Binding of Laminin and Fibronectin by the Trypsin-resistant Major Structural Domain of the Crystalline Virulence Surface Array Protein of Aeromonas salmonicida*

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The surface of Aeromonas salmonicida is covered by a tetragonal paracrystalline array (A-layer) composed of a single protein (A-protein, $M_r = 50,778$). This array is a virulence factor. Cells containing A-layer and isolated A-layer sheets specifically bound laminin and fibronectin with high affinity. Binding by cells was inhibited by selective removal of A-layer at pH 2.2, and neither isogenic A-layer-deficient A. salmonicida mutants nor tetragonal paracrystalline array producing Aeromonas hydrophila and Aeromonas sobria strains bound either matrix protein. Laminin binding was by a single class of high affinity interactions (cell $K_d = 1.52 \text{ nM}$), whereas fibronectin bound via two classes of interactions, one being similar to that of laminin (cell Class 2 interaction $K_d = 6.6 \text{ nM}$). This interaction with both proteins was partly hydrophobic. The Class 1 fibronectin interaction was of lower affinity (cell $K_d = 218 \text{ nM}$) and distinct. Purified A-protein inhibited binding of both matrix proteins to A-layer, and trypsin cleavage localized the matrix-protein binding region to the N-terminal major trypsin-resistant structural domain of A-protein. Monoclonal antibody inhibition studies showed that A-protein was folded such that Fab's of only one or two antibodies with epitopes mapping C-terminal to this trypsin-resistant peptide was capable of blocking binding.

Fibronectin and laminin are large, multidomain glycoproteins of the extracellular matrix that are important in cellular organization (1, 2). Laminin (molecular weight ($M_r$) about 1,000,000) is composed of three polypeptide chains connected by disulfide bonds and is the most abundant noncollagenous protein of basement membranes, while fibronectin is a disulfide-linked dimeric glycoprotein ($M_r$ about 450,000) present in a soluble form in blood plasma and other body fluids, as fibrils in extracellular matrices, and on the surfaces of certain cells. These proteins have a variety of biological functions, including important roles associated with cell-cell contact and adhesion and in promoting cell adhesion to the basement membrane.

Adhesion of bacteria to host tissues is a prerequisite of many types of bacterial infections (3). Although most studies have focussed on bacterial adhesion to eucaryotic cell surfaces, a number of pathogenic bacteria have been shown to be capable of binding matrix proteins such as fibronectin and/or laminin to their surfaces via specific, high affinity receptors (4–9). The binding of these extracellular matrix proteins to the bacterial surface may determine or enhance the ability of the bacterium to adhere to and colonize a surface, possibly by exploiting host cell extracellular matrix protein receptors. In the case of soluble fibronectin, binding may also serve to mask bacteria from normal immunological recognition mechanisms. The ability of bacteria to bind these extracellular matrix proteins is, therefore, likely a virulence factor. However, with the exception of the membrane-bound fibronectin binding protein of Staphylococcus aureus ($M_r = 200,000$–$210,000$) (10–12) and the coiled fibronectin binding surface structure of certain strains of Escherichia coli known as curli ($M_r = 17,000$) (13), most of the bacterial proteins with the capacity to bind fibronectin and/or laminin have neither been identified nor characterized.

S-layers, or paracrystalline surface protein arrays, constitute the outermost component of the cell envelope of a wide variety of bacteria (14–16). These regularly arranged two-dimensional assemblies of protein monomers virtually enclose the entire bacterial cell, and, in the case of pathogenic bacteria, they are ideally situated to play a role in the colonization process. Indeed, in the case of the Gram-negative fish pathogenic bacterium Aeromonas salmonicida (17), the tetragonally arrayed surface protein layer known as A-layer plays a major role in the ability of the bacterium to produce disease in fish, since mutants isogenic in their ability to produce A-layer display a $>10^5$-fold reduction in virulence (18). Certain strains (termed typical strains) cause the fatal systemic disease, furuncolosis, in salmonid fish, while “atypical” strains cause chronic, ulcerative, and systemic diseases in both salmonid and non-salmonid fish (17). Clinical isolates of both groups invariably produce A-layer (18, 19). A-layer contributes to protection against the bactericidal activities of both nonimmune and immune serum (21). A-layer also facilitates binding of A. salmonicida to macrophages (22) and binds immunoglobulins (23) and porphyrins (24).

A-layer is comprised of a single species of protein (A-protein) of $M_r = 50,778$ (25) which has been purified and subjected to biophysical and biochemical analysis (26, 27), as well as immunological characterization. The structural gene for A-protein ($uapA$) has also been cloned and sequenced (25, 28), and three-dimensional image reconstruction analysis has shown that the subunits comprise two morphological domains, a heavy mass domain with a linker arm to a domain of lesser

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1 P. Doig and T. J. Trust, unpublished observations.
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| Strain       | S-layer | Binding % |
|--------------|---------|-----------|
|              |         | Laminin  | Fibronectin |
| A. salmonicida |         |          |            |
| A449         | Untreated | +        | 48.7 34.2  |
|              | Glycine-extracted | -        | 5.8   2.9  |
|              | A449-TM1 | -        | 14.4  2.4 |
|              | A449-TM5 | Washed cells | 6.5  6.9  |
|              |          | Supernatant | + 78.4 40.6 |
|              | A449-3   | -        | 7.3   1.0  |
|              | A450     | Untreated | + 62.2 33.7 |
|              |          | Glycine-extracted | - 7.3  2.5 |
|              | A460-3   | -        | 3.3   2.1  |
|              | A451     | Untreated | + 62.2 22.7 |
|              |          | Glycine-extracted | - 5.6  2.6 |
|              | A451-3   | -        | 2.2   2.7  |
|              | A461     |          |        |
|              | A460     | +        | 14.9  2.6  |
|              |          | A400     | + 77.3 33.7 |
|              |          | A461     | + 70.0 15.2 |
|              | A. hydrophila | TF7 | + 1.0  2.2  |

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|              | A449-TM1 | -        | 14.4  2.4 |
|              | A449-TM5 | Washed cells | 6.5  6.9  |
|              |          | Supernatant | + 78.4 40.6 |
|              | A449-3   | -        | 7.3   1.0  |
|              | A450     | Untreated | + 62.2 33.7 |
|              |          | Glycine-extracted | - 7.3  2.5 |
|              | A460-3   | -        | 3.3   2.1  |
|              | A451     | Untreated | + 62.2 22.7 |
|              |          | Glycine-extracted | - 5.6  2.6 |
|              | A451-3   | -        | 2.2   2.7  |
|              | A461     |          |        |
|              | A460     | +        | 14.9  2.6  |
|              |          | A400     | + 77.3 33.7 |
|              |          | A461     | + 70.0 15.2 |
|              | A. hydrophila | TF7 | + 1.0  2.2  |

mass (29). Consistent with this three-dimensional ultrastructural evidence, trypsin cleavage of isolated A-protein has provided evidence for the presence of two structural domains, a larger mass trypsin-resistant N-terminal domain and a lesser mass C-terminal domain with intermediate resistance to trypsin (25).

In this report, we show that the substrate binding capabilities of the surface array protein of A. salmonicida are even wider than previously reported. We present data that both fibronectin and laminin bind to the tetragonally arranged A-layer and purified A-protein of A. salmonicida in a specific manner, but do not bind to the morphologically similar S-layer of other species of pathogenic Aeromonas (30, 31). Furthermore, we show that the binding activity for both glycoproteins is carried on the major trypsin-resistant N-terminal domain of A-protein. In addition, binding of fibronectin and laminin to A-layer is shown to differ, with fibronectin possessing both a shared binding mechanism with laminin and a unique binding mechanism. Such conserved activities associated with S-layer functions have not been previously reported and indicate that the A. salmonicida A-layer is a novel surface-located binding protein with broad substrate specificity.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Binding of Fibronectin and Laminin to A. salmonicida—The ability of wild type A-layer-containing cells of A. salmonicida to bind laminin and fibronectin was first determined by liquid phase assay. The results in Table I show that cells of typical strains A449, A450, and A451 and atypical strains A400 and 461 bound high levels of both 125I-radiolabeled glycoproteins, while significantly lower levels were bound by cells of A-layer-negative A. salmonicida 438 and tetragonal S-layer producing A. hydrophila TF7. This suggested that the surface component responsible for binding was the A-layer produced by virulent strains of A. salmonicida, and this possibility was tested by determining the binding ability of cells from which A-layer was selectively removed by extraction with 0.2 M glycine buffer at pH 2.2 and 4 °C (29). Table I shows that removal of A-layer resulted in a dramatic reduction in the ability of cells of strains A449, A450, and A451 to bind laminin and fibronectin. Similarly, isogenic A-layer-deficient derivatives A449-3, A450-3, and A451-3 (18) bound markedly lower levels of fibronectin and laminin than the respective parent strains (Table I).

The binding ability of two different single insertion Tn5 mutants of strain A449 was also determined. Mutant A449-TM1 is a periplasmic accumulator of A-protein and, while producing normal smooth LPS, appears to be incapable of assembling an intact A-layer on the cell surface (32). Mutant A449-TM5 is deficient in O-polysaccharide chains, and is incapable of anchoring assembled A-layer on the cell surface (32). Intact sheets of A-layer can be removed from the surface of agar-grown cells of this mutant by washing in phosphate-buffered saline. Table I shows that cells of A449-TM1 and washed cells of A449-TM5 bound significantly lower levels of laminin and fibronectin than parent A449, while the A-layer-containing supernatant obtained by washing A449-TM5 cells bound high levels of both proteins.

Both fibronectin and laminin also bound to wild type A. salmonicida strains A449, A450, A451, A460, A461, and purified A450 A-layer using the solid phase binding assay. Neither protein was observed to bind to BSA, a A-layer-negative derivative A449-TM1, A449-3, A450-3, and A451-3, or tetragonal S-layer-producing mesophilic Aeromonas strains TF7, A80-140, P77-155, Ah423, and A700 using this assay. Taken together, these results strongly suggested that the A-layer was responsible for laminin and fibronectin binding.

Parameters of Laminin and Fibronectin Binding—Since the deduced primary amino acid sequence of the A-layer protein of strain A450 and information on the higher order structure of this protein was known (25, 29), the binding of laminin and fibronectin to cells of strain A450 and A-layer purified from A450 was characterized further using the solid phase assay system. Saturation of binding of both laminin and fibronectin was reached within 2 h in this assay system (Fig. 1). These kinetics appeared to be a reflection of the assay procedure because in the liquid phase system binding of laminin and fibronectin proteins reached saturation within 5 min (data not shown). Binding was reversible in the solid phase assay (Fig. 2), and both glycoproteins bound to whole cells of strain A450, and A-layer purified from strain A450, in a concentration dependent manner (Figs. 3, and 4). Scatchard analysis (Fig. 3) of the binding of fibronectin to strain A450, as well as purified A450 A-layer, gave a biphasic curve indicating that there were two classes of binding interactions occurring (termed Class 1 interaction and Class 2 interaction, respectively). In contrast, laminin bound via a single class of interaction (Fig. 4). Binding of both glycoproteins to native A-layer on the cell surface as well as purified A-layer sheets was of high affinity with binding constants in the 1.52–218 nM range (Table II).

**Competition of Fibronectin, Laminin, and Nonspecific Immunoglobulin for Binding Sites**—Either laminin or fibronectin was able to inhibit the binding of the other glycoprotein to whole cells or purified A-layer (Fig. 5). This inhibition was concentration-dependent. Laminin was unable to inhibit fi-
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branectin binding completely, while fibronectin could completely inhibit laminin binding. Nonspecific rabbit immunoglobulin G gave virtually no inhibition of either fibronectin or laminin binding.

**Effect of pH and Salt Concentration on Binding**—Changes in the pH of the buffer in which the binding was conducted had a marked effect on the binding constant of fibronectin and laminin binding (Fig. 6A). In the case of the fibronectin Class 2 interaction and laminin binding, increasing the pH had little effect until pH 7.4 was obtained. At this point, the $K_d$ rose dramatically, followed by a gradual decrease in the $K_d$ as the pH was increased further. A similar effect on $K_d$ was seen in the case of the fibronectin Class 1 interaction, except that the highest $K_d$ value was obtained at pH 8.5.

TABLE II

| Fibronectin          | Laminin       |
|----------------------|---------------|
|                       | Class 1       | Class 2       |
| Purified A-layer      | 43.2          | 6.62          | 7.93          |
| Strain A450          | 218.0         | 6.90          | 1.52          |

The concentration of salt in the buffer had a less dramatic effect. Class two interaction of fibronectin binding was unaffected by salt concentration (Fig. 6B). However, both Class 1 fibronectin and laminin binding exhibited an increase in the $K_d$ when the salt concentration reached 0.15 M with best binding at 0.5 M (Fig. 6B). The salt effect was most pronounced in the case of laminin binding. Further increases in the salt concentration resulted in a decrease in both of these binding constants.

**Identification of Binding Domain**—Purified A-protein could inhibit the binding of either extracellular matrix protein to whole bacteria (data not shown) and to A-layer sheets (Fig. 7) in a concentration-dependent manner. Consistent with this finding was the observation that cleavage of layer protein by CNBr resulted in a decreased ability of A-layer to bind either fibronectin or laminin (Table III). Nonspecific modification by formaldehyde treatment and reductive alkylation of free amino groups accessible on the amino acid residues of A-protein when assembled into A-layer sheets also resulted in a reduced ability to bind glycoprotein (Table III). In contrast, treatments designed to modify histidine or tryptophan residues produced relatively small reductions in binding capacity when compared to controls (Table III). This slight inhibition is probably not significant and likely reflects a degree of denaturation of the A-layer sheet that occurred during the chemical modification procedure rather than modification of a residue within or near the binding domain.

Fabs prepared from certain specific antibodies were also capable of blocking fibronectin and laminin to A-layer. This was true in the case of a polyclonal anti-A-protein antiserum containing antibodies to A-layer surface epitopes and monoclonal antibody AA6 whose epitope is also exposed on the native A-layer surface (Table III). In contrast, Fabs of a second monoclonal antibody to a surface-exposed A-layer
epitope, Mab AA1, did not block fibronectin and laminin binding (Table III). Together, these data suggested that specific surface-exposed region(s) of A-protein were involved in fibronectin and laminin binding.

To further localize the domain of A-protein responsible for glycoprotein binding, we took advantage of the fact that trypsin cleaves A-protein into two structural domains, a C-terminal domain (25). Both peptides were purified, and, when tested in binding inhibition studies, the larger mass trypsin-resistant peptide was found to inhibit binding of both fibronectin and laminin to A-layer (Fig. 7).

**DISCUSSION**

This study provides compelling evidence for the ability of the surface array protein of the bacterium *A. salmonicida* to bind the extracellular matrix proteins laminin and fibronectin via high affinity interactions. This is the first report of such binding by a surface array protein, and, in the case of S-layers produced by species of *Aeromonas*, the property appears to be unique to the A-protein of *A. salmonicida* because the S-layers of the mesophilic strains of *A. hydrophila* and *A. sobria* tested failed to bind either glycoprotein. This is in keeping with previous observations that the S-layers of mesophilic *Aeromonas* lack other binding abilities exhibited by the *A. salmonicida* array protein (22-24) and is consistent with the significant differences in amino acid sequences of the nonbinding *Aeromonas* S-layers proteins and A-protein as judged by differences in N-terminal amino acid sequences (27, 41), differences in antigenicity (30), and the failure of the *A. hydrophila* and *A. sobria* S-layer protein genes to cross-hybridize to the A-protein gene (25). Some of these sequence differences are clearly of primary importance in determining the ability of the *A. salmonicida* array protein to bind fibronectin and laminin because, with respect to ultrastructure, the various *Aeromonas* S-layers are very similar (29, 31, 42-44).

Binding of fibronectin and laminin by A-protein differed significantly from the previously reported immunoglobulin binding property of this protein (23). Not only was fibronectin and laminin binding of significantly higher affinity (K<sub>a</sub> = 1.52-250 nm) than immunoglobulin binding (K<sub>a</sub> = 1.0-3.3 mm) (23), but the binding of these extracellular matrix proteins could be inhibited by purified A-protein. Immunoglobulin binding on the other hand requires an intact supramolecular array and cannot be inhibited by purified A-protein (23).

One or more amino groups on A-protein appear to be important components in maintaining the fibronectin and laminin binding function since nonspecific modification of accessible amino groups on assembled A-layer resulted in significant inhibition of binding activity. The makeup of the primary amino acid sequence precluded attempts by chemical modification to implicate specific individual amino acid residues in the binding activity, and the 38-residue sequence which is repeated completely three times and partially once in the *S. aureus* fibronectin-binding protein and functions as the fibronectin binding domain (12) is not present in the sequence of A-protein (25). However, we were able to localize the binding domain for these extracellular matrix proteins to the N-terminal M<sub>1</sub> = 37,600 trypsin-resistant domain of A-protein. Ultrastructural analyses have shown that A-layer contains a major tetragonal core of the array which is composed of the heavy mass domains of four subunits, contains a large depression, and is located toward the inside of the layer (29). The N-terminal M<sub>1</sub> = 37,600 trypsin-resistant peptide is proposed to comprise this heavy mass core-forming domain (25), and immunochemical analysis has shown that the peptide carries at least 7 surface exposed sequences containing 75 residues.

Fabs prepared from polyclonal anti-A-protein antiserum containing antibodies to these epitopes blocked the binding of both glycoproteins to A-layer, as did the Fabs of monoclonal antibody AA6 whose surface exposed epitope maps C-terminal to the M<sub>1</sub> = 37,600 matrix protein binding peptide. In contrast, Fabs of monoclonal antibody AA1 whose surface-exposed epitope maps closer to the M<sub>1</sub> = 37,600<sup>1</sup> peptide than that of AA6 failed to inhibit binding. These results indicate that the blocking of laminin and fibronectin binding by AA6 Fabs resulted from steric interference. Presumably, the folding characteristics of A-protein allow bound AA6 Fab to physically interfere with the access of the matrix proteins to their binding sequences, while the steric position of bound AA1 Fab provides no such physical impediment. Similar spatial considerations probably contribute to the correct positioning of the matrix protein binding sequences of the M<sub>1</sub> = 37,600<sup>1</sup> peptide because this domain has been shown to contain a significant amount of folding. For example, the peptide carries 27 potential trypsin sites which appear to be totally protected from trypsin activity (25). Also, CNBr cleavage of A-layer produced a large inhibition of binding activity. While this could indicate that a methionine at residue 291 or 352 of the M<sub>1</sub> = 37,600 peptide is in the A-protein binding site for fibronectin and laminin, the inhibition most probably results because cleavage of the protein at methionine residues destroys the folding properties of the protein and in so doing alters the surface topography of A-layer and positioning of the matrix protein binding sequences. Three-dimensional structural analysis has shown that the folding of A-protein provides the surface of A-layer with considerable architecture, including depressions or pits (29), which in concert with the specific binding sequences should facilitate binding of large molecules such as laminin and fibronectin.

Equilibrium analysis of binding of laminin and fibronectin to *A. salmonicida* using a modified Scatchard methodology revealed a difference in binding mechanisms. Binding to the A-layer/bacterium was at saturation and was reversible at the time of assessment, hence in equilibrium. Thus, the criteria for using this method of analysis were fulfilled. Using this analysis, laminin was observed to bind to whole cells and

| Treatment | Percent of control |
|-----------|-------------------|
| **Fibronectin binding** | | |
| MeSO/HCl<sup>a</sup> | 74.0 ± 5.0<sup>b</sup> | 77.0 ± 1.5 |
| Diethylpyrocarbonate<sup>a</sup> | 82.3 ± 2.2 | 89.0 ± 1.9 |
| Formaldehyde/borohydride<sup>a</sup> | 45.2 ± 0.5 | 48.5 ± 3.4 |
| Formaldehyde<sup>a</sup> | 47.4 ± 3.7 | 52.5 ± 4.7 |
| Perturbant | 15.1 ± 2.1 | 20.1 ± 3.3 |
| Antibody | | |
| Polyclonal anti-A-protein Fab | 32.7 ± 7.8 | 41.1 ± 2.6 |
| Polyclonal nonspecific IgG | 90.0 ± 5.6 | 93.4 ± 10.6 |
| Monoclonal antibody AA6 Fab | 50 ± 10.1 | 43.7 ± 6.1 |
| Monoclonal antibody AA1 Fab | 106 ± 8.9 | 94.2 ± 8.3 |

<sup>a</sup> Tryptophan modification.
<sup>b</sup> Mean ± S.D.
<sup>c</sup> Histidine modification.
<sup>d</sup> Amino group modification.
<sup>e</sup> Lysine (tyrosine, tryptophan, histidine, asparagine, cysteine) modification.
purified A-layer, via a single class of high affinity interactions, whereas fibronectin bound via two distinct classes of interactions. Two classes of fibronectin binding have also been reported in the case of E. coli (11), with 30 nM and 4 nM binding constants compared to the 218 and 6.6 nM Kd values obtained for A. salmonicida. In the E. coli case, the two binding constants were ascribed to the presence of two different binding sites for the fibronectin molecule (11), and a molecule as large and complex as fibronectin could also contain several different binding sites. Highest affinity in this study was obtained when laminin bound to native A-layer on A. salmonicida cells, and, at 1.52 nM, this binding was of slightly higher affinity than the 2.9 nM laminin binding constant reported for S. aureus (7) and significantly higher than the 40–80 nM values reported for species of streptococci (6, 8). Interestingly, the binding constants for purified A-layer and whole cells of strain A450 differed with the Class I interaction of fibronectin and laminin binding interaction. While this may indicate that a conformational change occurs when the A-layer is present on the surface of the bacterium compared to purified material (29, 42), it probably represents a technical effect associated with the assay procedure.

The ability of fibronectin and laminin to act as competitors against each other when the competed protein is at saturating concentrations indicates that at least one of the fibronectin-A-layer classes of binding interactions is similar to that of laminin. Indeed, based on competition data, approximately 80% of the fibronectin binding sites on A-protein appear to be identical with those for laminin. While this value may be an overestimate because of the large size of laminin and the possibility that it may sterically block access of fibronectin to another site, the effect of pH and salt concentration on the binding constant indicate that it is the laminin and fibronectin Class 2 interactions which are similar. Furthermore, the effect of salt on the binding constants for both laminin and fibronectin Class 2 interactions indicates that these interactions share a hydrophobic component, consistent with the hydrophobicity conferred on the A. salmonicida cell surface by A-layer (22, 45). The lower affinity fibronectin Class 1 interaction is clearly different, being unaffected by salt, and with a significantly higher pH optimum. The dramatic changes seen in each of the binding constants at a discrete pH value are likely caused by a sudden change in the net charge of the extracellular matrix proteins and/or the A-layer rather than protonation of a discrete amino acid.

The ability of the A. salmonicida A-layer to bind extracellular matrix proteins probably plays a role in the pathogenesis of the disease process this species exhibits in fish. In contrast to A. hydrophila and A. sobria which are associated with acute opportunistic extracellular infections in which the nonbinding S-layer appears to play a relatively minor role, A. salmonicida is an obligate parasite of fish capable of establishing a carrier state within the host for long periods until stress to the host precipitates an acute terminal fulminant septicemia (17). The extracellular matrix protein binding ability of the A. salmonicida A-layer could allow the bacterium to persist within the host and thus contribute to virulence. For example, binding of extracellular matrix proteins to the surface of the bacterium via the A-layer could block or reduce the host’s immune response to the bacterium by sterically masking immunogenic epitopes. Indeed, fibronectin or laminin bound to the surface of A. salmonicida can inhibit the binding of immune sera to the bacterium. The ability to bind extracellular matrix proteins might also facilitate adhesion of this pathogen to host cells such as macrophages via fibronectin or laminin receptors.

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REFERENCES

1. Ruoslathi, E. (1988) Annu. Rev. Biochem. 57, 375–413
2. Beck, K., Hunter, L., and Engel, J. (1990) FASEB J. 4, 148–160
3. Switalski, L., Höök, M., and Beachey, E. H. (1989) Molecular Mechanisms of Microbial Adhesion, Springer Verlag New York Inc., New York
4. Speciale, P., Höök, M., Wadström, T., and Timpl, R. (1982) FEBS Lett. 146, 55–58
5. Fitzgerald, T. J., Repesh, L. A., Blanco, D. R., and Miller, J. N. (1984) Br. J. Vener. Dis. 60, 357–363
6. Switalski, L., Speciale, P., Höök, M., Wadström, T., and Timpl, R. (1984) J. Biol. Chem. 259, 3734–3738
7. Lopes, J. D., Dos Reis, M., and Brentani, R. R. (1985) Science 229, 275–277
8. Switalski, L., Murchison, H., Timpl, R., Curtians, R., III, and Höök, M. (1987) J. Bacteriol. 169, 1095–1101
9. Höök, M., Switalski, L. M., Wadström, T., and Lindberg, M. (1988) in Fibronectin (Mosher, D. E., ed) pp. 295–305, Academic Press, New York
10. Espsersen, F., and Clemmensen, I. (1982) Infect. Immun. 37, 595–591
11. Friman, G., Switalski, L., Speciale, P., and Höök, M. (1987) J. Biol. Chem. 262, 6564–6571
12. Signas, C., Raucci, G., Jönsson, K., Lindgren, P.-E., Anantharamaiah, G. M., Höök, M., and Lindberg, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 699–703
13. Olesen, A., Jönsson, A., and Normark, S. (1989) Nature 338, 652–655
14. Koval, S. F. (1988) Can. J. Microbiol. 34, 407–414
15. Sleytr, U. B., Messner, P., Sara, M., and Pun, D. (1986) Syst. Appl. Microbiol. 7, 310–313
16. Sleytr, U. B., and Messner, P. (1988) J. Bacteriol. 170, 2891–2897
17. Timpl, T. J. (1986) Annu. Rev. Microbiol. 40, 479–502
18. Ishiguro, E. E., Kay, W. W., Ainaworth, T., Chamberlain, J. B., Buckley, J. T., and Trust, T. J. (1981) J. Bacteriol. 148, 333–340
19. Trust, T. J., Howard, P. S., Chamberlain, J. B., Austen, R. A., Ishiguro, E. E., and Buckley, J. T. (1980) FEMS Microbiol. Lett. 9, 35–38
20. Kay, W. W., Buckley, J. T., Ishiguro, E. E., Phipps, B. M., Monette, J. P. L., and Trust, T. J. (1981) J. Bacteriol. 147, 1077–1084
21. Munn, C. B., Ishiguro, E. E., Kay, W. W., and Trust, T. J. (1982) Infect. Immun. 36, 1069–1075
22. Trust, T. J., Kay, W. W., and Ishiguro, E. E. (1983) Curr. Microbiol. 9, 315–318
23. Phipps, B. M., and Kay, W. W. (1988) J. Biol. Chem. 263, 9298–9303
24. Kay, W. W., Phipps, B. M., Ishiguro, E. E., and Trust, T. J. (1985) J. Bacteriol. 164, 1332–1338
25. Chu, S., Cavaignac, S., Feutrier, J., Phipps, B. M., Koutzynska, M., Kay, W. W., and Trust, T. J. (1991) J. Biol. Chem. 266, 15258–15265
26. Phipps, B. M., Trust, T. J., Ishiguro, E. E., and Kay, W. W. (1985) Biochemistry 24, 2934–2939
27. Kay, W. W., Phipps, B. M., Ishiguro, E. E., Olafson, R. W., and Trust, T. J. (1984) Can. J. Biochem. Cell Biol. 62, 1064–1071
28. Belland, R. J., and Trust, T. J. (1987) J. Bacteriol. 169, 4086–4091
29. Ebert, J. S. G., Engelhardt, H., Baumeister, W., Kay, W. W., and Trust, T. J. (1989) J. Bacteriol. 171, 190–197
30. Dooley, J. S. G., and Trust, T. J. (1988) J. Bacteriol. 170, 499–506
31. Kokka, R. P., Vedros, N. A., and Janda, J. M. (1990) J. Clin. Microbiol. 28, 2240–2247
32. Belland, R. J., and Trust, T. J. (1985) J. Bacteriol. 163, 877–881

4 P. Doig, L. Emody, and T. J. Trust, unpublished data.
33. Leemli, U. K. (1970) Nature 227, 680–685
34. Pearson, T. W., Funder, M., Roelants, G. E., Kar, S. K., Lundin, L. B., Mayor-Whitney, L. S., and Shewett, R. (1980) J. Immunol. Methods 34, 141–154
35. Kostrzynska, M., Schalen, C., and Wadstrom, T. (1989) FEMS Microbiol. Lett. 59, 229–234
36. Emody, L., Heesemann, J., Wolf-Watz, H., Skurnik, M., Kappelrud, G., O’Toole, P., and Wadstrom, T. (1989) J. Bacteriol. 171, 6674–6679
37. Doig, P., Todd, T., Sastry, P. A., Lee, K. K., Hodges, R. S., Paranchych, W., and Irving, R. T. (1988) Infect. Immun. 56, 1641–1646
38. Means, G. E. (1977) Methods Enzymol. 47, 469–478
39. Savige, W. E., and Fontana, A. (1977) Methods Enzymol. 47, 442–455
40. Miles, E. W. (1977) Methods Enzymol. 47, 431–442
41. Dooley, J. S. G., McCubbin, W. D. M., Kay, C. M., and Trust, T. J. (1987) J. Bacteriol. 170, 2651–2658
42. Stewart, M., Beveridge, T. J., and Trust, T. J. (1986) J. Bacteriol. 166, 120–127
43. Murray, R. G. E., Dooley, J. S. G., Whippie, P. W., and Trust, T. J. (1988) J. Bacteriol. 170, 2625–2630
44. Al-Karadagli, S., Wang, D. N., and Hovmöller, S. (1988) J. Ultrastruct. Mol. Struct. Res. 101, 92–97
45. Van Alstine, J. M., Trust, T. J., and Brooks, D. E. (1986) Appl. Environ. Microbiol. 51, 1309–1313

Supplemental Material

Binding of laminin and fibronectin by the crystalline surface protein array of Aeromonas salmonicida

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Fibronectin and Laminin Binding by *A. salmonicida* Surface Array Protein

Modification of the A-layer - Purified A-layer sheets were suspended at a protein concentration of 0.1 mg/ml. Modification of free amino groups on A-layer was performed by treatment for 1 h with 1% (w/v) formaldehyde (BDH Inc., Toronto, ON, Canada), then washed 2 times with 0.1 M carbonate buffer pH 9.4 before being used in ELISA plates. Reductive modifications of amino groups carried on the A-layer sheets was performed using the formaldehyde/hydrazine protocol described by Means (38). Nε-Aminoethyl modification to lysine residues using ethylenediamine hydrochloride and was performed as described by Savage and Pantanella (21). Maleyl residues modifications using diethylpyrocarbonate (Sigma) was performed by the method described by Mills (40). The A-layer was also derivatized using peridinin bromo-dPEG6 (T. Baker Chemical Co., Phillipsburg, NJ). For this, the A-layer was suspended in 50% formic acid and CNBr was added to a concentration of 200 mM excess with respect to methionine. After 24 h at room temperature, the solution was diluted to 10 times its original volume with water and lyophilized. Cleavage was confirmed by 30% PAGGE.

All modified or unmodified A-layer components were resuspended in 0.1 M carbonate buffer at a concentration of 10 µg/ml and coated on ELISA plates as described above. Fibronectin and laminin binding were assayed as above. A protein binding to the wells was confirmed using a conventional ELISA using rabbit anti-*A. salmonicida* immunoglobulin.

Protein assay - Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad), using BSA as a standard.

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**Figure 1.** Time course of fibronectin and laminin binding to *A. salmonicida* strain A50 (fibronectin □ and laminin ▲) and A-layer purified from strain A50 (fibronectin ○ and laminin ■). Error bars represent one standard deviation.

**Figure 2.** Inhibition of fibronectin and laminin binding to *A. salmonicida* strain A50 (fibronectin □ and laminin ▲) and A-layer purified from strain A50 (fibronectin ○ and laminin ■). L ■.

**Figure 3.** Inhibition of laminin binding to *A. salmonicida* strain A50 (■) receptor purified from strain A50 (□) by fibronectin (●). L ■. Error bars represent one standard deviation.