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 fibronectin, 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.
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 NFO 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::Tn5, the virulence plasmid of *Y. enterocolitica* O:9 with an inactivated YadA gene (strain NFpCB9::Tn5); and (iv) pRK290B-4, a hybrid plasmid consisting of the vector pRK290B and BamHI fragment 4 of the virulence plasmid of *Y. enterocolitica* O:9 encoding YadA (1) (strain NFpRK290B-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 NFO, NFpRK290B, and NFpCB9::Tn5) 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.

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 anticolonagen 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-ßl-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. Extractions and purification experiments 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 anticolonagen 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
Y. enterocolitica binding to cellular fibronectin

**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 anticalcigen antibody B1, used to detect collagen contaminations. Enzyme-linked immunosorbent assays (ELISA) with the B1 antibody revealed collagen contamination of less than 2.5 µg 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::Tn5) 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.
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).

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-
Binding of glass coated with cFN (●), pFN (○), or BSA (□) to YadA-positive yersiniae (NFPBK290B9-4). 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.

FIG. 5. Binding of glass coated with cFN (●), pFN (○), or BSA (□) to YadA-positive yersiniae (NFPBK290B9-4). 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 facilitations (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 αβ1 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 segments therefore might confer specific binding functions to cFN. However, despite 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 αβ1-integrins occurs via an R-G-D-S motif in the type III repeat 111-10 (36). The 111-10 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 to 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.

ACKNOWLEDGMENTS

We thank Eva Bauer for expert technical assistance and Klaus von der Mark for critical reading of the manuscript.

H. S.-K. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (Schu 786/1-1). This work was partly supported by the Deutsche Forschungsgemeinschaft, SFB 263, project C3. The Max-Planck-Arbeitsgruppen in Erlangen are funded by the German Ministry for Research and Technology (BMBF, grant 01VM87020).

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