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Outer Membrane Protein YadA of Enteropathogenic Yersiniae Mediates Specific Binding to Cellular but Not Plasma Fibronectin

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The binding of bacteria or bacterial products to host proteins of tissue extracellular matrix may be a mechanism of tissue adherence. We investigated interactions of the plasmid-encoded outer membrane protein YadA, which confers pathogenic functions on enteropathogenic yersiniae, with fibronectin. Attachment of YadA-positive and YadA-negative recombinant *Yersinia enterocolitica* strains to cartilage-derived human cellular fibronectin and human plasma fibronectin in the solid phase revealed that YadA mediates binding of yersiniae to cellular fibronectin in a saturable, concentration-dependent manner. The interaction could be inhibited by an anti-YadA-specific anti-serum. An anti-β1-integrin antibody and the synthetic peptide G-R-G-D-S-P, representing the binding site for α5β1-integrin on fibronectin, did not block attachment of YadA-positive yersiniae to cellular fibronectin, indicating a binding site for YadA on cellular fibronectin independent of the R-G-D-S-containing site. By contrast, YadA failed to mediate binding to plasma fibronectin immobilized on nitrocellulose or plastic surfaces. These observations provide evidence for the hypothesis that the binding region for YadA in cellular fibronectin is not present in plasma fibronectin. This study is the first report on differential binding of bacteria to splicing variants of fibronectin. Further experiments might answer the question whether binding of YadA to cellular fibronectin contributes to the pathogenesis of yersiniae, both to the initial adhesion of the bacteria to the matrices of the host and to the arthritogenic potential of enteropathogenic yersiniae.

The interaction between bacteria or bacterial products and host tissues or soluble proteins is crucial during infectious diseases, both for primary adhesion and invasion of the microorganism into the host and for tissue-specific colonization and disease tropism. A variety of bacteria have been shown to bind host proteins, in particular, the extracellular matrix (ECM) proteins collagen (8, 40, 47), laminin (30, 46, 48), and fibronectin (10, 24, 43, 50) (for a review, see reference 18). The importance of these interactions for tissue adhesion has been demonstrated previously (9, 21).

Fibronectins are found in tissue-associated and soluble forms. The tissue-associated forms (cellular fibronectins [cFN]) are virtually insoluble in nonionic or mildly ionic detergents (19, 53). They are produced by a variety of cells and are incorporated into the basement membranes and connective tissue matrices (7, 19, 53). The vast majority of soluble fibronectin is found in plasma (plasma fibronectin [pFN]) (33) and is produced predominantly by hepatocytes (19). However, different body fluids (e.g., synovial fluid [51]) contain distinct forms of soluble fibronectins, which are made by various cellular sources (for reviews, see references 19 and 53). Fibronectins are disulfide-linked multidomain dimeric glycoproteins (M_r, ~480,000). They are involved in cellular adhesion to the ECM, platelet function, cell migration, interaction with the cytoskeleton, and reticuloendothelial functions (19). The main functions are related to their ability to interact with a variety of other molecules, e.g., collagen, heparin, fibrin(ogen), proteoglycans, and thrombospondin in the matrix and integrins and proteoglycans on cell surfaces (40). Moreover, fibronectin also mediates adhesion between certain microorganisms and eukaryotic cells as well as the ECM (2, 21). Since the first report on the ability of fibronectin to bind *Staphylococcus aureus* (24), numerous pathogenic organisms have been found to bind fibronectin (10, 18, 43, 50), and in a recent study the interaction of pFN to yersiniae was investigated (49).

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are enteropathogenic for humans (6). The intestinal infection caused by these microorganisms can cause disease sequelae such as reactive arthritis, erythema nodosum, and uveitis (6). It has been shown that in yersiniae, a 70-kb plasmid (pYV) is necessary for full expression of virulence (4, 38). Several plasmid-encoded proteins, including outer membrane proteins have been found (3, 4, 13, 15). The most prominent plasmid-encoded outer membrane protein, YadA, forms multimeric fibrillae on the surface of the bacteria (22, 27) and is associated with several virulence functions such as resistance to complement lysis (37), resistance to phagocytosis, and adherence to mammalian cells (15, 17). Recently, we reported on YadA-mediated specific binding to collagen types I, II, III, IV, V, and XI (40). In the present study, we investigated YadA-mediated binding to both pFN and cFN.

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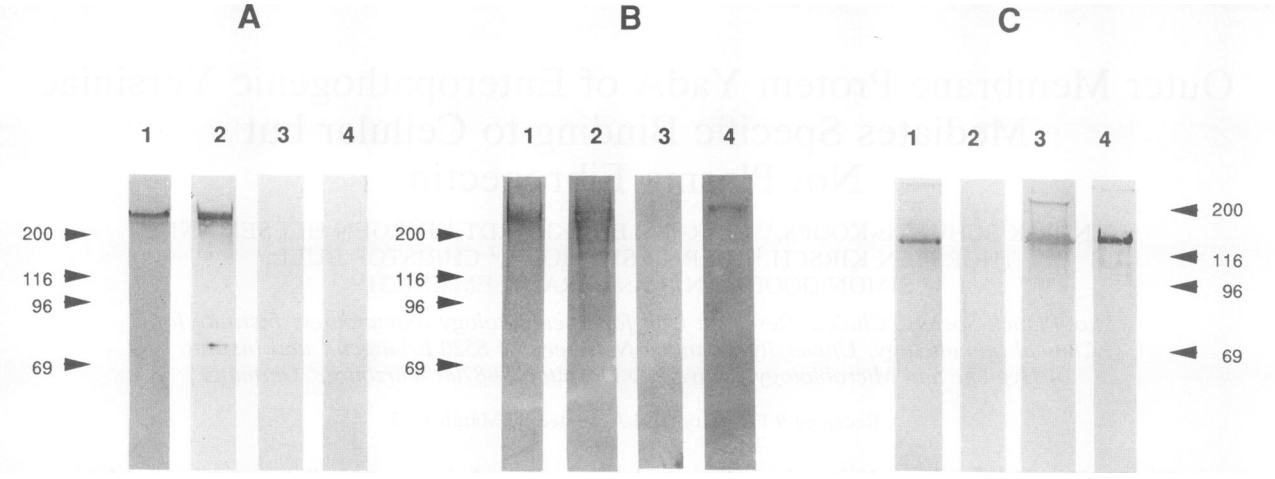

FIG. 1. SDS-PAGE and immunoblotting of pFN (A), cFN (B), and collagen type II (C) and binding of YadA-positive yersiniae to the proteins. Lanes: 1, SDS-PAGE under reducing conditions; 2 and 3, proteins were electroblotted to nitrocellulose filters after SDS-PAGE, and antifibronectin anti-serum (lanes 2) or the anticollagen monoclonal antibody B1 (lanes 3) was added, with an anti-rabbit peroxidase conjugate as the detection system; 4, for *Yersinia* affinity blots, proteins were electroblotted onto nitrocellulose filters after SDS-PAGE, and YadA-positive yersiniae (NFpRK290B9-4) were added. Filter-bound yersiniae were assessed with a rabbit anti-*Y. enterocolitica* O:5 anti-serum. An anti-rabbit peroxidase conjugate was used as the detection system. Standard proteins of known molecular mass (in kilodaltons) are indicated by arrowheads.

In adhesion assays with YadA-positive and YadA-negative yersiniae and with pFN and cFN coated onto microtiter plates, as well as in affinity blot experiments with fibronectin immobilized on nitrocellulose filters, we could demonstrate that YadA was involved in binding of yersiniae to cFN purified from cartilage. In contrast, YadA-mediated binding of yersiniae to pFN could not be detected in these assays.

MATERIALS AND METHODS

Bacterial strains. Four *Y. enterocolitica* strains of serotype O:5 were used in this study: (i) strain NF0 has an environmental origin, is plasmid free and YadA negative, and was used as the host strain for the plasmids; (ii) pRK290B, a vector mobilizable in yersiniae (17) (strain NFpRK290B); (iii) pCB9::Tn*5*, the virulence plasmid of *Y. enterocolitica* O:9 with an inactivated YadA gene (strain NFpCB9::Tn5); and (iv) pRK290B9-4, a hybrid plasmid consisting of the vector pRK290B and *Bam*HI fragment 4 of the virulence plasmid of *Y. enterocolitica* O:9 encoding YadA (1) (strain NFpRK290B9-4). It has been previously shown that the last strain expresses YadA on its surface (17). All strains have been described previously (8, 15, 17). No strain lacking YadA expression (e.g., strains NF0, NFpRK290B, and NFpCB9::Tn*5*) was found to bind fibronectin throughout the study. Bacteria were grown in Luria-Bertani-Bouillon at 37°C for 24 h, washed, and resuspended in phosphate-buffered saline (PBS) immediately before the experiments were performed. For some experiments, bacteria were heat inactivated at 60°C for 45 min and stored in PBS–0.01% NaN_3 (pH 7.3) at 4°C.

Antibodies. Polyclonal rabbit antifibronectin anti-serum and the synthetic G-R-G-D-S-P peptide were purchased from Telios Inc. (San Diego, Calif.). The antiserum was obtained by immunization with purified pFN and solid phase absorbed with human plasma proteins except fibronectin. The monoclonal mouse anticollagen antibody B1 was kindly provided by R. Holmdahl, Uppsala, Sweden. This antibody binds to an epitope conserved in different collagen types, i.e., types I, II, IX, and XI. Its affinity for binding is not affected by heat denaturation of the collagen molecule (unpublished data). Rabbit anti-*Y. enterocolitica* O:5 anti-serum was raised against formalin-fixed plasmidless *Y. enterocolitica* O:5 grown at 22°C (14a). The rabbit anti-YadA anti-serum was obtained by immunization with electroeluted YadA (16). The anti-β1-integrin antibody AIIB2 was a kind gift from C. Damsky, University of California at San Francisco, San Francisco. The optimal concentration for each antibody was determined by dilution experiments.

Purification of ECM proteins. Type II collagen was prepared from chicken xiphoid process. Extraction with guanidinium hydrochloride for removal of proteoglycans, digestion with pepsin, and subsequent fractional salt precipitation were performed as described by Miller and Rhodes (32). pFN was purified from human plasma by affinity chromatography on gelatin-Sepharose followed by affinity chromatography on heparin-Sepharose (44). cFN was extracted from adult human cartilage with 4 M guanidinium hydrochloride–50 mM Tris–1 mM EDTA (pH 7.2) containing 1 mM *N*-ethylmaleimide–phenylmethylsulfonyl fluoride. The extract was subjected to a CsCl equilibrium density gradient centrifugation (starting density, 1.35 g of CsCl per ml) at 18°C for 60 h at 105,000 × *g*. Fibronectin was further purified from the nonproteoglycan-containing fraction by gel filtration chromatography on a Bio-Gel A-5m Gel column (Bio-Rad Laboratories), followed by anion-exchange chromatography on a DEAE column (Whatman) and affinity chromatography on heparin-Sepharose. The purity of the proteins was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7% acrylamide minigels with the buffer system of Laemmli (28) and by Western blot (immunoblot) analysis with antifibronectin and anticollagen antibodies (Fig. 1).

Affinity blot. SDS-PAGE was performed with the purified ECM proteins, with 10% acrylamide minigels. The proteins were electrophoretically transferred to nitrocellulose filters. After blocking of unspecific protein-binding sites with 5% dried milk powder in PBS for 2 h at room temperature, the

filter was incubated with a particulate yersinia suspension (3×10^9 bacteria per ml in PBS) for 2 h at room temperature. Filter-bound yersiniae were immunostained with a rabbit anti-*Y. enterocolitica* O:5 anti-serum, with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 in PBS–1% bovine serum albumin [BSA]) as the detection system and 4-chloro-1-naphthol as the color substrate.

Adhesion assay. Nunc-Immuno Maxisorp 96-well plates (A/S Nunc, Kamstrup, Denmark) were coated with different amounts of protein in 50 μl of PBS for 1 h at 37°C. The coating solution was removed, and the remaining binding sites in each well were blocked by incubation with 100 μl of PBS–2% BSA for 2 h at 37°C. The wells were washed five times with PBS–0.02% Tween 20 and incubated with 100 μl of the particulate bacterium suspension (4×10^8 bacteria per ml in PBS) for 60 min at room temperature. After washing, bound yersiniae were immunostained with a rabbit anti-*Y. enterocolitica* O:5 anti-serum, with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Bio-Rad Laboratories) as the detection system and 3,3′-5,5′-tetramethylbenzidine as the substrate. The A_{450} was determined. The same assay was performed with fibronectin coated onto glass tubes. For the blocking experiments with antisera, the YadA-positive yersiniae were preincubated with increasing concentrations of anti-YadA anti-serum, an irrelevant rabbit anti-mouse anti-serum, or the anti-β1-integrin antibody AIIB2 for 1 h at room temperature prior to adding the bacteria to the plates (coating concentration of the ECM proteins, 10 μg/ml). In the inhibition experiments with the synthetic G-R-G-D-S-P peptide, increasing amounts of the peptide were added simultaneously with the bacteria to the plates. Human fibrosarcoma cells (HT-1080) were used to control cell binding of fibronectins and collagen coated to plastic surfaces (52). Nunc-Immuno Maxisorp 96-well plates were coated with different concentrations of proteins, and the remaining binding sites were blocked with PBS–2% BSA. A total of 5×10^4 cells were added to each well and incubated for 1 h at 37°C. After washing with PBS, a hexosaminidase substrate was added and allowed to react with endogenous lysosomal hexosaminidase for 1 h at 37°C (29). After adding 0.2 M NaOH–5 mM EDTA, the A_{405} was determined. In the blocking experiments, the anti-β1-integrin antibody AIIB2 in increasing concentrations was added simultaneously with the cells to the wells for 1 h at 4°C. The plate was then incubated for 1 h at 37°C, and staining was performed as described above. Each experiment was performed in triplicate.

RESULTS

Purity of fibronectin preparations. SDS-PAGE and Coomassie blue staining of the fibronectins used in this study revealed bands with a molecular mass of ~240 kDa under reducing conditions. The antifibronectin anti-serum reacted with both cFN and pFN in the Western blot analysis (Fig. 1). Figure 1 demonstrates the purity of our fibronectin preparations. No fibronectin preparation reacted with anticollagen antibody B1, used to detect collagen contaminations. Enzyme-linked immunosorbent assays (ELISA) with the B1 antibody revealed collagen contamination of less than 2.5 pg of collagen per 1 μg of fibronectin in both fibronectin preparations. The B1 antibody binds to an epitope conserved in different collagen types, i.e., types I, II, IX, and XI.

Binding of YadA-positive yersiniae to filter-bound ECM proteins. As assessed by a *Yersinia* affinity blot, YadA-positive yersiniae (NFpRK290B9-4) bound cFN and collagen type II (Fig. 1). Heat inactivation of the bacteria (45 min, 60°C) did not affect the binding abilities of YadA to cFN immobilized on nitrocellulose. In contrast, pFN did not bind either native or heat-inactivated YadA-positive yersiniae. YadA-negative yersiniae (e.g., strains NF0, NFpRK290B, and NFpCB9::Tn*5*) did not bind to any of the proteins (data not shown).

Binding of YadA-positive yersiniae to plastic-bound ECM proteins in various concentrations. For quantitation of YadA binding to cFN and pFN, ECM proteins in a wide range of concentrations were coated onto microtiter plates. Collagen type II was used as a positive control for binding of YadA-positive yersiniae. As shown in Fig. 2, native YadA-positive yersiniae (NFpRK290B9-4) bound cFN and collagen type II in a concentration-dependent manner. Heat treatment of the bacteria did not influence YadA-mediated binding of yersiniae to cFN and collagen type II (Fig. 2b). The binding was saturable for both proteins. Only at higher concentrations (above 2 μg of fibronectin per ml in the coating buffer) were bacteria found to bind pFN. No marked difference between YadA-positive and YadA-negative yersiniae could be detected on pFN. The curves shown in Fig. 2 for YadA-binding to pFN were similar to the curves for background binding of the YadA-negative *Y. enterocolitica* strains to collagen type II, pFN, and cFN and therefore do not demonstrate YadA-mediated binding. As revealed with an antifibronectin antiserum, coating of both fibronectin preparations to the plastic surfaces was equally effective (data not shown). No binding at all was found with BSA-coating or plasma vitronectin, an ECM protein which mediates cell attachment over different integrins (39) (Fig. 2b). Preincubation of YadA-positive yersiniae with increasing amounts of anti-YadA anti-serum prior to adding the bacteria to the microtiter plates inhibited binding of the bacteria to cFN and collagen type II (Fig. 3). Fifty percent blocking was achieved with a 1:1,000 dilution of the antiserum. An irrelevant rabbit anti-mouse antiserum failed to block yersinia-ECM protein interaction. Attachment of YadA-positive yersiniae to cFN was not affected by an anti-β1-integrin antibody (AIIB2) (Fig. 4) or the synthetic peptide G-R-G-D-S-P (data not shown), indicating that the YadA-cFN interaction is independent of the amino acid motif R-G-D-S, which is the classical binding site for α5β1-integrins of eukaryotic cells on fibronectin (36). In order to test the adhesive activities of the ECM proteins coated onto plastic, human fibrosarcoma cells (HT-1080) were added to the microtiter plates. It could be demonstrated that pFN, cFN, and collagen type II all effectively supported the attachment of HT-1080 cells. BSA did not bind fibrosarcoma cells. In contrast to YadA-mediated binding of yersiniae to cFN, binding of HT-1080 cells to pFN and cFN could be inhibited by the anti-β1-integrin antibody AIIB2 in a concentration-dependent manner (Fig. 4).

Binding of YadA-positive yersiniae to glass-bound fibronectins in various concentrations. The surface chosen for adsorption of fibronectin is crucial for the conformation of the attached fibronectin (12). In order to elucidate whether the differences in binding abilities of cFN compared with those of pFN were due to conformational differences of the fibronectins induced by the plastic surfaces, we performed adhesion assays with fibronectins in a wide range of concentrations coated onto glass. The binding curve for YadA-positive yersiniae (NFpRK290B9-4) to cFN coated onto glass was identical to the curve for yersiniae binding to plastic-coated cFN (Fig. 5). In contrast to the results obtained with plastic-bound pFN, binding of yersiniae to pFN coated onto glass was dependent on YadA expression.

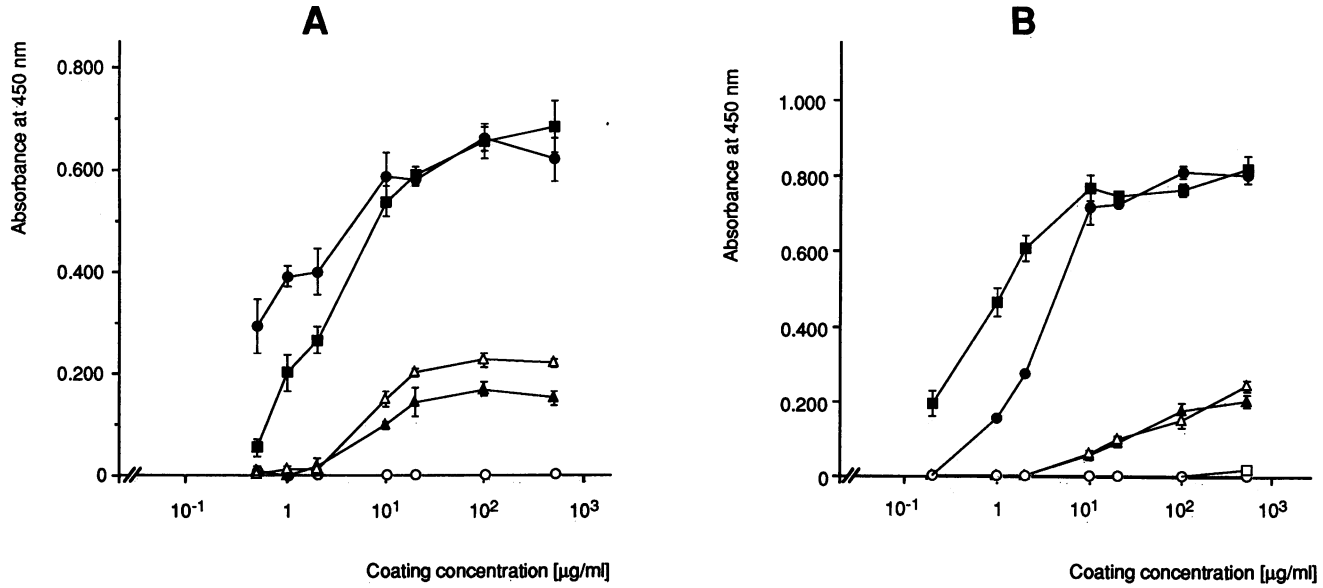

FIG. 2. Binding of plastic coated with cFN (●), collagen type II (■), pFN (▲), vitronectin (□), and BSA (○) to YadA-positive native (A) or heat-inactivated (B) yersiniae (NFpRK290B9-4). Yersiniae were added to microwells precoated with the proteins in different coating concentrations. Yersiniae attached to the proteins were assessed with a rabbit anti-*Y. enterocolitica* O:5 anti-serum and an anti-rabbit peroxidase conjugate as the detection system. Binding of YadA-negative yersiniae (△) to cFN and pFN (resulting in similar binding curves) is also shown. No obvious differences in binding abilities of YadA-negative *Y. enterocolitica* strains (e.g., strains NF0, NFpRK290B, and NFpCB9::Tn*5*) could be detected. Each value is the mean and standard deviation of at least two (A) or four (B) independent triplicate assays.

However, compared with that of cFN, a marked difference in relative binding affinities was seen. As revealed by ELISA, the coating efficiencies of pFN and cFN to glass were equally effective (data not shown).

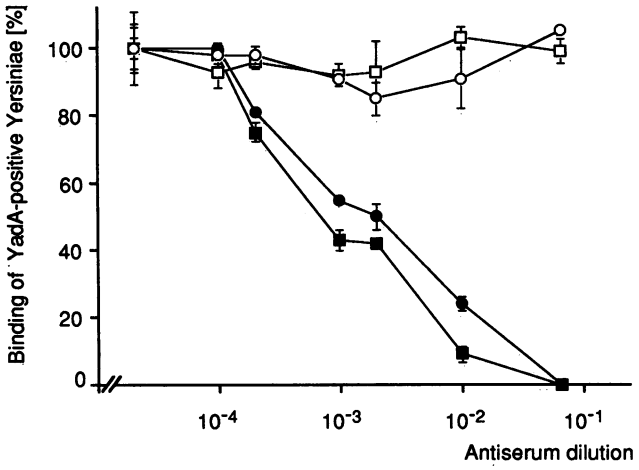

FIG. 3. Blocking effect of increasing amounts of anti-YadA anti-serum (filled symbols) and irrelevant rabbit anti-mouse anti-serum (open symbols) of the YadA-mediated binding to collagen type II (■ and □) and cFN (● and ○) which are coated to plastic surfaces. Inactivated YadA-positive yersiniae (NFpRK290B9-4) were incubated with increasing amounts of the antiserum prior to adding the bacteria to microwells coated with the ECM proteins (10-µg/ml coating concentration). Yersiniae attached to the proteins were assessed as described in the legend to Fig. 2. Values obtained after preincubation of the bacteria with PBS were defined as 100% binding of yersiniae. Each value is the mean and standard deviation of at least three independent triplicate assays.

DISCUSSION

Fibronectin is produced by almost all mammalian cells (19) and is a major component of the ECM of most tissues (11). Although fibronectins differ in size, solubility, and structure, they all are derived from a single fibronectin-

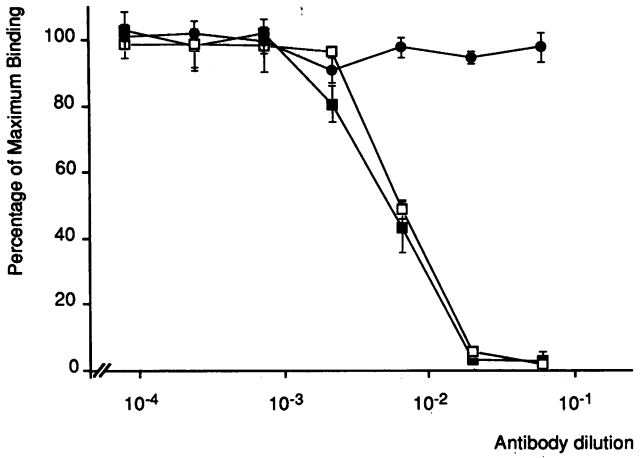

FIG. 4. Effect of anti-β1-integrin antibody AIIB2 on binding of YadA-positive yersiniae (NFpRK290B9-4) and HT-1080 cells to fibronectin. Bacteria and the fibrosarcoma cells were preincubated with increasing amounts of the antibody prior to allowing binding of YadA-positive yersiniae to cFN (●) and of HT-1080 cells to cFN (■) and pFN (□). Yersiniae attached to cFN were assessed as described in the legend to Fig. 2. Bound HT-1080 cells were quantified by an endogenous lysosomal hexosaminidase reaction. Values obtained after preincubation of the bacteria and the cells, respectively, with PBS were defined as 100% binding. Each value is the mean and standard deviation of at least three independent triplicate assays.

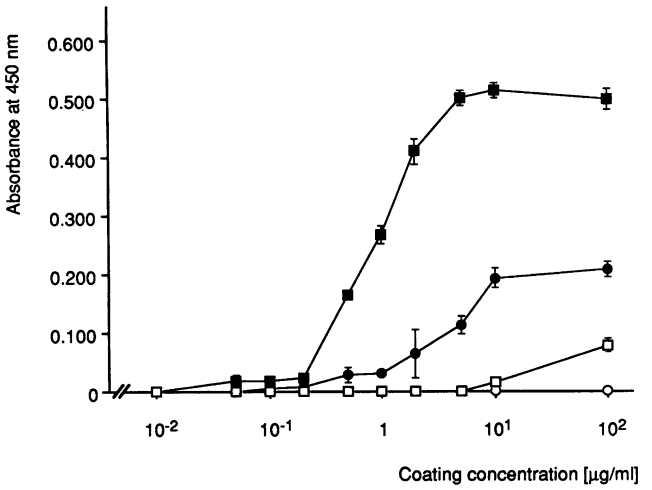

FIG. 5. Binding of glass coated with cFN (■), pFN (●), or BSA (○) to YadA-positive yersiniae (NFpRK290B9-4). Heat-inactivated yersiniae were added to glass tubes precoated with different concentrations of the proteins. Yersiniae attached to the proteins were assessed as described in the legend to Fig. 2. Binding of YadA-negative yersiniae to cFN and pFN (□) (resulting in similar binding curves) is also shown. No differences in binding abilities of the YadA-negative *Y. enterocolitica* strains could be detected. Each value is the mean and standard deviation of at least four independent triplicate assays.

encoding gene (23, 42), which can give rise to several different proteins by alternative processing of the primary RNA transcript. These differences might account for differential binding of the fibronectin variants. In particular, some domains are found in cFN but not pFN. The central 180- to 190-kDa region of fibronectin consists of 15 type III repeats, which are found in all fibronectins (19). One type III repeat mediates binding to cell surfaces (35), and it is speculated that every segment has its own binding facilities (19). At three positions, extra segments may be included in some but not all fibronectin subunits. The V- or III-CS region can undergo complex patterns of alternative splicing and contains sequences encoding binding sites for α4β1 integrins (20). The EIIIA and EIIIB segments can be inserted in cFN but are generally not found in pFN (14, 23, 41). III-CS, EIIIA, and EIIIB therefore might confer specific binding functions to cFN. However, despite of these structural differences, unique functional properties of the fragments specific for cFN have not yet been identified (19).

Since the interplay of fibronectin with products of *Y. enterocolitica* might contribute to diseases caused by this microorganism by serving as substrata for tissue adhesion, we investigated binding of cFN purified from cartilage to the major outer membrane protein of yersiniae, YadA. We could show that YadA mediates binding of yersiniae to cFN in a saturable and concentration-dependent manner. The YadA-cFN interaction was not altered by heat inactivation of the bacteria. Binding occurred independent of the solid phase used for immobilization of fibronectin, e.g., nitrocellulose, plastic, or glass. By contrast, pFN did not support binding of yersiniae in affinity blots. When it was coated onto plastic surfaces, pFN bound only unspecifically and at higher concentrations to yersiniae, irrespective of YadA expression. Only when pFN was coated onto glass was a slight YadA-dependent binding detectable at higher coating concentrations (Fig. 5). However, comparison with cFN indicated a marked difference in the binding affinities for YadA. As revealed by ELISA with antifibronectin anti-serum, differences in coating efficiencies of cFN and pFN could not account for the different relative affinities of YadA to the fibronectin preparations; the adsorption of both fibronectins to glass and plastic surfaces was similarly effective. Therefore, the type of solid surface used for immobilization of fibronectin may be responsible for the discrepancy in binding affinities of pFN coated onto glass or plastic for YadA-positive yersiniae. Grinnell and Feld found that fibronectin immobilized on different surfaces may display different epitopes, and they proposed that different conformations of fibronectin exist on different surfaces (12). In binding studies with staphylococci and streptococci, soluble pFN inhibited binding of the bacteria to pFN coated onto microwells but failed to block binding of pFN coated onto glass (5, 25, 26, 31). Taken together, our observations provide unequivocal evidence for the hypothesis that the binding region for YadA in cFN is not present in pFN. This is the first report of differential binding of bacteria to splicing variants of fibronectin.

Most bacteria capable of binding to fibronectin interact with the 29-kDa N-terminal region of the subunits (10, 34, 45, 50). Variations in the N-terminal region between cFN and pFN have so far not been described (19). Thus, the assumption that YadA interacts with the N-terminal region of fibronectin cannot explain the observed differences in binding to cFN and pFN and is, therefore, rather unlikely. For some bacteria, alternative binding sites located outside the N-terminal region have been found in studies with pFN; however, they have not yet been located to specific domains (10, 25, 26). Binding of cells to fibronectin involving α5β1-integrins occurs via an R-G-D-S motif in the type III repeat III-10 (36). The III-10 repeat is conserved in both types of fibronectin (19) and is, therefore, unlikely to be critical for YadA binding. In agreement with the hypothesis of a binding independent of the R-G-D-S-containing binding site, a synthetic G-R-G-D-S-P peptide failed to inhibit binding of YadA-positive yersiniae to cFN.

Further experiments are required to localize precisely the binding site of YadA in the splicing segments of fibronectin in order to answer the question whether binding of YadA to cFN may contribute to the pathogenesis of yersiniae. The binding of YadA to cFN might be involved in the initial adhesion of the bacteria to the basal membranes of the host tissue. Moreover, since the cFN used in our study was purified from cartilage, the interaction could also contribute to the arthritogenic potential of enteropathogenic yersiniae.

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