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YadA Mediates Specific Binding of Enteropathogenic *Yersinia enterocolitica* to Human Intestinal Submucosa

MIKAEL SKURNIK,[1,2]* YASMIN EL TAHIR,[1,2] MARJA SAARINEN,[2] SIRPA JALKANEN,[3] AND PAAVO TOIVANEN[2]

Turku Centre for Biotechnology[1] and Department of Medical Microbiology,[2] University of Turku, and National Public Health Institute,[3] SF-20520 Turku, Finland

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The binding of live *Yersinia enterocolitica* to frozen sections of human intestine was investigated qualitatively by monitoring the binding of bacteria by using Gram or immunoperoxidase staining as well as quantitatively by a new enzyme immunoassay-on-slide method. We have demonstrated that the binding of various *Y. enterocolitica* serotypes and *Escherichia coli* clones to frozen sections of human intestine is mediated by the *Yersinia* adhesin, YadA. The YadA-mediated binding occurs mainly at the submucosal layer of the intestinal wall and only to a limited extent at the mucosal layer; there binding is mostly to the mucin threads. In addition, partially purified YadA binds to frozen sections with a pattern similar to that of intact bacteria. Collagen, laminin, or partially purified YadA only partially inhibited the YadA-mediated binding of bacteria, presumably because YadA is multifunctional. A combination of collagen and laminin inhibited the binding more efficiently. Therefore, YadA may be involved in the interactions with the extracellular matrix molecules after the invasion of the intestinal tissue.

Enteropathogenic *Yersinia* species can cause different types of diseases, ranging from mild diarrhea to septicemia; mesenteric lymphadenitis and related diseases that mimic the symptoms of appendicitis are common (10). Very mild enteropathogenic *Yersinia* infections appear to be associated with reactive arthritides and autoimmune thyroid diseases (46, 48). Infection usually occurs when contaminated food is eaten (7). *Yersinia enterocolitica* invades through the M cells of the distal small intestine and penetrate the lamina propria (12, 47). The bacteria (apparently) multiply in lymphoid follicles and Peyer's patches, drain into mesenteric lymph nodes, and may even give rise to a systemic infection.

Cellular penetration of enteropathogenic *Yersinia* species is mediated mainly by invasin, the product of a chromosomal *inv* gene (15, 30) that initiates entry by binding to mammalian cell receptors belonging to the integrin family (16). *Y. enterocolitica* also has the chromosomal *ail* gene product, which facilitates adhesion and/or invasion of bacteria (23, 24). Invasin is expressed early in infection and is probably important for initiation of gastrointestinal infection; Ail is induced later and is important for extracellular spread (14).

In addition to chromosomally encoded factors, the *Yersinia* virulence plasmid (pYV)-encoded factors also play a role in the bacterium's adhesive properties; pYV-positive *Y. enterocolitica* strains adhere better to intestinal tissue in vitro than the corresponding isogenic pYV-negative strains do (25–27). The pYV plasmid encodes a number of *Yersinia* outer membrane proteins (Yops) which are important virulence determinants. One of these proteins, YadA (*Yersinia* adhesin, formerly called Yop1 or P1), is largely responsible for the adhesion of *Y. enterocolitica* to intestinal tissue in vitro (28). YadA also mediates internalization of *Y. pseudotuberculosis* into tissue culture cells (4, 50). Furthermore, YadA has been associated with numerous phenomena: autoagglutination of *Y. enterocolitica* and of *Y. pseudotuberculosis* (3, 39), serum resistance of *Y. enterocolitica* (3, 9, 20, 31, 38), adherence to and inhibition of internalization in HEp-2 cells of *Y. enterocolitica* (8, 13), expression of fibrils on the bacterial surface (18), binding to collagens (11, 35, 43), laminin (43), and immobilized fibronectin (34, 43, 45), and inhibition of the anti-invasive effect of interferon (8).

Our aim in this study was to characterize the YadA-mediated binding of *Y. enterocolitica* to intestinal tissue. For this purpose, we tested the binding of YadA-positive and -negative *Y. enterocolitica* strains and of *Escherichia coli* YadA clones to frozen sections of human intestine. We demonstrate that YadA mediates adhesion of bacteria to intestinal tissue, mainly to submucosa, and that this binding was most efficiently inhibited by a combination of collagen and laminin.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are shown in Table 1. In addition, *E. coli* JM103 (22) was used for cloning the *yadA* gene (see below). For the binding experiments, the bacteria were grown in 10 ml of MedECa (0.1 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid, 10 g of K_2HPO_4, and 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$ per liter) supplemented with 0.2% glucose, 0.2% Casamino Acids, 1 mg of vitamin B_1 per liter, and 2.5 mM $CaCl_2$ (37). The cultures were incubated stationary at 37°C overnight.

The optical density at 600 nm of the overnight culture was determined spectrophotometrically; then the bacteria were centrifuged for 15 min at 3,000 rpm and suspended in the appropriate amount of RPMI 1640 medium (Biological Industries, Kibbutz Beth Haemek, Israel) to obtain an optical density of between 0.19 and 0.21. An optical density of 0.2 was equivalent to a bacterial concentration of about 2×10^8 CFU/ml, as determined by the dilution plating method. Different bacterial concentrations, ranging from 10^4 to 10^9 bacteria per ml, were prepared from this suspension. For the binding and inhibition assays, the inhibitor solutions were added to 800 μl of bacterial suspensions, and the final volume

* Corresponding author. Mailing address: Turku Centre for Biotechnology, P.O. Box 123, 20521 Turku, Finland. Phone: +358-21-633 8035. Fax: +358-21-633 8000. Electronic mail address: MSKURNIK@FINABO.ABO.FI.

TABLE 1. Binding of *Yersinia* strains and *yadA* clones grown at 37°C[a] onto intestinal frozen sections

Bacterial strain	Description	Expression of YadA[b]	Binding of bacteria to[c]:		Reference
			Mucosa	Submucosa	
E. coli					
C600	Host strain	−	−	−	1
C600/pYMS4514	$yadA_{YeO3}$ and $lcrF_{YeO3}$ cloned into pTM100	+	+	+++	41
PM191	Host strain, *recA*	−	−	−	21
PM191/pYMS1	*yadA* of *Y. pestis* cloned into pBR322	−	−	−	42
PM191/pYMS2	*yadA* of *Y. pseudotuberculosis* cloned into pBR322	+	+	+++	42
PM191/pYMS3	$yadA_{8081}$ cloned into pBR322	+	+	+++	42
PM191/pYMS4	$yadA_{YeO3}$ cloned into pBR322	+	+	+++	42
Y. enterocolitica					
6471/76 (YeO3)	Serotype O:3, pYV^+ patient isolate grown at 22°C	−	−	−	36
YeO3	Grown at 37°C	+	+	+++	
6471/76-c (YeO3-c)	pYV^- derivative of YeO3 (6471/76)	−	−	−	36
YeO3-028	YeO3 *yadA*::*kan* (kanamycin GenBlock)	−	−	−	38
YeO3-c/pYL8	$yadA_{YeO3\text{-}\Delta 83\text{-}104}$ and $lcrF_{YeO3}$ cloned into pTM100	+	+	+	43
YeO3-c/pYMS4514	$yadA_{YeO3}$ and $lcrF_{YeO3}$ cloned into pTM100	+	+	+++	41
8081	Serotype O:8, pYV^+	+	+	+++	32
8081-c	pYV^- derivative of 8081	−	−	−	32
YeO8-116	8081 *yadA*::*kan* (kanamycin GenBlock)	−	−	+	17

[a] Strains were grown at 37°C unless otherwise indicated.
[b] Assessed by SDS-PAGE and/or autoagglutination test.
[c] Based on visual examination of the Gram- or immunoperoxidase-stained sections. At least 20 microscopic fields on the mucosal and submucosal regions of each section were examined. −, no bacteria or at most a few bacteria seen occasionally; +, a few bacteria seen in most of the microscopic fields studied; +++, numerous bacteria seen in every field studied (see Fig. 1).

was brought to 1 ml with an appropriate amount of phosphate-buffered saline, pH 7.4 (PBS).

Special reagents. Laminin (catalog no. L-2020), fibronectin (F-2006), and collagen type I (C-7774) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin was shipped at a concentration of 1 mg/ml in a 0.15 M NaCl–0.05 M Tris solution; fibronectin was dissolved in distilled water to a concentration of 1 mg/ml; collagen type I was dissolved into 0.1 M acetic acid to a concentration of 1 mg/ml.

Recombinant DNA techniques and construction of strains. Standard protocols were applied to manipulate DNA (2). Construction and characterization of *yadA* mutants YeO3-028 and YeO8-116 (Table 1) will be described elsewhere (17, 38). Briefly, in YeO3-028, the protein-coding sequence of the *yadA* gene was replaced with the kanamycin resistance gene GenBlock (Pharmacia, Uppsala, Sweden), while in YeO8-116, GenBlock was inserted between the promoter and the open reading frame of the *yadA* gene.

The *yadA* gene is not ordinarily expressed in *E. coli* from its own promoter (3, 41). Plasmid pYMS4 contains the *yadA* gene cloned into pBR322, so that one of the pBR322 promoters directs the transcription of the *yadA* gene (5, 42). The expression of YadA by pYMS4 is, however, poor. To achieve good expression in *E. coli*, the *yadA* gene was cloned downstream of a strong inducible promoter, P_{tac}, resulting in plasmid pYMS4450. Plasmid pYMS4450 was constructed as follows. Plasmid pL2.1, a derivative of pBR322 with the P_{tac} promoter cloned into the *Eco*RI site (49), was digested with *Cla*I and *Sph*I, and the DNA fragments were dephosphorylated with bacterial alkaline phosphatase. A 2-kb *Cla*I-*Sph*I fragment from pYMS4 which contains the *yadA* gene of *Y. enterocolitica* 6471/76, without its promoter region, was ligated with the *Cla*I-*Sph*I-digested pL2.1. The resulting plasmid, pYMS4450, thus contained the P_{tac} promoter followed by the promoterless *yadA* gene in the correct orientation with respect to the P_{tac} promoter. The plasmid was transformed into *E. coli* JM103, in which *yadA* expression should have been inducible with IPTG (isopropyl-β-D-thiogalactoside). For some still unknown reason, the *yadA* gene was not repressed in the absence of IPTG; instead, huge amounts of YadA were synthesized constitutively. This was verified by positive autoagglutination reactivity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). In addition, colonies of JM103/pYMS4450 and JM103/pL2.1 had different morphologies. Colonies of the former were flat and had waxy and ruffled surfaces, as opposed to smooth and concave colonies of JM103/pL2.1.

Purification of YadA by using Tx-114 extraction. JM103/pYMS4450 extracts (Tx-YadA) and control JM103/pL2.1 extracts (Tx-103) were prepared as follows. Cells were grown overnight at 37°C in 800 ml of Luria broth supplemented with 50 μg of ampicillin per ml. The bacteria were pelleted, and the pellets were suspended in 20 ml of lysis buffer (10 mM EDTA, 50 mM glucose, 25 mM Tris-HCl [pH 8.0], 5 mg of lysozyme per ml). The suspensions were incubated on ice for 1 h. Triton X-114 (Tx-114; catalog no. 93421; Fluka Chemie AG, Buchs, Switzerland), prepared as described previously (6), was added to a final concentration of 5% in a total volume of about 80 ml. The extraction was carried out in a test tube at 4°C for 72 h with slow rocking, and the suspension was incubated overnight at 37°C to separate the water and Tx-114 phases. After centrifugation (8,000 × *g* for 10 min) to clear the phases, the lower phase containing Tx-114 was retained and stored at 4°C. The precipitates which formed during storage contained most of the extracted YadA protein and some proteins of *E. coli* origin. They were pelleted, washed with ethanol, and suspended in 10 mM Tris-HCl (pH 8.0)–0.15 M NaCl. They were not readily soluble in water or physiological buffers at milligram-per-milliliter concentrations but dissolved at 20 μg/ml. The protein concentrations of suspended Tx-YadA and Tx-103 preparations were about 0.2 and 1.2 mg/ml, respectively, as determined by the Lowry method (19).

SDS-PAGE and immunoblotting. Standard SDS-PAGE protocols were used (2); the acrylamide concentration was 4% in the stacking gel and 10% in the separation gel. After electrophoresis, the gels were either stained with Coomassie brilliant

blue or processed for immunoblotting. For immunoblotting, the bands were transferred onto a supported nitrocellulose membrane (BAS85; Schleicher & Schuell, Keene, N.H.), using a semidry blotting device (Milliblot-SDE system; Millipore, Bedford, Mass.). Blocking of the empty spaces was achieved by incubating the membranes in 3% bovine serum albumin (BSA; Sigma) in PBS for at least 1 h at 37°C. For mouse monoclonal immunoglobulin detection, horseradish peroxidase (HRP)-conjugated antibodies specific for mouse immunoglobulin (P260; Dakopatts, Glostrup, Denmark) was used at a 1:500 dilution in 3% BSA–PBS. After incubation overnight, the nitrocellulose filters were washed four times with PBS, and the specifically bound HRP was visualized by using diaminobenzidine as the substrate.

MAbs specific for YadA. BALB/c mice were immunized by injecting Tx-YadA in Freund's incomplete adjuvant into the footpads once a week for 3 weeks. Lymphocytes extracted from popliteal lymph nodes were fused with NS-1 cells, and hybridomas were screened by enzyme immunoassay (EIA). Four monoclonal antibodies (MAbs), 2A9, 2G12, 3G6, and 3G12, were characterized further by immunoblotting on the basis of strong reactivity in EIA. The YadA-specific MAbs were all immunoglobulin G1 class antibodies.

Preparation of frozen sections. Pieces of human distal ileum or proximal colon, removed at surgery, were frozen into blocks by using optimal cutting temperature compound (Miles Inc. Diagnostics Division, Elkhart, Ind.). Eight-micrometer frozen sections were cut from the blocks and mounted inside water-repellent circles on sterile microscope slides. The water-repellent circles were drawn with a peroxidase-antiperoxidase pen (Daido Sangyo Co. Ltd., Tokyo, Japan).

Gram staining and binding conditions. Gram staining was initially used to find conditions which promote the virulence plasmid-mediated binding of bacteria to the frozen sections. Bacterial concentrations of up to 2×10^8 bacteria per ml, incubation times of between 5 and 20 min, and rotation speeds of between 30 and 60 rpm were tested. Satisfactory binding was obtained when the frozen sections were overlaid with 100 μl of bacterial suspension containing about 2×10^7 bacteria per ml, and the bacteria were allowed to interact with the sections for 15 min under gentle rotation at 60 rpm at 4°C. The suspension with unbound bacteria was poured away, and the slides were fixed in 1% glutaraldehyde–PBS for 30 min at 4°C. After fixing, the slides were Gram stained and observed with a light microscope.

Immunohistochemical staining. For immunohistochemical stainings, the frozen sections were fixed for 30 min with methanol containing 2% H_2O_2, washed three times with PBS (all washes, 5 min each, were performed in PBS), and blocked with 3% BSA–PBS for 30 min at room temperature. The sections were washed twice, overlaid with 100 μl of bacterial suspension, placed in a wet chamber, and incubated for 15 min at 4°C under gentle rotation at 60 rpm. The unbound bacteria were poured away, and the sections were washed three times. The sections were then incubated for 15 min at room temperature with 100 μl of 1:10-diluted MAb A6 (specific for the O antigen of *Y. enterocolitica* serotype O:3 [29]) in 3% BSA–PBS, washed twice, and incubated for 15 min at room temperature with 100 μl of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts P260) diluted 1:500 in 3% BSA–PBS. After two washes, the sections were incubated with a substrate solution (0.05% diaminobenzidine–0.01% H_2O_2 in PBS) for 3 min and washed twice. The sections were finally incubated with 5% $CuSO_4$ solution for 2 to 3 min, washed twice, and stained with hematoxylin. After being washed with tap and distilled water, the slides were dried in increasing concentrations of ethanol and cleared in xylene. The sections were covered with coverslips, using a few drops of mounting medium (BDH Ltd., Poole, England), and examined with a light microscope.

Binding of Tx-YadA to frozen sections. Binding YadA to frozen sections was performed similarly to the immunohistochemical staining described above except that the sections were incubated for 30 min at 4°C with 100 μl of Tx-YadA (about 2 μg/ml) instead of being overlaid with bacteria. Tx-103 (about 12 μg/ml) and buffer alone were used as controls. Bound YadA was detected by using undiluted tissue culture supernatant of the YadA-specific MAb, 3G12.

EIA-on-slide assay. For quantitative binding analysis, we developed a new method that we call the EIA-on-slide assay. The bacterial binding assay was similar to that for immunohistochemical staining (see above) except for the blocking and substrate steps. The sections were blocked first with 3% BSA–PBS for 30 min at room temperature, washed twice, and blocked for another 30 min at room temperature with 10% sheep serum in 3% BSA–PBS. Additional blocking with sheep serum decreased background absorbance. In the substrate step, the sections were incubated for 10 min at room temperature with 100 μl of EIA substrate solution and 3 mg of 1,2-phenylendiamine per ml dissolved in citrate buffer supplemented just before use with 10 μl of 30% H_2O_2 per 15 ml of substrate solution. From each section, 75 μl of the substrate solution was pipetted into EIA plate wells (Dynatech Immulon), and the reactions were stopped with 125 μl of 1 M HCl. A_{492} was recorded with a Labsystems Multiskan Plus spectrophotometer. The control experiments had sections without added bacteria. For each bacterial dose and experimental setting, at least four parallel sections were used.

Inhibition experiments. In experiments to inhibit bacterial binding by neutralizing the binding sites on YadA, bacteria were incubated at 37°C for 30 min with different concentrations of collagen type I, laminin, and fibronectin before bacterial binding, and EIA-on-slide assays were done as described above. In experiments to block the frozen section binding sites, the sections were overlaid with 100 μl of the Tx-YadA or Tx-103 preparation at different concentrations and incubated at 4°C for 1 h before the bacterial suspension was added, and the EIA-on-slide assay was done as usual.

RESULTS

Enteropathogenic *Y. enterocolitica* binds to cultured epithelial cells and various extracellular matrix molecules (8, 11, 13, 34, 35, 43). In all cases, the binding is mediated by the virulence plasmid-encoded outer membrane protein YadA. We wanted to determine whether *Y. enterocolitica* would also bind frozen sections of human intestine and, if so, where it bound. We first performed simple binding assays by incubating frozen sections with suspensions of bacteria expressing YadA and by detecting the bound bacteria by Gram staining.

Binding assay. In the preliminary binding experiments, the wild-type *Y. enterocolitica* strain 6471/76 (YeO3), its virulence plasmid-cured derivative, YeO3-c, and *E. coli* C600 were tested. Wild-type YeO3 grown at 37°C bound the section very rapidly; a 15-min incubation of the bacterial suspension was sufficient for significant binding to occur. YeO3-c and *E. coli* C600 did not adhere to the sections with such a short incubation.

Since YadA is known to be the virulence plasmid-encoded adhesion molecule, we wanted to determine whether binding was YadA dependent; for this purpose, we used YadA-positive and -negative *Y. enterocolitica* strains and YadA-expressing *E. coli* clones (Table 1). Wild-type YeO3, grown at 37°C and

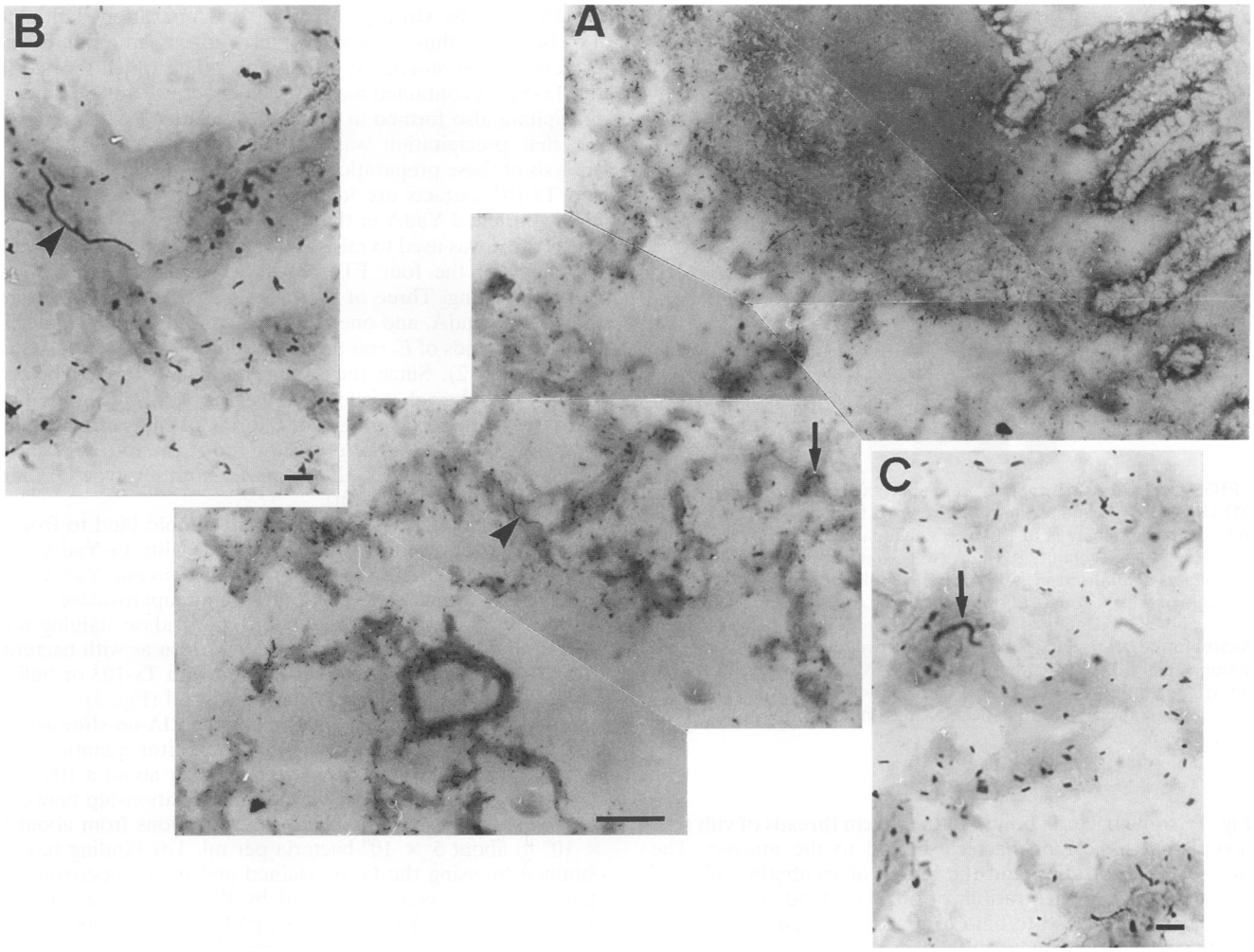

FIG. 1. Micrographs of immunoperoxidase-stained human intestinal tissue showing the binding of YadA-expressing *Y. enterocolitica* 6471/76 (YeO3) to frozen sections. Bound YeO3 bacteria were stained by using YeO3 O-antigen-specific mouse MAb and HRP-conjugated rabbit anti-mouse antibodies. (A) Composite micrograph at low magnification (bar, 100 μm) of human intestinal tissue showing bacterial binding to mucosa and submucosa. Mucosa and a few villi (top right) and a lymphoid follicle (top center) are seen; the lower and left parts of the figure are occupied by submucosa. (B and C) Micrographs at higher magnification (bar, 10 μm) of submucosal parts of panel A. Identical structures in the different micrographs are indicated by arrows and arrowheads. Bacteria can be seen as dark spots, mostly as coccobacilli, and some very long filamentous forms are present (e.g., arrowhead in panel B). Strains that did not express YadA did not bind to the sections in detectable numbers under the same conditions.

expressing YadA, bound to the sections; the same strain, grown at 22°C and not expressing YadA, did not. YeO3-c, which does not have the *yadA* gene, did not bind, nor did YeO3-028, a strain carrying a GenBlock in the *yadA* gene but able to express the other pYV-encoded proteins. YeO3-c/pYMS4514, which expresses a cloned *yadA* gene, bound as efficiently as the wild-type strain. YeO3-c/pYL8 expresses a YadA molecule with a 22-amino-acid deletion (amino acid residues 83 to 104 [43]). The mutant YadA has lost its collagen binding ability but retains its laminin and fibronectin binding ability (43). YeO3-c/pYL8 bound poorly to submucosa but clearly more than YeO3-c under the same binding conditions. The results obtained with *E. coli* PM191 carrying plasmids pYMS1, pYMS2, pYMS3, and pYMS4 were in accordance with the results presented above. A cloned *yadA* gene expressed in PM191 conferred binding capacity to these normally nonbinding bacteria. Among these strains are two that differ from each other minimally: PM191/pYMS2, a strain with the *yadA* gene of *Y. pseudotuberculosis*, bound to sections, whereas PM191/pYMS1, a strain that carries the *yadA* gene of *Y. pestis*, did not. The only relevant difference between these two strains is the 1-bp deletion in the *yadA* gene of *Y. pestis* (33, 42). The deletion leads to a premature stop codon, and so YadA cannot be expressed. YadA is clearly important in binding.

YadA mediates bacterial binding preferentially to submucosa. In Gram-stained sections, especially the mucosal tissue strongly stained red; since bound bacteria were not easy to distinguish, we could not analyze the tissue distribution of bound YadA-expressing bacteria accurately. Therefore, we visualized the bound bacteria with a MAb specific for the *Y. enterocolitica* O:3 O antigen (29) and immunoperoxidase staining. All of the *Y. enterocolitica* serotype O:3 strains listed in Table 1 were tested by this method. Again, only the YadA-expressing strains could bind the sections. The YadA-expressing *Y. enterocolitica* bound in great numbers to the submucosal region of intestine on both cellular and noncellular material

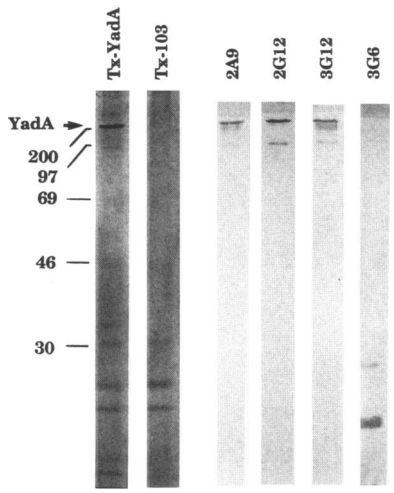

FIG. 2. SDS-PAGE analysis of YadA extracted by Tx-114 from JM103/pYMS4450 and of the control extract from JM103/pPL2.1, and immunoblotting analysis of MAbs. Lanes marked with Tx-YadA and Tx-103 contained about 100 μg (wet weight) of ethanol-washed precipitates solubilized in SDS-PAGE sample buffer at 37°C for 1 h and stained with Coomassie brilliant blue. Lanes labeled 2A9, 2G12, 3G12, and 3G6 contained whole cell lysate of JM103/pYMS4450, electroblotted after electrophoresis to nitrocellulose membranes and immunostained by using the appropriate MAb followed by HRP-conjugated anti-mouse antibodies. The ≈200-kDa YadA band and the sizes (in kilodaltons) of the molecular mass markers are indicated on the left.

(Fig. 1); some bacteria bound to the mucin threads of villi (not shown), but almost no bacteria bound to the mucosa. The bacterial concentration and the length of incubation affected binding: at high concentrations or during extended incubation times, bacteria started to bind to mucosa as well, but binding was always most abundant to the submucosal region.

Partial purification of YadA, MAbs specific for YadA, and binding of Tx-YadA to frozen sections. Tx-114 is used in studies of amphipathic molecules because amphipathic molecules partition into the Tx-114 phase. Analysis of the amino acid sequence of YadA revealed hydrophobic and hydrophilic domains (42), suggesting that YadA is an amphipathic protein. This prompted us to try Tx-114 extraction of the live JM103/pYMS4450 cells. During extraction, YadA partitioned into the Tx-114 phase, thus confirming that YadA is an amphipathic protein. Under storage at 4°C, a precipitate which formed in the Tx-YadA contained most of the extracted YadA. A similar precipitate also formed in the control extract Tx-103, indicating that precipitation was not YadA specific. SDS-PAGE analysis of these preparations is shown in Fig. 2. The Tx-YadA and Tx-103 extracts are identical except for the presence of highly enriched YadA in the former (Fig. 2).

Tx-YadA was used to raise MAbs specific for YadA, and the specificity of the four EIA-reactive MAbs was assessed by immunoblotting. Three of these, 2A9, 2G12, and 3G12, were specific for YadA, and one, 3G6, recognized two low-molecular-weight bands of *E. coli* origin present both in Tx-YadA and Tx-103 (Fig. 2). Since the samples were not boiled before loading onto the gel, it is feasible that the bands recognized by 3G6 are monomeric and dimeric forms of the same protein. MAbs 2A9 and 2G12 were specific for *Y. enterocolitica* serotype O:3 YadA, while 3G12 recognized both serotype O:3 and O:8 YadA (data not shown).

To determine whether purified YadA could bind to frozen sections, frozen sections were incubated with Tx-YadA and with Tx-103 or buffer as a control. The bound YadA was visualized by using MAb 3G12 and immunoperoxidase staining. In the Tx-YadA-treated sections, peroxidase staining was seen in the submucosa with the same pattern as with bacteria (Fig. 3). In the control sections treated with Tx-103 or buffer alone, no peroxidase staining was seen at all (Fig. 3).

Quantitation of bacterial binding by the EIA-on-slide assay. The EIA-on-slide assay allowed us to monitor quantitatively bacterial binding to the frozen sections over about a 100-fold range (Fig. 4 and 5). There was a linear relationship between absorbance values and bacterial concentrations from about 5×10^6 to about 5×10^8 bacteria per ml. The binding results obtained by using the Gram-stained and immunoperoxidase-stained sections were confirmed by this assay (Fig. 4); i.e., binding requires YadA. YeO3-c/pYL8, which expresses a collagen binding-deficient derivative of YadA (43), did bind but much more weakly than strains expressing the wild-type YadA.

To determine what proportion of the bacteria in the suspension bound to the sections during the binding assay, two approaches were used. First, known numbers of YeO3 and YeO3-c were bound on slides by drying 100 μl of bacterial suspensions on slides and fixing the bacteria with methanol.

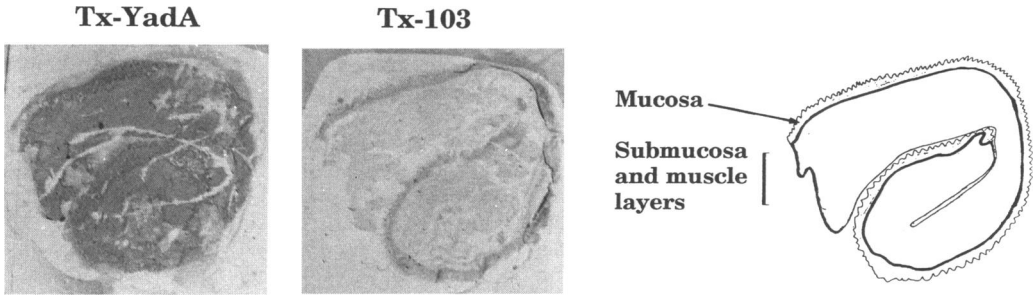

FIG. 3. Immunoperoxidase staining of sequential frozen sections of human intestine incubated with Tx-YadA and Tx-103. To visualize YadA, the sections were immunostained by using MAb 3G12 (see Fig. 1). The sections were lightly background stained with hematoxylin, which mainly stained the mucosal region and which is easier to distinguish in the Tx-103-treated section. Note that the darker appearance of the mucosal part in both sections is due to bluish background staining performed with hematoxylin. Examination at higher magnification revealed that YadA-specific staining was restricted mainly to the submucosal layer. A diagram of the sections is shown at the right, with an overall magnification of about threefold. To prepare the frozen sections, the intestine was opened longitudinally, part of the muscular layer and the serosa was removed, a rectangular piece was excised and rolled so that mucosa was directed outward, and the roll was frozen into a block by using the OCT compound.

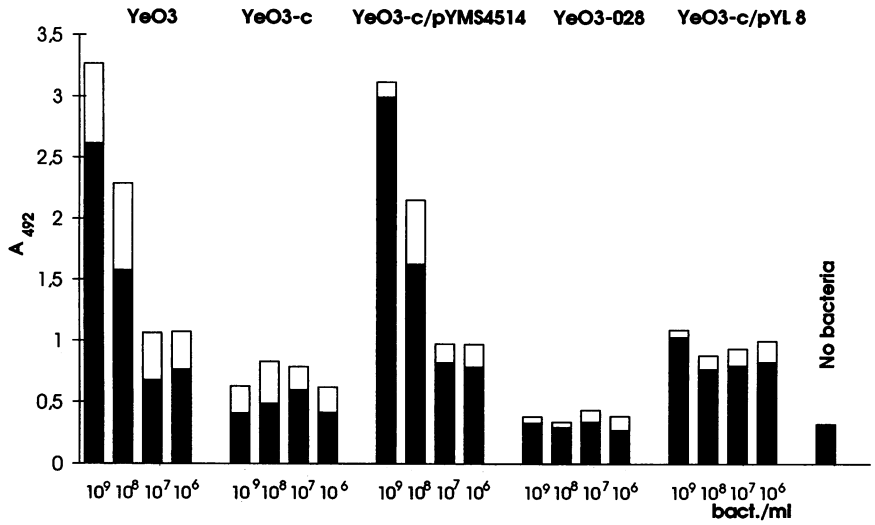

FIG. 4. EIA-on-slide assay showing the binding of different *Y. enterocolitica* strains to frozen sections. The binding is shown as A_{492}. Filled columns show the mean absorbances calculated from a minimum of four parallel sections, and open columns represent the respective standard deviations. One hundred microliters of bacterial suspensions of 0.5 to 0.7 times the indicated bacterial concentrations was incubated on the sections, and then bound bacteria were detected by the EIA-on-slide assay. *Y. enterocolitica* YeO3 and YeO3-c/pYMS4514 expressed YadA, and strains YeO3-c and YeO3-028 did not. YeO3-c/pYL8 expressed the collagen binding-deficient form of YadA. Absorbances obtained from sections without added bacteria (bact.) but otherwise treated identically (no bacteria) are included to show background absorbances. (The standard deviation for the no-bacteria column was so small that it is not visible in this drawing.)

The slides were then processed according to the EIA-on-slide protocol (Fig. 5). In this assay, the two strains gave identical results, showing that the detection method used in the EIA-on-slide protocol does not discriminate between YadA-expressing and -nonexpressing bacteria. Assuming that drying and methanol fixation of bacteria on slides had been 100% effective, then under our experimental conditions, at most 4×10^6 bacteria were needed to generate absorbances above 2.0 (Fig. 5). Using approximately the same number of YadA-expressing bacteria in the binding assays (Fig. 4, the 10^8 column of YeO3), we obtained only slightly lower absorbances, suggesting that many of the added bacteria had bound. We verified this by determining the number of bacteria not bound to the sections during the binding assays. Using 3.4×10^6 or 3.4×10^5 bacteria per 100-μl droplet, we followed the EIA-on-slide protocol up to the binding step. Then, instead of washing the unbound bacteria away, we removed samples from the droplets on the sections and determined the numbers of unbound bacteria by serial dilution plating. With the higher bacterial concentration, 27.5% (9×10^5) bound, and with the lower, about 47% (1.6×10^5) bound (data not shown). As 9×10^5 bacteria bound with the higher concentration of bacteria, all of the 3.4×10^5 bacteria added to the sections should have been able to bind. Apparently, under the conditions of the

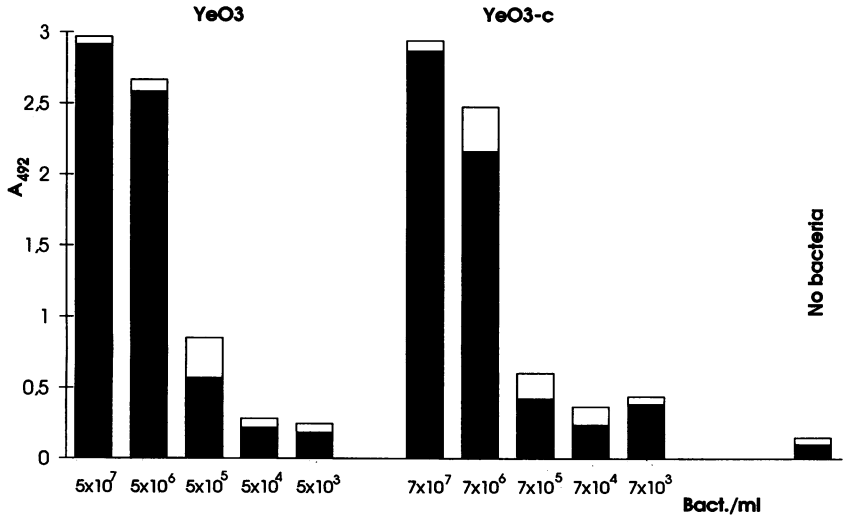

FIG. 5. EIA-on-slide assay showing absorbances obtained with the indicated numbers of *Y. enterocolitica* strains YeO3 and YeO3-c fixed directly onto the slides by drying and methanol. Filled columns show the mean absorbances, and open columns show the respective standard deviations. bact., bacteria.

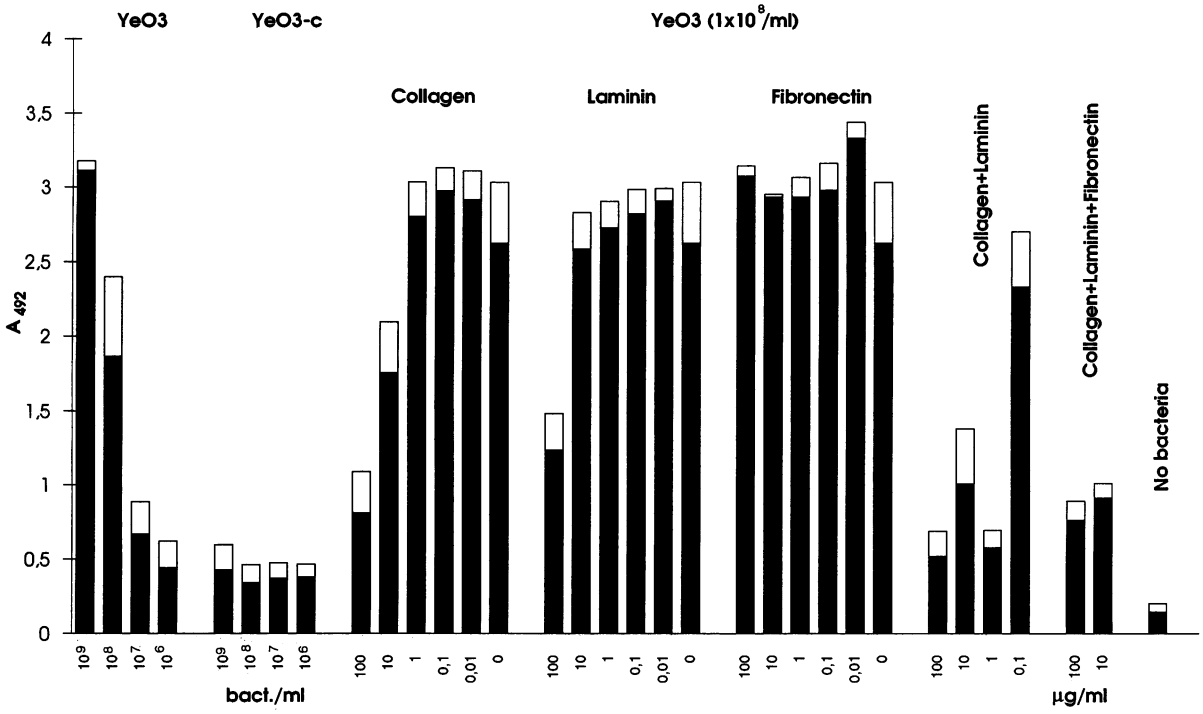

FIG. 6. Inhibition experiments with extracellular matrix molecules, using the EIA-on-slide assay. YadA-expressing *Y. enterocolitica* YeO3 at a concentration of 10^8/ml was incubated with different concentrations and combinations of collagen, laminin, and fibronectin before the binding assay was performed. Also shown are absorbances obtained in the same experiment with YeO3 and YeO3-c used at 0.5 to 0.7 times the indicated bacterial concentrations without any inhibition. Filled columns show the mean absorbances, and open columns show the respective standard deviations. bact., bacteria.

binding assay, not all binding-competent bacteria were able to bind.

Inhibition experiments. To monitor the effects of various treatments on bacterial binding, we used in the inhibition experiments bacterial concentrations (about 10^8/ml) which gave absorbances of 2 to 2.5 in the EIA-on-slide assay, thus being close to the upper limit of the linear range of this assay. Any inhibition of bacterial binding should result in decreased absorbance values; under the experimental conditions used here, absorbances under 0.8 would be equivalent to >90% inhibition.

(i) By extracellular matrix molecules. To inhibit YadA-mediated binding, the YadA-expressing bacteria were incubated before the binding assay with different concentrations and combinations of collagen, laminin, and plasma fibronectin. After incubation of the bacterial suspensions with these reagents for 30 minutes at 37°C, 100-µl portions of these mixtures were pipetted on the fixed and blocked frozen sections. The number of bacteria bound to the sections was quantitated by the EIA-on-slide method (Fig. 6). Collagen produced a marked but not complete inhibition of binding at a concentration of 100 µg/ml. Inhibition was dose dependent; no inhibition was seen with ≤1 µg of collagen per ml. Laminin showed an inhibition pattern similar to that observed for collagen, although the inhibition seemed less efficient; laminin concentrations of ≤10 µg/ml were not inhibitory. Fibronectin did not affect the binding of bacteria. The combination of collagen and laminin, on the other hand, inhibited the binding most efficiently, as shown by the significant decrease in absorbances even at a concentration of 1 µg/ml. The combination of all three matrix molecules, collagen, laminin, and fibronectin, did not increase inhibition.

(ii) By antibodies. MAbs specific for YadA, as well as rabbit polyclonal anti-*Y. enterocolitica* O:3 antiserum (37, 40), were not inhibitory (data not shown).

(iii) By Tx-YadA. The YadA binding sites were blocked before the bacteria were added to the sections by incubating the frozen sections with different concentrations of Tx-YadA or Tx-103. Tx-YadA treatment of the sections partially inhibited binding in a dose-dependent manner, whereas Tx-103 treatment did not inhibit the binding even at the highest concentration used in the experiment (Fig. 7).

DISCUSSION

Binding of *Y. enterocolitica* to epithelial cells, to several types of collagens, to fibronectin, and to laminin is YadA mediated (5, 11, 13, 34, 43, 45). In addition to having YadA-mediated binding properties, *Y. enterocolitica* possesses other molecules, such as invasin, Ail, and type 3 fimbriae, which mediate binding to a number of targets (24, 44). The present study was initiated to find the targets for binding of *Y. enterocolitica* in intestinal tissue. We expected to find bacteria binding to mucosa and the luminal epithelial cells of intestine. Surprisingly, the bacteria bound mostly to submucosa, not to mucosa. Several lines of evidence suggested that YadA plays a central role in the binding to submucosal intestinal tissue. (i) pYV-positive *Y. enterocolitica* strains bound to frozen sections, while pYV-negative *Y. enterocolitica* strains did not. (ii) *Y. enterocolitica* grown at 37°C bound to frozen sections, while bacteria grown at 22°C did not. YadA is pYV encoded and expressed only at 37°C (39, 41). (iii) *E. coli* strains with the cloned *yadA* gene from *Y. enterocolitica* O:3, from *Y. enterocolitica* O:8, or from *Y. pseudotuberculosis*, but not that from *Y. pestis*, had binding

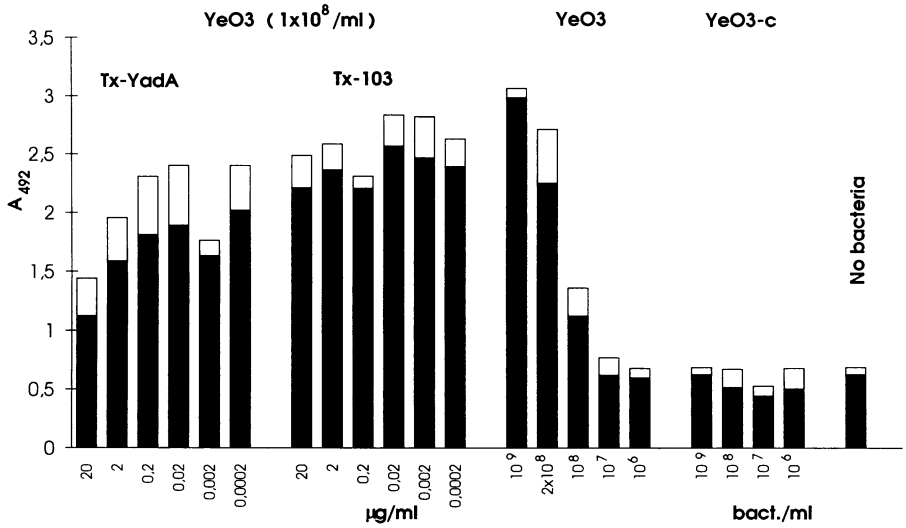

FIG. 7. Inhibition experiments with Tx-YadA and Tx-103, using the EIA-on-slide assay. The sections used in the binding assays were preincubated with the indicated concentrations of Tx-YadA and Tx-103 before YadA-expressing YeO3 at a concentration of 10^8/ml was used to monitor the YadA-mediated binding of bacteria (bact.) to frozen sections. Also shown are absorbances obtained in the same experiment with YeO3 and YeO3-c used at 0.5 to 0.7 times the indicated bacterial concentrations without any inhibition. Filled columns show the mean absorbances, and open columns show the respective standard deviations.

properties similar to those of *Y. enterocolitica*. (iv) YadA mutants of *Y. enterocolitica* had no binding capacity. (v) Tx-YadA, but not Tx-103, bound to frozen sections with distribution identical to that of the bacterial binding. Our binding assay was set up to specifically study the pYV-mediated binding. Increasing the incubation time of the binding assay increased only a little the binding of the pYV-negative strain. It is possible that this binding was specific and mediated by the other adhesion factors of *Y. enterocolitica*. In this system, YadA-mediated binding was clearly the most important.

The target molecules for YadA-mediated binding in the frozen sections seemed to be collagen and laminin, since a combination of these molecules almost totally inhibited binding (Fig. 6). Of these two, collagen seemed to be the major target. First, smaller concentrations of collagen than of laminin were inhibitory (Fig. 6). Second, a mutant of YadA that does not bind collagen but binds laminin and fibronectin (expressed by YeO3-c/pYL8) bound sections much more weakly than did wild-type YadA (Fig. 4). These results are in accordance with the differences in the relative affinities of YadA for these molecules (43).

Anti-YadA MAbs or *Y. enterocolitica*-specific antiserum did not inhibit bacterial binding to frozen sections. We recently identified the region of YadA involved in the collagen binding phenomenon, and this region did not include the epitopes recognized by the MAbs (43). Thus, the antibodies do not block the collagen binding domain of YadA, which may explain why they do not inhibit binding. Moreover, we were not able to totally inhibit the binding of bacteria with Tx-YadA, possibly because YadA, which also causes autoagglutination of bacteria, functions similarly when bound to submucosa.

Ingestion of contaminated food or water precedes infection by enteropathogenic *Y. enterocolitica* (3). After that, the bacteria have to pass through the acidic stomach to the ileum, where invasion to M cells takes place. M cells are epithelial cells overlying the lymphoid follicles of Peyer's patches. M cells endocytose the bacteria through receptor-mediated phagocytosis and excrete the bacteria into the lamina propria, where a nonspecific local host defense (complement and phagocytes) meets the bacteria and starts the inflammatory reaction. For bacterial virulence, the bacteria must be able to resist or evade this host defense. Some of the properties of pathogenic *Y. enterocolitica* are obviously mediated through YadA, which is an essential virulence determinant in *Y. enterocolitica* (17, 43). YadA is associated, for instance, with serum resistance (3, 20, 31, 38) and with inhibition of the anti-invasive effect of interferon (8). The major role of YadA in *Y. enterocolitica* virulence is not yet clearly understood, but it at least partly mediates the bacterium's adhesive properties. Our study implies that YadA-mediated adhesion does not play a role in binding to and invasion of the intestinal epithelium but is important once the bacteria have reached the lamina propria and submucosa. What, then, is the significance of this binding? We hypothesize that the bacteria may protect themselves against phagocytosis by adhering to the structures of submucosa and thus making it more difficult for the phagocytes to reach them. Sticking there might also provide the bacteria with a place where they can multiply before spreading further and, in some cases, producing systemic infections.

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REFERENCES

1. **Appleyard, R.** 1954. Segregation of new lysogenic types during growth of doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39**:440–447.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. Green Publishing Associates and John Wiley

& Sons, New York.
3. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48:**782–786.
4. **Bliska, J. B., M. C. Copass, and S. Falkow.** 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment and entry into HEp-2 cells. Infect. Immun. **61:**3914–3921.
5. **Bölin, I., Å. Forsberg, L. Norlander, M. Skurnik, and H. Wolf-Watz.** 1988. Identification and mapping of the temperature-inducible plasmid-encoded proteins of *Yersinia* spp. Infect. Immun. **56:**343–348.
6. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256:**1604–1607.
7. **Brubaker, R. R.** 1972. The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. Immunol. **57:**111–158.
8. **Bukholm, G., G. Kapperud, and M. Skurnik.** 1990. Genetic evidence that the *yopA* gene-encoded *Yersinia* outer membrane protein Yop1 mediates inhibition of anti-invasive effect of interferon. Infect. Immun. **58:**2245–2251.
9. **China, B., M.-P. Sory, B. T. N'Guyen, M. de Bruyere, and G. R. Cornelis.** 1993. Role of the YadA protein in prevention of opsonization of *Yersinia enterocolitica* by C3b molecules. Infect. Immun. **61:**3129–3136.
10. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
11. **Emödy, L., J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadström.** 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. J. Bacteriol. **171:**6674–6679.
12. **Hanski, C., U. Kutschka, H. P. Schmoranzer, M. Naumann, A. Stallmach, H. Hahn, H. Menge, and E. O. Riecken.** 1989. Immunochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O:8 with intestinal mucosa during experimental enteritis. Infect. Immun. **57:**673–678.
13. **Heesemann, J., and L. Gruter.** 1987. Genetic evidence that the outer membrane protein YOP1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. FEMS Microbiol. Lett. **40:**37–41.
14. **Isberg, R. R.** 1990. Pathways for the penetration of enteroinvasive *Yersinia* into mammalian cells. Mol. Biol. Med. **7:**73–82.
15. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317:**262–264.
16. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
17. **Ismail, N., L. Pelliniemi, P. Toivanen, and M. Skurnik.** Unpublished data.
18. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbakken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55:**2247–2254.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
20. **Martinez, R. J.** 1989. Thermoregulation-dependent expression of *Yersinia enterocolitica* protein P1 imparts serum resistance to *Escherichia coli* K-12. J. Bacteriol. **171:**3732–3739.
21. **Meacock, P., and S. Cohen.** 1980. Partitioning of bacterial plasmids during cell division: a cis-acting locus that accomplishes stable plasmid inheritance. Cell **20:**529–542.
22. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9:**309–321.
23. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci from *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
24. **Miller, V. L., J. T. Farmer, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57:**121–131.
25. **Pærregaard, A., F. Espersen, and N. Baker.** 1990. The influence of properties encoded by the Yersinia virulence plasmid on adhesion of *Yersinia enterocolitica* to ileal brush border vesicles. APMIS **98:**927–932.
26. **Pærregaard, A., F. Espersen, J. Hannover Larsen, and N. Høiby.** 1990. Adhesion of *Yersinia enterocolitica* to human epithelial cell lines and to rabbit and human small intestinal tissue. APMIS **98:**53–60.
27. **Pærregaard, A., F. Espersen, O. M. Jensen, and M. Skurnik.** 1991. Interactions between *Yersinia enterocolitica* and rabbit ileal mucus: growth, adhesion, penetration, and subsequent changes in surface hydrophobicity and ability to adhere to ileal brush border membrane vesicles. Infect. Immun. **59:**253–260.
28. **Pærregaard, A., F. Espersen, and M. Skurnik.** 1991. Role of *Yersinia* outer membrane protein YadA in adhesion to rabbit intestinal tissue and rabbit intestinal brush border membrane vesicles. APMIS **99:**226–232.
29. **Pekkola-Heino, K., M. K. Viljanen, T. H. Ståhlberg, K. Granfors, and A. Toivanen.** 1987. Monoclonal antibodies reacting selectively with core and O-polysaccharide of *Yersinia enterocolitica* O:3 lipopolysaccharide. APMIS **95:**27–34.
30. **Pepe, J., and V. Miller.** 1990. The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. J. Bacteriol. **172:**3780–3789.
31. **Piltz, D., T. Vocke, J. Heesemann, and B. Brade.** 1992. Mechanism of YadA-mediated serum resistance of *Yersinia enterocolitica* serotype O3. Infect. Immun. **60:**189–195.
32. **Portnoy, D., S. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31:**775–782.
33. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature (London) **334:**522–525.
34. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, T. Kirsch, B. Swoboda, C. Bull, S. Goodman, and F. Emmrich.** 1993. Outer membrane protein YadA of enteropathogenic yersiniae mediates specific binding to cellular but not plasma fibronectin. Infect. Immun. **61:**2513–2519.
35. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, K. von der Mark, and F. Emmrich.** 1992. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. Infect. Immun. **60:**2153–2159.
36. **Skurnik, M.** 1984. Lack of correlation between the presence of plasmids and fimbriae in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. J. Appl. Bacteriol. **56:**355–363.
37. **Skurnik, M.** 1985. Expression of antigens encoded by the virulence plasmid of *Yersinia enterocolitica* under different growth conditions. Infect. Immun. **47:**183–190.
38. **Skurnik, M., A. Al-Hendy, and P. Toivanen.** Unpublished data.
39. **Skurnik, M., I. Bölin, H. Heikkinen, S. Piha, and H. Wolf-Watz.** 1984. Virulence plasmid-associated autoagglutination in *Yersinia* spp. J. Bacteriol. **158:**1033–1036.
40. **Skurnik, M., and K. Poikonen.** 1986. Experimental intestinal infection of rats by *Yersinia enterocolitica* O:3. A follow-up study with specific antibodies to the virulence plasmid specified antigens. Scand. J. Infect. Dis. **18:**355–364.
41. **Skurnik, M., and P. Toivanen.** 1992. LcrF is the temperature-regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. J. Bacteriol. **174:**2047–2051.
42. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. **3:**517–529.
43. **Tamm, A., A.-M. Tarkkanen, T. K. Korhonen, P. Kuusela, P. Toivanen, and M. Skurnik.** 1993. Hydrophobic domains affect the collagen-binding specificity and surface polymerization as well as the virulence potential of the YadA protein of *Yersinia enterocolitica*. Mol. Microbiol. **10:**955–1011.
44. **Tarkkanen, A.-M., B. L. Allen, B. Westerlund, H. Holthöfer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen.** 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. **4:**1353–1361.

45. **Tertti, R., M. Skurnik, T. Vartio, and P. Kuusela.** 1992. The adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectin. Infect. Immun. **60:**3021–3024.
46. **Toivanen, A., and P. Toivanen (ed.).** 1988. Reactive arthritis, p. 1–186. CRC Press, Boca Raton, Fla.
47. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. I. Experimental infection in rabbits. Microbiol. Immunol. **21:**349–363.
48. **Valtonen, V. V., M. Leirisalo, P. J. Pentikäinen, T. Räsänen, I. Seppälä, U. Larinkari, M. Ranki, S. Koskimies, M. Malkamäki, and P. H. Mäkelä.** 1985. Triggering infections in reactive arthritis. Ann. Rheum. Dis. **44:**399–405.
49. **Viitanen, A.-M., P. Toivanen, and M. Skurnik.** 1990. The *lcrE* gene is part of an operon in the *lcr* region of *Yersinia enterocolitica* O:3. J. Bacteriol. **172:**3152–3162.
50. **Yang, Y., and R. R. Isberg.** 1993. Cellular internalization in the absence of invasin expression is promoted by the *Yersinia pseudotuberculosis yadA* product. Infect. Immun. **61:**3907–3913.