in 0.5 M NaCl with 5 mM EDTA was added, and the preparation was incubated for 30 min at 30°C. The extracted A-layer/OM pellet was separated by centrifugation at 10,000 × *g* for 10 min at room temperature and washed with 20 mM phosphate buffer, pH 7.3. This preparation was called 1× DOC. The DOC extraction steps were repeated to obtain the corresponding 2×, 3×, and 4× DOC preparations.

A protein from A450 was purified to homogeneity by previously published methods (31). A-layer sheets from the A-layer-secreting mutant A450-1 were purified from culture supernatants by centrifugation at 100,000× *g* for 1 h and a single DOC extraction.

Coating of latex beads. Latex beads (3.1-μm diameter; SERADYN Particle Technology Division) were washed extensively with sterile glycine-buffered saline (0.1 M glycine, 0.85% NaCl [pH 8.4]). The suspension of beads was adjusted to a density of 1% solids ($\sim 5.6 \times 10^8$ beads per ml), and an A-layer preparations from A450-1 was added at a final concentration of 50 μg/ml. Coating was allowed to occur at room temperature with gentle agitation for 3 h. The beads were then washed twice with sterile PBS and stored on ice.

A-layer reconstitution. A450-3 was cocultivated with A450-1 in a mixed lawn on TSA plates. Suspensions of the two strains to an OD_{650} of 1 were prepared in PBS and mixed

in an A450-3/A450-1 ration of 1.5:1. One hundred microliters of this mixture was spread on a TSA plate and incubated for 72 h at 20°C. Bacterial growth was scraped off the plate and washed twice in PBS. The bacterial cell density was adjusted as needed. Reconstitution in the liquid phase was done by a method similar to that of Griffiths and Lynch (15). Washed A450-3 cells suspended to an OD_{650} of 1 in an A450-1 culture supernatant were gently agitated for 3 h at room temperature. Alternatively, 0.4 mg of the 4× DOC A-layer preparation was added to 1 ml of the A450-3 cell suspension and agitated gently for 1 h at room temperature.

Bacterium-Mϕ association assays. Mϕ were gently washed twice with HBSS, and adherent cells from two coverslips were stained and counted. Based on the Mϕ count, the bacterial cell suspensions were adjusted so that 0.5 ml contained a number of bacteria equal to 50 times the number of Mϕ per coverslip. Coverslips were covered with 0.5 ml of the corresponding bacterial suspension in HBSS or in tissue culture medium. At each sampling time, coverslips were gently washed three times with 3 ml of HBSS and air dried. Mϕ were fixed and stained for examination by light microscopy.

The number of Mϕ per well in 24-well plates was estimated from the average yield of adherent versus plated cells, determined previously by direct microscopy. Bacteria were inoculated into the wells in these plates in sequence (at the corresponding sampling times), so that at the end of the experiment, all the wells in a plate were processed together by shaking off the well supernatants and washing the plate three times with HBSS by using a wash bottle. The plates were then air dried and stained. Well bottoms were cut with a cork boring machine and mounted on a glass slide for examination by light microscopy.

Phagocytosis experiments with latex beads were done in 24-well tissue culture plates at a beads-to-Mϕ ratio of 6:1 and by the protocol described for the bacterium-Mϕ association assays.

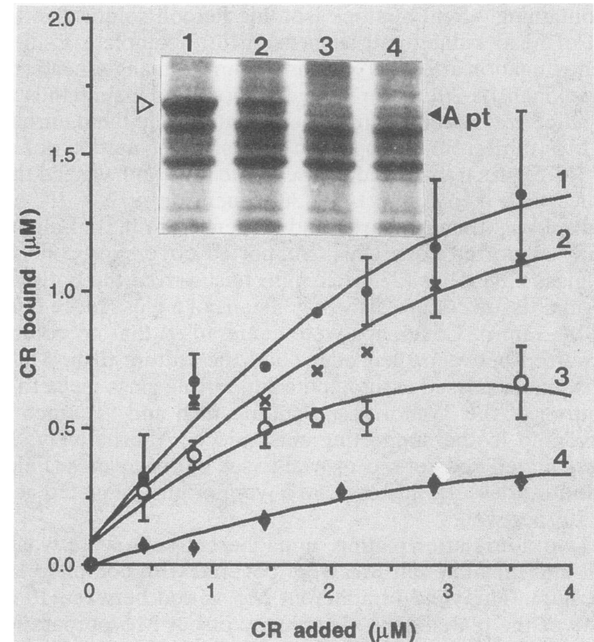

FIG. 1. Congo red binding of the A^+ reconstituted cocultured mixture (×) compared with that of the wild-type A450 (●) and the separate reconstitution partners A450-1 (○) and A450-3 (◆). Curves represent the averages of three independent assays. For clarity, standard deviation bars are shown for only two of the curves. (Inset) SDS-PAGE analysis of whole-cell lysates of the strains used in the CR-binding assays. The lanes in the inset and curves in the graph have the same numbers for direct identification: 1, A450; 2, reconstituted mixture; 3, A450-1; 4, A450-3. A distinct band at the A protein position (A-pt, indicated by the open and solid arrowheads) is observed in the reconstituted bacteria.

RESULTS

Culture of Mϕ. Isolation of Mϕ from head kidney tissue by the original method of Braun-Nesje et al. (4) or modifications of it is a widely used technique (36). The cells we isolated were adherent, capable of phagocytosing bacteria, were viable in culture for up to 1 week, and had a cell morphology very similar to that reported by Braun-Nesje et al. (4) and Secombes (35) and therefore were confidently considered to be Mϕ. The use of supported coverslips with free edges proved to have many quantitative advantages. The main cellular contaminant of Mϕ prepared from salmonid head kidney is lymphocytes (4). These nonadherent cells were easily and completely eliminated by washing the supported coverslips. In contrast, it was more difficult to eliminate nonadherent cells from cultures kept in walled devices (i.e., tissue culture dishes or multiwell plates). Moreover, at the time of plating, the surface tension of the tissue culture medium in contact with the coverslip edges forced the cells away from the edges, preventing their accumulation in regions that were difficult to observe. Adherent Mϕ, confined to the surface of the coverslip, were 100% observable and recoverable for counting purposes. Furthermore, all added bacterial cells also remained confined to the surface of the coverslip and interacted only and exclusively with the Mϕ on the coverslip. Bacterial cells were presumably more available to Mϕ in coverslip cultures because of the low depth of the liquid phase covering the culture. Mϕ on supported coverslips could be maintained with only 0.5 ml of buffer or tissue culture medium covering them.

Reconstitution of an A-layer on A^- LPS^+ cells. A functionally competent A-layer was successfully reconstituted onto the surface of A450-3 by a novel coculturing technique on agar plates. A450-1 produces A-layer sheets but cannot tether them to its cell surface because of the lack of LPS O-chains. These A-layer sheets, formed and released as the culture grew, attached to neighboring A450-3 (A^- LPS^+) cells, whose surface acted as an immobilized reconstitution template, producing a mixture of A^- and reconstituted A^+ bacteria. The presence of cell-associated A-layers in reconstituted bacteria was confirmed by the appearance of a distinct band at the A protein position on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 1, inset). The reconstituted A^+ mixture was shown to be still competent in Congo red-binding assays (20) (Fig. 1). Coculturing the two strains in liquid medium did not provide an effective reconstitution, as judged by SDS-PAGE (not shown).

A. salmonicida **surface changes and Mϕ association.** The use of different *A. salmonicida* strains, each with a different cell surface, allowed us to evaluate the role of predominant surface structures in the bacterium-Mϕ interaction.

Mϕ association with wild-type A^+ LPS^+ bacterial cells or with cocultured A-layer-reconstituted cells was significantly greater than with the A^- strains A450-1 and A450-3 (Fig. 2a

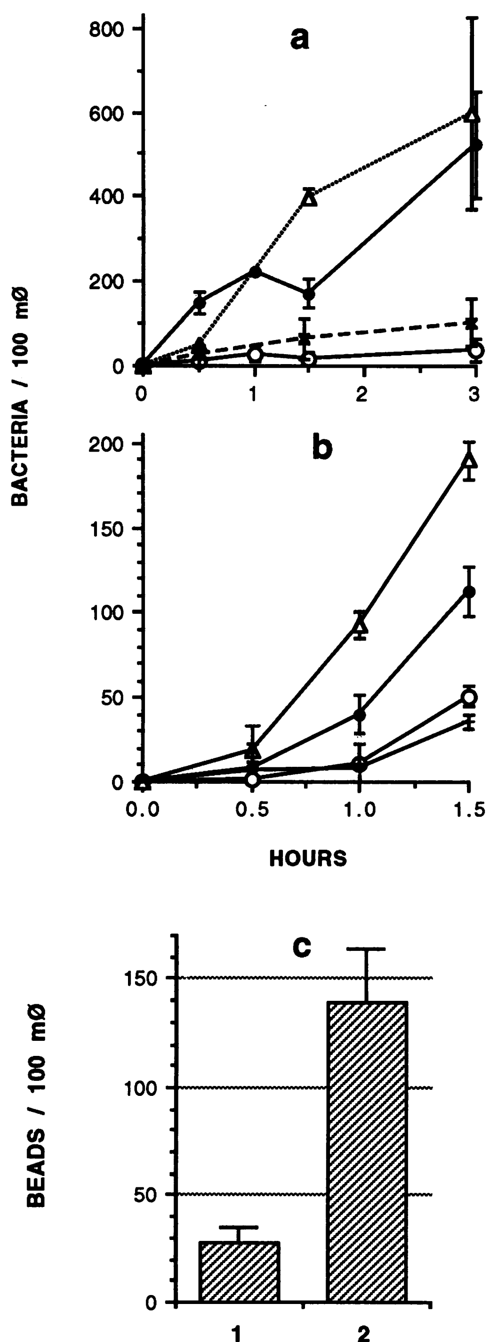

FIG. 2. (a) Mϕ association of the A^+ reconstituted cocultured mixture (△) compared with that of the wild-type A450 (●) and the separate reconstitution partners A450-1 (×) and A450-3 (○). Curves represent the averages of two independent experiments run in duplicate. (b) Effect of different A-layer reconstitution methods on Mϕ association. The A-layer was reconstituted in A450-3 by coculturing with A450-1 on TSA plates, as in panel a (△); in the liquid phase with the 4× DOC preparation (●); or in the liquid phase with the supernatant from an A450-1 culture (+). A control consisting of a 1:1.5 mixture of the A450-1 and A450-3 strains (○) was included. (c) Results from a 3-h Mϕ association assay with plain uncoated latex beads (column 1) and beads coated with A-layer sheets from A450-1 (column 2). Each column represents the average of three measurements in a single experiment. Error bars in panels a to c represent standard deviations.

and b). Phenotypically, A450-1 behaves as an A^- strain, since it is unable to tether the A-layers it produces. A^- LPS^+ cells (A450-3) reconstituted in the liquid phase with supernatant from an A450-1 culture were still unable to associate with Mϕ (Fig. 2b). In contrast, reconstitution in the liquid phase with the 4× DOC A-layer preparation resulted in the appearance of a cell-associated A protein band in SDS-PAGE (not shown) and slightly enhanced Mϕ association (Fig. 2b). Mϕ association with a mixture of A450-3 and A450-1 cells (mixed in a 1.5:1 ration just before the association assay) remained low (Fig. 2b), demonstrating that the enhanced association of the reconstituted mixture with Mϕ was due specifically to the reconstitution of an A-layer and not to the mere presence of both cell types. Furthermore, coating latex beads with A-layer sheets from A450-1 resulted in a fivefold increase in adherence compared with adherence to plain uncoated beads (Fig. 2c).

Effect of A-layer structural modifications. The A-layers of strains A450-10S and A450-10SR were previously shown to exhibit structural alterations (40). Electron microscopy of negatively stained specimens shows that the former exhibits a pattern of unstained units running at 45° to the regular tetragonal array and the latter exhibits a pattern of discontinuous A-layer aggregates (Fig. 3b, inset). Furthermore, A-layer aggregation on the surface of A450-10SR is known to occur only after 3 days in culture (40). Younger A450-10SR cultures produce A-layers similar to those of the parental strain A450. In reference to wild-type A450 and young A450-10SR cultures, A450-10S consistently showed a reduced capacity to associate with Mϕ (Fig. 3). However, dramatically decreased association of Mϕ with older A450-10SR cultures was observed, presumably associated with the A-layer aggregation process (Fig. 3b).

The A-layers assembled by A^+ bacteria grown in FPM displayed the novel BS (big squares) altered pattern (Fig. 4, inset). In contrast to the normal A-layer structure, formed by two morphological units (39), the altered BS pattern is formed by a single morphological unit (13). This effect has been associated with the lower level of calcium in FPM (7.5 μM Ca^{2+}) than in TSB (173 μM Ca^{2+}) (13). Other than this major change in the A-layer structure, no major changes in the protein or LPS profile of FPM-grown cells were detected on Coomassie blue-stained SDS-PAGE gels of whole-cell lysates or on Western immunoblots. It was striking that only A^+ strains grown in FPM (including A450-1) had a markedly enhanced ability to associate with Mϕ (Fig. 4); the A^- strain A450-3 grown in the same medium was still unable to associate with Mϕ (not shown).

Effect of hemin on Mϕ interaction. Bacterial cells coated in the liquid phase with hemin or the hemin analog CR (20) were used to study the effect of these coatings on their ability to associate with Mϕ. While A^+ bacteria coated with hemin associated with Mϕ better than uncoated cells (Fig. 5a), a CR coating was ineffective (not shown). Furthermore, A450-3 cells exposed to either of these compounds were still unable to effectively associate with Mϕ (Fig. 5a). Bacteria grown on TSA-blood plates exhibited much higher levels of Mϕ association (Fig. 5b) than did uncoated or hemin-coated bacteria (compare ordinates of Fig. 5a and b). The effect of trout serum components was eliminated as the cause of enhanced adherence by carrying out a simple association assay with bacteria opsonized in 10% fresh trout serum in incomplete L-15. None of the three bacterial strains tested (A450, A450-3, and A450-10SR) showed a significant increase in Mϕ association mediated by the presence of serum alone (data not shown).

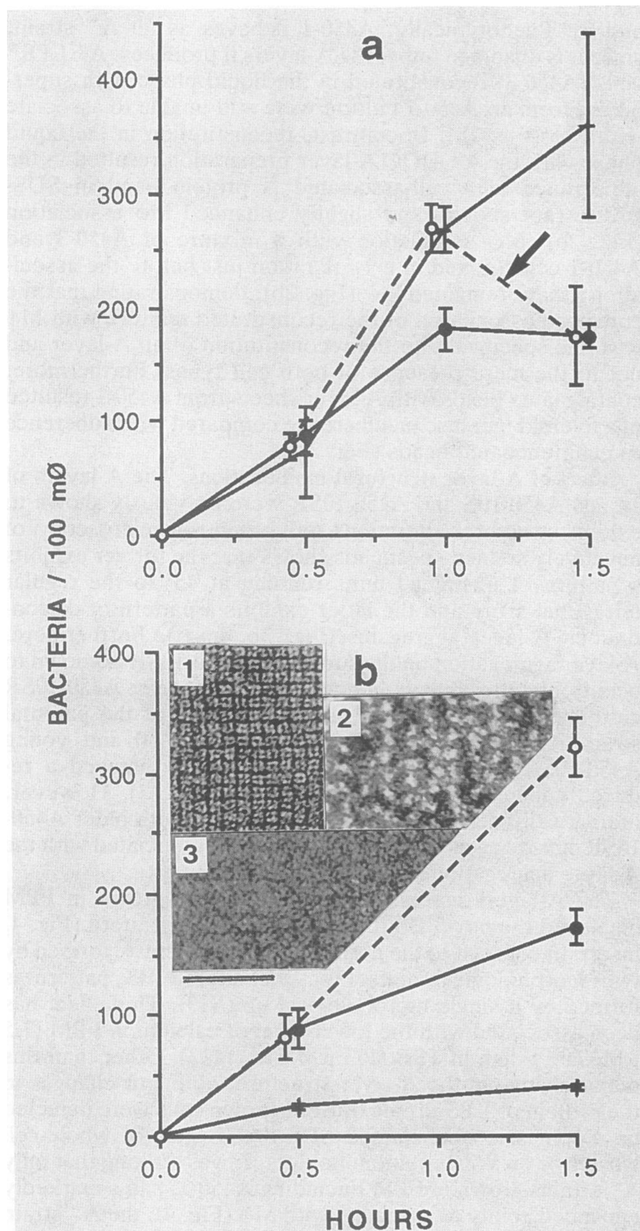

FIG. 3. Mϕ association of two different A450 mutants with structural alterations in their A-layers. (a) Average results from two independent bacterium-Mϕ association experiments in which fresh TSA cultures were used. Arrow indicates a drop in the association level due to a loss of heavily infected Mϕ as a result of toxic effects. Error bars represent standard deviations. (b) Results from an experiment in which 2-week-old TSA cultures were used. Symbols: ○, A450; ●, A450-10S; +, A450-10SR. Error bars represent standard deviations of the number of bacteria per Mϕ. (b, inset) Negatively stained specimens of: 1, normal A-layer from A450; 2, aggregated layers from A450-10SR; 3, disorganized layer form A450-10S. Bar, 50 nm.

Cytopathic effects of *A. salmonicida* on trout Mϕ. During bacterium-Mϕ association assays, we observed that an increase in the incubation time as well as the bacterium-Mϕ cell ratio (with a consequent increase in Mϕ association levels) was invariably accompanied by an increase in Mϕ detachment from the substratum and Mϕ lysis. As observed before in a more detailed microscopic study of the cytopathic effects of *A. salmonicida* on murine Mϕ (12), most of the Mϕ exposed to the A^- strains A450-3 and A450-1 had well-conserved morphological features similar to those of control Mϕ not exposed to bacteria (not shown). Cytopathic effects were observed mostly in association with A^+ bacteria, accounting for the abrupt reduction in the Mϕ association index of A^+ strains (450, 10S, and reconstituted A450-3), usually seen at the latest sampling time during the association assays (e.g., arrow in Fig. 3a). It is of interest that, in spite of being an A^+ strain capable of associating with Mϕ, A450-10SR never showed this reduction in Mϕ association, suggesting that it was probably not severely cytopathic to trout Mϕ.

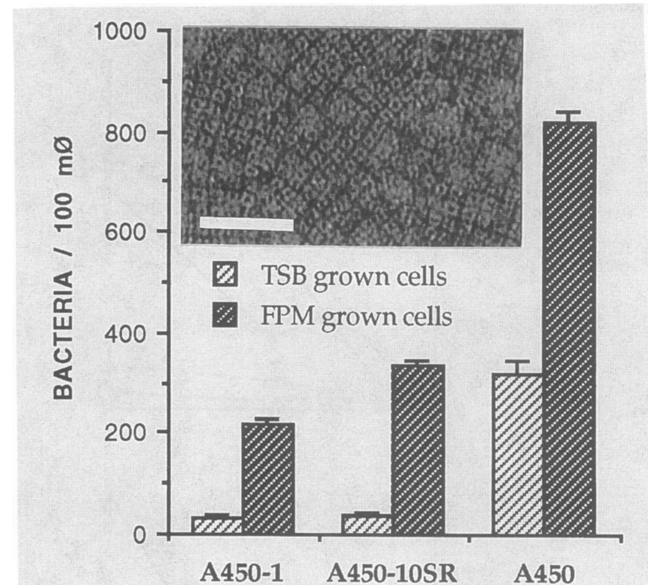

FIG. 4. Mϕ association of different A^+ strains grown in FPM or TSB. Association levels were determined after an incubation period of 1.5 h. (Inset) Micrograph depicting the unusual BS A-layer pattern displayed by FPM-grown A^+ cells. Bar, 50 nm.

It was evident, from microscopic observations, that *A. salmonicida* also produced morphological changes and lysis in trout Mϕ (Fig. 6). It was common to observe pronounced cell rounding and compaction (R in micrograph 2 and arrow in micrograph 6). Macrophages exposed to *A. salmonicida* grown in the presence of hemin presented a particularly peculiar shape (Fig. 6, panel 8), interpreted as massive lysis due to cell fragility. In some instances, Mϕ cell nuclei were also observed to lyse (Fig. 6, arrows in micrographs 7 and 8). Macrophages observed by SEM had a bizarre shape, presumably the result of lysis due to increased susceptibility to chemical or physical stress. Almost the entire surface of the coverslips prepared for SEM was analyzed to determine the frequency of lysis, and we found that the majority of Mϕ cells exposed to A450 cells had the morphological features presented in micrographs 2 and 3 (Fig. 6). Similarly, some Mϕ exposed to A450-3 presented the same features but at a lower frequency. It was interesting that lysed Mϕ revealed A^+ cells that had been internalized (arrows in Fig. 6, micrograph 3). Further support for bacterial cell internalization came from studies on the replication of *A. salmonicida* inside trout Mϕ in the presence of the aminoglycoside gentamicin (11).

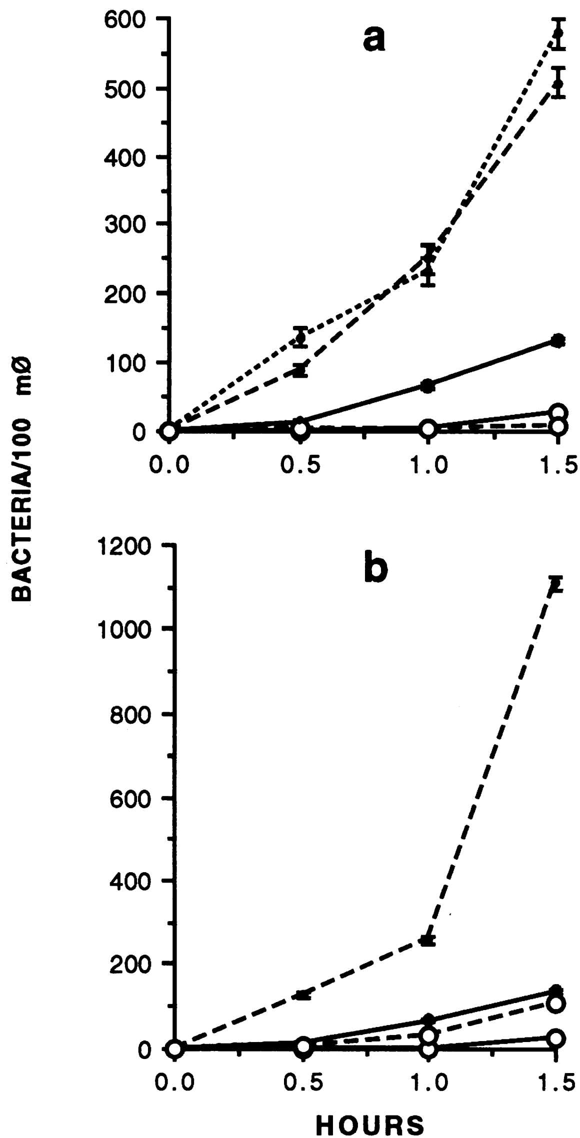

FIG. 5. Effect of different surface coatings on the Mϕ association levels of A^+ A450 (●) and A^- A450-3 (○) bacterial cells. (a) Effect of hemin coating. Coating was carried out by mixing bacterial cell suspensions (OD_{650} of 1) with hemin at room temperature for 10 min. Hemin was used at 1 μg/ml (---) and 0.2 μg/ml (· · · ·); ——, uncoated strains. (b) Effect of growth on TSA-blood plates (---) and on TSA alone (——). All experiments were performed with Mϕ from the same batch. Error bars represent standard deviations of the number of bacteria per Mϕ.

DISCUSSION

Previous studies with the murine Mϕ cell line $P388D_1$ demonstrated that the A-layer is involved in mediating a specific interaction between Mϕ and *A. salmonicida* (12). We have extended these studies to the more relevant salmonid (*O. mykiss*) Mϕ system and further examined the effects that some A-layer structural modifications have on this mediated interaction.

Our experimental system of rainbow trout Mϕ cultured on supported coverslips proved to be particularly useful, and we think it could easily be adapted and applied to other quantitative studies in which adherent cells in culture are used.

It was confirmed that the predominant bacterial surface structure, the A-layer, acts by facilitating an enhanced interaction with Mϕ, whereas an intact, smooth, hydrophilic LPS layer prevented Mϕ recognition. The *A. salmonicida* strain A450-1, which lacks both of these two main surface components, has a hydrophobic surface, as judged by adherence to hydrocarbons (33) and salting-out (23) and polymer partition (44) tests (not shown). The intermediate to low level of Mϕ association shown by A450-1 (Fig. 2a and b) suggests that nonspecific hydrophobic interactions played a minor role in the association with Mϕ.

To ideally demonstrate that the A-layer has an adhesin-like role, it would be necessary to follow the so-called molecular Koch's postulates (10), i.e., reintroduction of the cloned *vapA* gene into an A^- deletion mutant, with the subsequent regaining of adherence to host cells. This has not yet been possible because *vapA* has so far been refractile to expression in *vapA* deletion mutants (7). As an alternative approach, we used the in vitro reconstitution of A-layer onto the smooth LPS surface of the A^- deletion strain A450-3 (2) and demonstrated that the A-layer alone is capable of mediating efficient Mϕ recognition. An interesting finding was that reconstitution of A450-3 with culture supernatants containing A-layer sheets from the A-layer-secretory strain A450-1 was not particularly effective, as these cells did not recover the ability to associate with Mϕ (Fig. 2b). From a non-functional perspective, Griffiths and Lynch (15) reported that this method of reconstitution in the liquid phase was effective, but the A^+ LPS^- strain that they used released into the culture supernatant what they described as floret-like materials. We, too, observed this kind of material in negatively stained specimens of the 4× DOC A-layer preparation but not in culture supernatants of A450-1; instead, these contained regularly arrayed A-layer sheets similar to the double layers described by Dooley et al. (9). The monolayers that form these double layers interact through their inner faces, and thus the normal A-layer polarity is lost (Fig. 7a), accounting for the failure to achieve complete reconstitution of A450-3 cells (Fig. 7b). However, these double-layered sheets efficiently coated hydrophobic latex beads (Fig. 7c) and mediated efficient interaction of the coated beads with Mϕ (Fig. 2c) (12). Coated beads therefore displayed the correct wild-type A-layer orientation on their surfaces (compare Fig. 7c with d), which seems to be critical for effective Mϕ recognition. Our coculturing reconstitution method proved to be functionally effective, as judged by CR binding and efficient Mϕ association, most likely because single A-layers released during growth of A450-1 were immediately adsorbed onto the A450-3 cell surface template, before they had a chance to form double layers.

The presence of A-layer has profound effects on the characteristics of the *A. salmonicida* cell surface (18, 27, 28, 30, 42, 44). Therefore, it is conceivable that different A-layer arrangements are critical to the bacterial surface topology and, consequently, to Mϕ recognition. Both the mutant A450-10S, in which the A-layer appears to be disorganized, and A450-10SR, with aggregated A-layers, had a reduced capacity to associate with Mϕ. On the other hand, it was striking that growth in FPM dramatically increased the capacity of all A^+ bacteria tested to associate with Mϕ, whereas cells of the A^- LPS^+ strain were still incapable of Mϕ association. Since no major changes were detected in the protein and LPS profiles of FPM-grown cells, we have ascribed the increase in Mϕ association to the major struc-

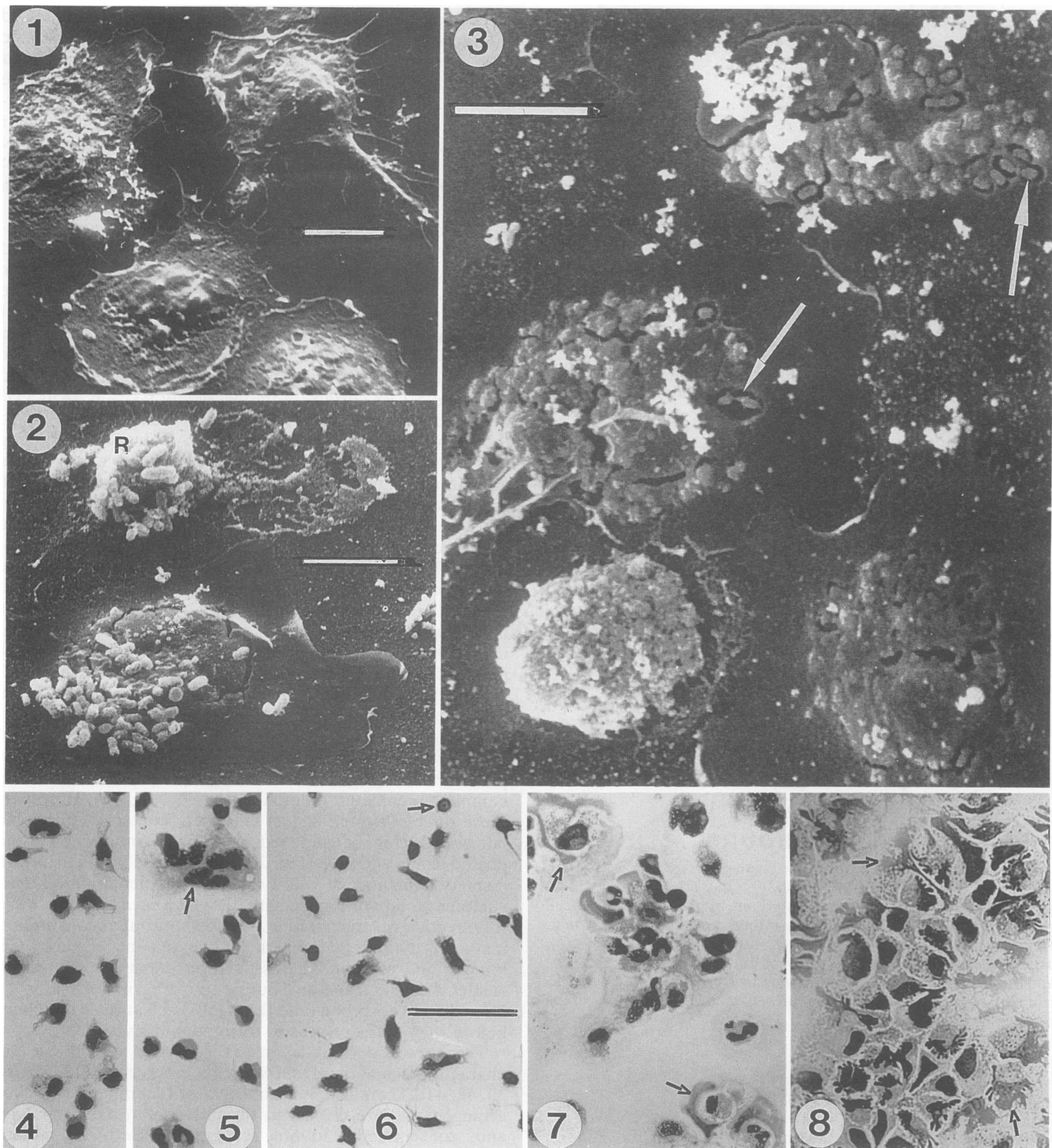

FIG. 6. Effects of *A. salmonicida* on Mϕ morphology. SEM of control, intact Mϕ (micrograph 1) and Mϕ exposed to A450 for 2 h, at a bacterium-to-Mϕ cell ratio of 50:1 (micrographs 2 and 3). R, rounded Mϕ covered with bacteria. Arrows point to exposed internalized bacteria. Bars, 10 μm. Light microscopy images of control Mϕ in L-15 (micrograph 4) and HBSS (micrograph 5). A multinucleated giant cell (35) is shown in the latter (arrow). Different Mϕ morphological changes induced by A450 are shown: pronounced Mϕ rounding (micrograph 6), lysis and cell distention (micrograph 7), and massive lysis (micrograph 8). All light micrographs have the same magnification, represented by the 50-μm bar in micrograph 6.

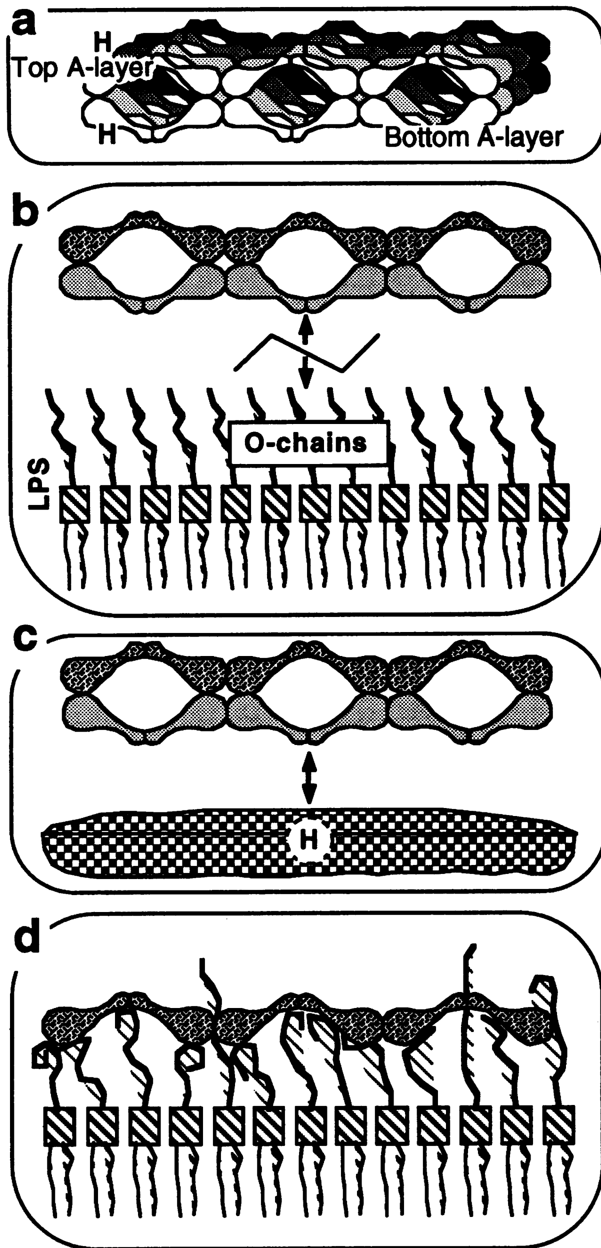

FIG. 7. Rationale for surface reconstitution with A-layer sheets from the A450-1 strain. (a) Simplified diagram showing the external hydrophobicity (H) and the lack of polarity of double A-layer sheets (according to Dooley et al. [9]). (b and c) Schematic explanation of why the bacterial surface of A450-3, covered with a smooth hydrophilic LPS layer, was not efficiently reconstituted (b), while a hydrophobic (H) latex bead surface was (c). (d) Single A-layer at the surface of a wild-type strain, showing its interaction with LPS O-chains. This is the putative arrangement obtained by reconstitution of A450-3 in coculture with A450-1.

tural change in the A-layer, from a normal to a BS pattern. It is possible that some protein domains, necessary for efficient adherence, are more accessible in the A protein subunits arranged in the BS pattern. We have not determined which natural environments or host compartments may contain low enough levels of calcium to induce a structural change in the A-layer, but the possibility that the A-layer exists in two functional states of organization cannot be ruled out. What is clear from our results is that structural modifications in the A-layer had important effects on the association of *A. salmonicida* with Mϕ, suggesting once more that the A-layer is indeed responsible for mediating this process.

Hemin coatings also had an important enhancing effect on adherence to Mϕ. Because A protein is the hemin-binding molecule on the surface of *A. salmonicida* (20), it appears (in its native A-layer-arrayed form) to be directly responsible for this effect. Surprisingly, growing bacterial cells on plates with whole rainbow trout blood had an even greater effect on adherence to Mϕ (Fig. 5b). This remains a partially unexplained effect because, although it is known that A^+ bacteria are capable of binding immunoglobulins (31) and complement (41), exposure to normal fresh trout serum alone (containing immunoglobulins and complement) did not increase adherence to Mϕ. Thus, we have attributed the effect of whole blood to hemin binding and bacterial cell surface modification through some as yet unidentified mechanisms. Interestingly, it has been reported that hemin- or CR-coated *Shigella flexneri* showed greater invasiveness in the HeLa cell system than noncoated bacteria (8).

Although it was not a major objective of our present study, it was impossible to ignore the cytopathic effects associated with A^+ *A. salmonicida*. Previously observed in murine Mϕ (12), these effects on trout Mϕ were confirmed in this study (Fig. 6). Cytotoxicity appeared not to be a direct effect of the A-layer, since the A-layer-possessing strain A450-10SR did not appear to be as cytotoxic as A450, and A-layer-coated latex beads did not induce major changes in Mϕ morphology. A marked effect of *A. salmonicida* on trout Mϕ, not previously observed with murine Mϕ, was the detachment of Mϕ from the substratum. Similar effects on Atlantic salmon peritoneal Mϕ have been reported by Olivier et al. (26). These authors also concluded that cytotoxicity was not a direct effect of the A-layer. The potentiating effect of hemin on the cytolytic activity of coated *A. salmonicida* remains to be explored.

In summary, we have shown that adherence of the fish pathogen *A. salmonicida* to trout Mϕ is mediated by the A-layer and that structural alterations in the A-layer have important effects on adherence. The significance of this phenomenon is being explored in an in vivo model and by studies on adherence to and penetration of nonphagocytic epithelial fish cell lines in vitro.

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