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The Major Fimbrial Subunit of *Bordetella pertussis* Binds to Sulfated Sugars

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Bordetella pertussis **fimbriae are composed of major and minor subunits, and recently it was shown that the minor fimbrial subunit binds to Vla-5, a receptor located on monocytes (W. Hazenbos, C. Geuijen, B. van den Berg, F. Mooi, and R. van Furth, J. Infect. Dis. 171:924-929, 1995). Here we present evidence that the major subunits bind to sulfated sugars, which are ubiquitous in the respiratory tract. Binding was observed to chondroitin sulfate, heparan sulfate, and dextran sulfate but not to dextran. Removal of the minor subunit from fimbriae did not significantly affect binding to sulfated sugars, indicating that the major subunit alone is sufficient for this binding. Fimbriae were also able to bind HEp-2 cells, which are known to display glycoconjugates on their surface. This binding was not dependent on the presence of the minor subunit. However, binding was dependent on the sulfation state of the glycoconjugates, since inhibition of the sulfation resulted in a significant reduction of fimbria binding. The specificity of fimbria binding was further characterized by using heparan sulfate-derived disaccharides in inhibition assays. Two disaccharides were highly effective inhibitors, and it was observed that both the degree of sulfation and the arrangement of the sulfate groups on the disaccharides were important for binding to fimbriae.** ***B. pertussis*** **bacteria also bound to sulfated sugars and HEp-2 cells, and analysis of** ***B. pertussis*** **mutants indicated that both filamentous hemagglutinin and fimbriae were required for this binding. A host protein present in the extracellular matrix, fibronectin, has binding activities similar to those of** ***B. pertussis*** **fimbriae, binding to both Vla-5 and sulfated sugars. Two regions in the major fimbrial subunit were identified which showed similarity with fibronectin peptides which bind to sulfated sugars. Thus,** ***B. pertussis*** **fimbriae exemplify molecular mimicry and may co-opt host processes by mimicking natural ligand-receptor interactions.**

To initiate infection, *Bordetella pertussis* must adhere to host tissues of the upper respiratory tract, and three adhesins have been implicated in this process: the filamentous hemagglutinin (FHA), pertactin, and fimbriae. FHA is a multifunctional adhesin with binding sites for integrin CR3, lactosylceramides, and sulfated sugars (6, 28, 29, 40, 45). Three binding domains have been mapped to distinct regions of the FHA molecule, which play different roles in the pathogenesis of pertussis. The interaction of FHA with integrin CR3 is important for invasion of macrophages, in which *B. pertussis* can survive for extended periods (39). FHA binding to lactosylceramides confers the ability to bind to ciliary cells (6). Sulfated sugars are ubiquitous in mammalian tissues, and FHA attachment to these receptors allows *B. pertussis* to colonize many regions of the respiratory tract (29). Indeed, studies in animal models have shown that FHA mutants are hampered in their ability to colonize the respiratory tract, confirming the important role of this adhesin in the pathogenesis of pertussis (19, 31).

Pertactin is a membrane-associated protein and is synthesized as a precursor with a molecular mass of 93 kDa (8). Pertactin contains an RGD sequence, which is involved in adherence (22, 41). *B. pertussis* invasion of HeLa cells can be inhibited by purified pertactin, suggesting that this adhesin can act as an invasin, like FHA (22). The exact role of pertactin in pathogenesis of pertussis, however, remains to be elucidated, since a *B. pertussis* pertactin mutant colonizes the trachea and lungs of mice just as well as the wild-type *B. pertussis* strain does (41).

B. pertussis produces two closely related but antigenically distinct fimbriae, designated serotype 2 and 3 fimbriae, which are composed of subunits with molecular sizes of 22.5 and 22 kDa, respectively (2, 16, 30, 55). *B. pertussis* strains show fimbrial phase variation, a process characterized by a random switch between high- and low-level expression of a particular *fim* gene, resulting in a serotype switch (51). The serotype 2 and 3 fimbriae carry a single minor subunit species, designated FimD, with a molecular size of 40 kDa (48). Fimbriae are important in pertussis immunity, since trials with whole pertussis vaccines have revealed that the agglutinogen response in mice and children correlates with protection (20, 38). Later, it was shown that agglutinating antibodies are directed primarily against fimbriae (3). Animal studies have also provided evidence that *B. pertussis* fimbriae are protective antigens (42, 55); therefore, fimbriae form part of several experimental acellular pertussis vaccines undergoing field trials (11).

Although the role of fimbriae in immunity has been studied for decades and their genetic organization and regulation have been resolved to great detail (30, 31, 47–50), their function has remained elusive. Early studies by Preston (38) with fimbrial phase variants provided evidence that fimbriae are important for persistent infection. When natural isolates devoid of fimbriae were used to inoculate rabbits or marmosets, the isolates acquired the ability to produce either type 2 or type 3 fimbriae, suggesting that in vivo there is a strong selection for fimbriated strains. Studies with strains which contained defined mutations

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or reference
B. pertussis strains		
BP536	Wild type, Fim2$^+$, Fim3$^-$	40
BP347	*bvgS*::Tn*5*	46
B52	*fim2*::*Sac*I *fim3*::*kan*	32
B172	*fimB*::*kan*	48
BPGR4	*fhaB* deletion mutant, Fim2$^+$, Fim3$^-$	25
E. coli strains		
TB1	*ara*δ(*lac-proAB*) *rpsL* (ϕ80 *lacZ*δM15) *hsdR*	New England Biolabs
BL21	*ompT* (r_B^- m_B^-) *lon*	44
Plasmids		
pMAL-cRI	*lacI*P_{tac} *malE*δ2-26-fx *lacZ*, Amp	New England Biolabs
pRIP642	pMAL-cRI containing an *Eco*RI-*Hin*dIII fragment within the *fimD* gene	This study

in fimbrial genes revealed that fimbrial mutants were less well able to colonize the upper respiratory tract of mice and rabbits than were wild-type strains (31, 32, 47). Although these studies showed that fimbriae are important for infection, they revealed little about the host cells or receptors involved. Recently, it was shown that the minor fimbrial subunit binds to the integrin Vla-5 (15), thereby facilitating the uptake of *B. pertussis* by monocytes. Until now, no function has been described for the major subunit. Here, we report that the major fimbrial subunit binds to sulfated sugars. The fine specificity of binding was studied by inhibition experiments with disaccharides derived from heparan sulfate.

MATERIALS AND METHODS

Reagents. Chondroitin sulfate A (from bovine trachea), heparan sulfate (from bovine kidney), glucuronic acid, *N*-acetylglucosamine (GlcNac), heparin disaccharides (I-A, II-A, III-A, IV-A, I-S, II-S, III-S, and IV-S), dextran (molecular weight, 526,000), and fibronectin (from bovine plasma) were purchased from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin (BSA) was obtained from Organon Teknika, Boxtel, The Netherlands; dextran sulfate (molecular weight, 500,000) was obtained from Pharmacia, Uppsala, Sweden; and fetuin was obtained from Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany. A preparation containing both serotype 2 and 3 fimbriae was kindly provided by A. Robinson and M. Matheson, Public Health Laboratory Service, Porton Down, United Kingdom. These fimbriae were purified from the Wellcome 28 strain by mechanical shearing as described by Robinson et al. (42). Serotype 2 fimbriae were purified from strain BP536 by the method of Cowell et al. (10). K88 fimbriae were kindly donated by H. van den Bosch, Intervet International B.V., Boxmeer, The Netherlands.

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *B. pertussis* strains were grown at 35°C for 3 days on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) supplemented with 15% defibrinated sheep blood without antibiotics. Subsequently, the cells were scraped off, suspended in phosphate-buffered saline (PBS), washed, and suspended in PBS containing 1% heat-treated BSA. Heat treatment of BSA consisted of incubating at 56°C for 60 min and filtering the solution through a 0.22-μm-pore-size nitrocellulose filter to remove insoluble particles (35). For adhesion studies with HEp-2 cells, *B. pertussis* strains were grown until mid-log phase in Verwey medium at 35°C.

Purification of MBP-FimD. FimD was produced in excess with the pMAL-cRI vector. Plasmid pRIP642 was derived from pMAL-cRI and contains a gene fusion between *fimD*, from which the signal sequence-encoding region was removed, and *malE*, which encodes the maltose-binding protein (MBP). The fusion protein (MBP-FimD) and MalE (encoded by pMAL-cRI) were affinity purified on amylose resin as recommended by the supplier (New England Biolabs, Beverly, Mass.). Briefly, *Escherichia coli* TB1 harboring plasmid pMAL-cRI and *E. coli* BL21 harboring plasmid pRIP642 were grown at 37°C in 500 ml of Terrific Broth medium supplemented with 200 μg of ampicillin per ml to an optical density at 600 nm of 1.0. Production of the recombinant proteins was then induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was grown for an additional 3 h at 30°C. The cells were harvested by centrifugation at 13,000 × *g* for 20 min and resuspended in 20 mM NaCl–1 mM EDTA–20 mM Tris-HCl (pH 7.4). To break the cells, they were frozen (−20°C), thawed, and subsequently sonicated four times for 30 s each (Branson sonifier; 50% output). NaCl was added to 200 mM, and the insoluble fraction was removed by centrifugation at 26,000 × *g* for 30 min. The soluble fraction was loaded on a 5-ml cross-linked amylose column (2-cm inner diameter) previously equilibrated with 50 ml of PBS. The column was washed with 60 ml of 200 mM NaCl–1 mM EDTA–20 mM Tris-HCl (pH 7.4). The bound proteins were eluted with 7.0 ml of 200 mM NaCl–1 mM EDTA–20 mM Tris-HCl (pH 7.4)–10 mM maltose. Between 7 and 10 mg of each protein was purified from 500 ml of cultured cells. The purified proteins were stored at −20°C until used.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (21) with a 13% polyacrylamide gel. After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (Serva).

Immunoblot analyses. Separated proteins were electroblotted onto a nitrocellulose membrane (BA 83; Schleicher & Schuell, Dassel, Germany). The blots were blocked for 1 h with PTBE (0.5% fat-free milk powder, 0.1% BSA, plus 0.1% Tween 20 in PBS) and were subsequently incubated with a 1:500 dilution of an anti-FimD monoclonal antibody (11a) in PTBE for 1 h. The blots were washed with PBS containing 0.1% Tween 20, and bound antibodies were detected with a 1:500 dilution of alkaline phosphatase rabbit anti-mouse immunoglobulin G (heavy plus light chains) (DAKO A/S, Glostrup, Denmark) in PTBE buffer for 1 h. The blots were developed in a solution of nitroblue tetrazolium chloride plus 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in dimethyl formamide.

Adherence of bacteria or proteins to immobilized sulfated sugars. Sulfated sugars, dissolved in PBS, were absorbed onto plastic 96-well plates (E.I.A./R.I.A. plates; Costar, Cambridge, Mass.) by incubation overnight at room temperature. After eight washes with tap water, the wells were blocked for 1 h at room temperature with 200 μl of PBS containing 1% heat-treated BSA (see above). The wells were washed with tap water, serial dilutions of a bacterial suspension or a protein solution were added to the wells, and the plates were incubated overnight at room temperature. Nonadherent bacteria or proteins were removed by eight washes with tap water. Subsequently, 100 μl of a dilution of antibodies to the antigen assayed (see below) was added to each well, and the plates were incubated for 60 min at 37°C. The wells were washed eight times with tap water and incubated with 100 μl of goat anti-mouse (or rabbit) peroxidase conjugate for 60 min at 37°C. Subsequently, the plates were washed and incubated with a peroxidase substrate (0.4 mM 3,3,5,5′-tetramethylbenzidine [Sigma] with 0.009% H_2O_2 in 110 mM sodium acetate buffer [pH 5.5]) for 10 min. Finally, the reaction was stopped by adding 50 μl of 3 M H_2SO_4 and optical densities at 450 nm were measured with an automated microplate reader. The fimbrial preparation used in this study was derived from the Wellcome 28 strain and contained both serotype 2 and 3 fimbriae. Binding of fimbriae was assayed with an anti-serotype 3 monoclonal antibody. Essentially identical results were obtained when a preparation containing only serotype 2 fimbriae in combination with an anti-serotype 2 monoclonal antibody was used (results not shown). The following monoclonal antibodies were used (37): anti-lipopolysaccharide (LPS), monoclonal antibody 36G3; anti-serotype 3 fimbriae, monoclonal antibody 72G10; and anti-serotype 2 fimbriae, monoclonal antibody 288B7. Polyclonal antibodies directed against K88 fimbriae were generously provided by H. van den Bosch.

Inhibition of fimbria binding to heparan sulfate. In competition assays, fimbriae were used at 10 μg/ml. Prior to incubation with immobilized heparan sulfate, fimbriae were incubated for 30 min at room temperature with different concentrations of the inhibitor. The amount of fimbriae bound to heparan sulfate was determined as described above. These experiments were performed with the preparation containing both serotype 2 and 3 fimbriae in combination with an anti-serotype 3 monoclonal antibody. Essentially identical results were obtained when a preparation containing only serotype 2 fimbriae in combination with an anti-serotype 2 monoclonal antibody was used (results not shown).

Adherence of proteins to epithelial cells. HEp-2 cells (ATCC CCL23) were grown at 37°C, in a humidified atmosphere containing 5% CO_2 and 95% air, in RPMI 1640 (Gibco-BRL Life Technologies) medium supplemented with 1% (vol/vol) heat-inactivated fetal calf serum (Hyclone, Logan, Utah), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Gibco-BRL). The cells were harvested by trypsinization, resuspended in RPMI 1640 medium–1% fetal calf serum, plated at 5×10^4 cells per well in 96-well tissue culture plates, and cultured until 90% confluent (~2×10^5 cells per well). Subsequently, the supernatant was removed and the cells were fixed with 200 μl of ethanol-acetic acid (95%/5%) per well at −70°C (12) and stored at −70°C until use. For the adherence assay, the fixation fluid was removed and the wells were air dried. After being washed with PBS, the wells were blocked for 60 min with 150 μl of block buffer (PBS, 10% fetal calf serum, 0.5% nonfat milk powder, 0.01% Tween 20). The wells were washed eight times with PBS and subsequently incubated with serial dilutions of protein. After overnight incubation at room temperature, the unbound proteins were removed by eight washes with PBS. Detection of bound protein was determined as described above.

Adherence of bacteria to HEp-2 cells. Bacteria were harvested from overnight, mid-log-phase cultures, washed twice with PBS, and suspended in PBS supplemented with 1% BSA (PBS-BSA). A suspension of 10^7 bacteria per ml was incubated with 10^7 HEp-2 cells per ml in PBS-BSA at 37°C under rotation (4

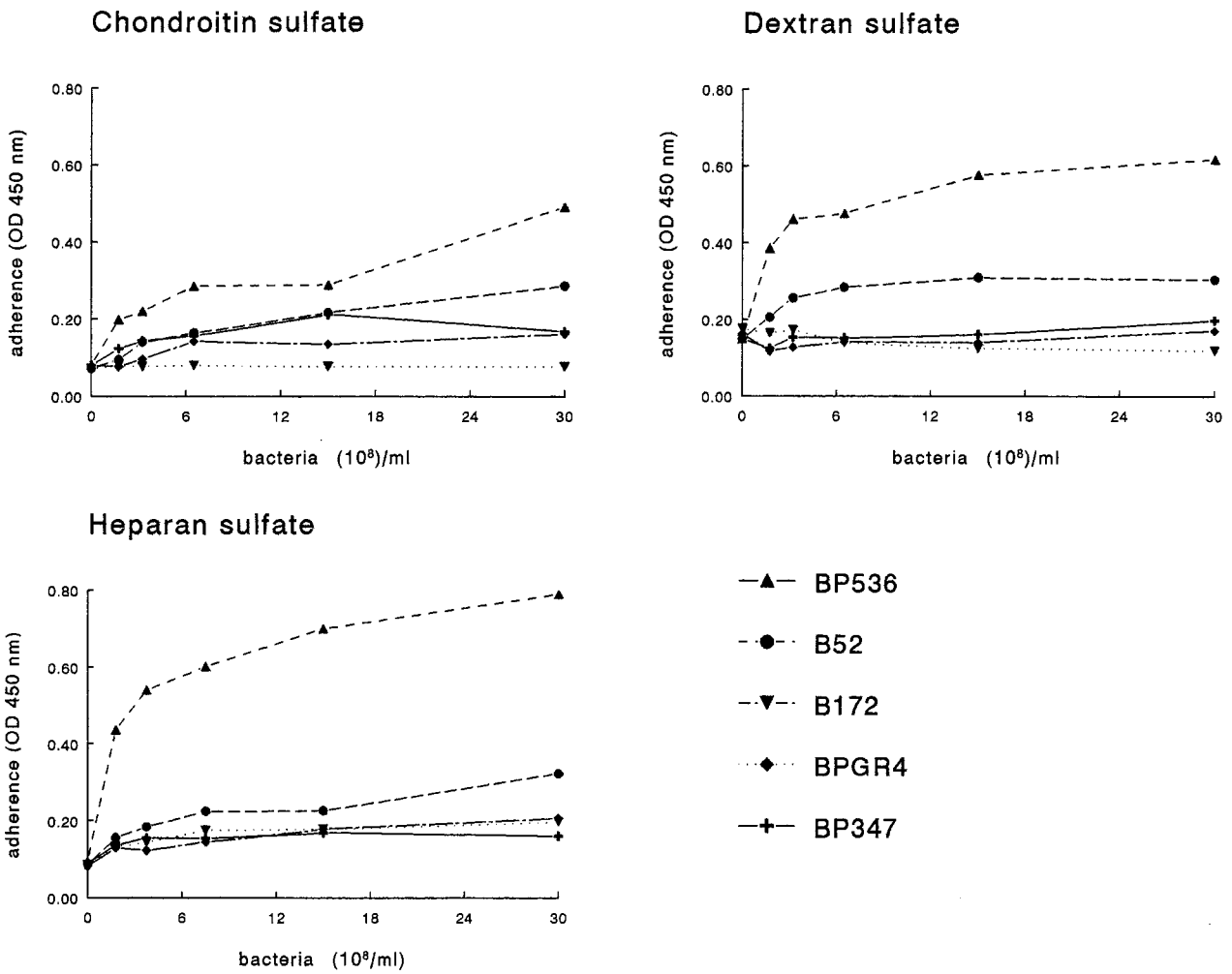

FIG. 1. Adherence of *B. pertussis* cells to immobilized sulfated sugars. Bacteria were allowed to adhere to immobilized heparan sulfate, chondroitin sulfate, and dextran sulfate. Unbound bacteria were removed by washing, and adherent bacteria were detected with a monoclonal anti-LPS antibody. The assay was carried out at least three times, and a representative result is shown. Strains: BP536, wild type; BP347, *bvg* mutant; B52, *fim2 fim3* mutant; B172, *fimB* mutant; BPGR4, *fhaB* mutant.

rpm). After 90 min, adherent bacteria were separated from nonadherent bacteria by differential centrifugation for 4 min at 110 × *g*. The supernatant, containing the nonadherent bacteria, was plated in serial dilutions onto Bordet-Gengou agar plates. Three days later the number of CFU was determined. The experiments were carried out three times.

Chlorate treatment of HEp-2 cells. To obtain undersulfation of proteoglycans, HEp-2 cells were grown in the presence of chlorate. The 96-well tissue culture plates (Gibco) were seeded with 12,000 HEp-2 cells per well, and the cells were grown in 1% Joklik modified minimal essential medium (Gibco-BRL). Twelve hours later, the monolayers were treated with 10 mM sodium chlorate (Merck, Darmstadt, Germany) for 48 h in Joklik modified minimal essential medium supplemented with 1% fetal calf serum. No growth or morphological differences were observed between the chlorate-treated cells and control cells as determined by light microscopy.

FHA and fimbria production by *B. pertussis* strains. The amount of FHA and fimbriae produced was determined by a whole-cell enzyme-linked immunosorbent assay (ELISA) essentially as described by Willems et al. (49). Briefly, microtiter plates were coated with 5×10^7 bacteria suspended in 0.1 M sodium carbonate buffer (pH 9.6), and the water was evaporated overnight. The plates were washed with running tap water and blocked for 1 h at 37°C with PBS–1% BSA–0.05% Tween 20. FHA and fimbrial antigens were detected by adding serial dilutions of the monoclonal antibodies 118E10, 31E2, and 36G3, raised against serotype 2, FHA, and LPS, respectively (37). Subsequently, the plates were washed and incubated with a sheep anti-mouse peroxidase conjugate. The amount of bound monoclonal antibodies was determined as described above. Incubation with LPS monoclonal antibody was performed to correct for differences in the coating efficiency of the different *B. pertussis* mutants. No significant differences were found between coating efficiencies among *B. pertussis* strains.

Statistical methods. Statistical significance was determined by the *t* test, using STATA software (Computing Resource Center, Santa Monica, Calif.).

RESULTS

Binding of *B. pertussis* cells to sulfated sugars. Five *B. pertussis* strains were tested for binding to sulfated sugars: a wild-type strain (BP536) and four mutant derivatives which contain mutations in the *bvg* (or *vir*) locus (BP347), the *fhaB* gene (BPGR4), and fimbrial genes (B52 and B172) (Table 1). The *bvg* locus plays a central role in the regulation of virulence factors in *B. pertussis*, and the *bvg* mutant does not produce any known adhesins. In strain B52, both the *fim2* and *fim3* genes were inactivated by allelic exchange; however, this strain is still able to express the minor fimbrial subunit, FimD, at its cell surface (unpublished results). Strain B172 contains a mutation in the chaperone gene and is devoid of both major and minor fimbrial subunits.

Binding to sulfated sugars was assayed by incubation of bacteria with immobilized chondroitin sulfate, dextran sulfate, or heparan sulfate. The amount of attached bacteria was determined with a monoclonal antibody directed against *B. pertussis* LPS. The wild-type strain was able to bind to all three

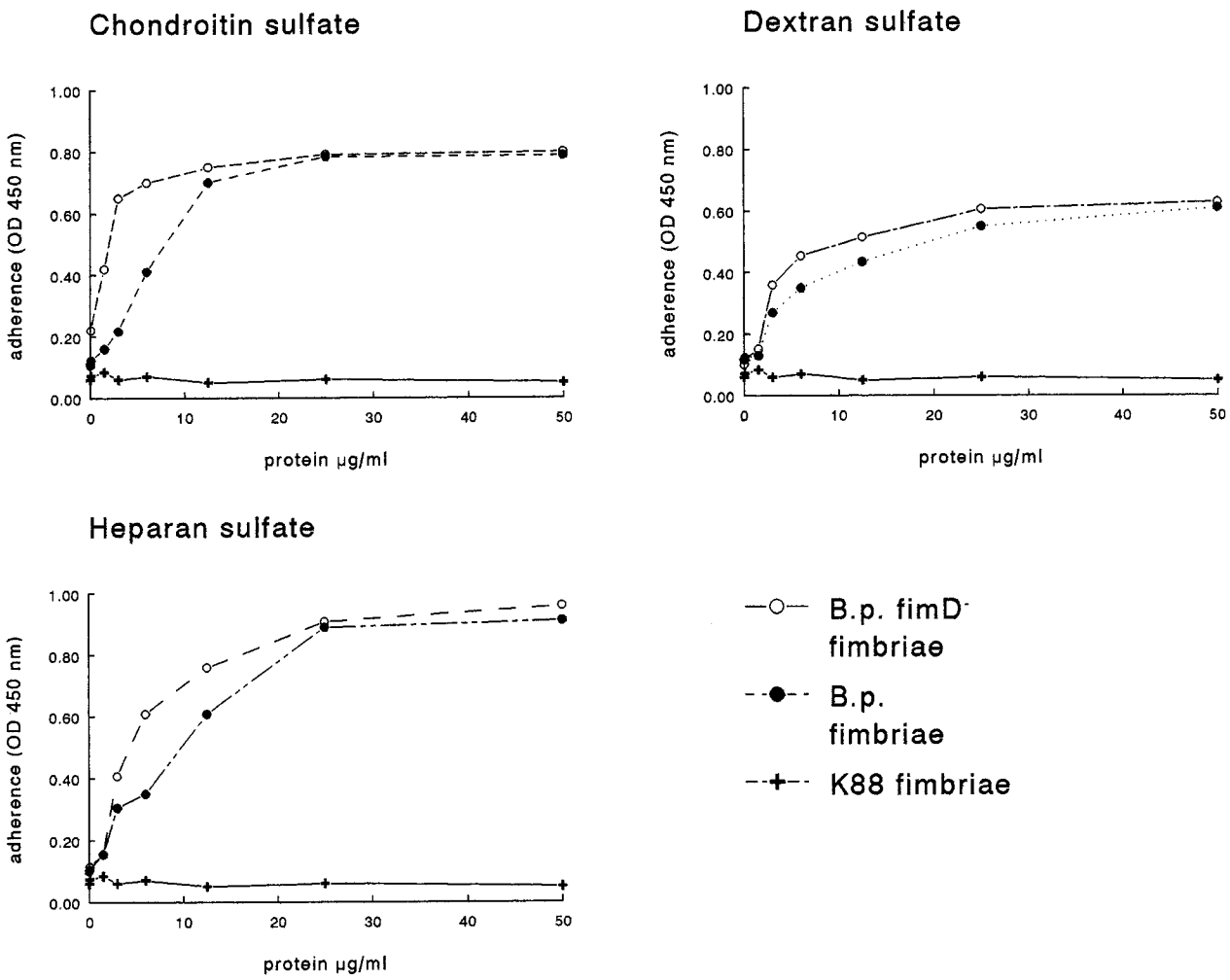

FIG. 2. Binding of fimbriae to sulfated sugars. Fimbriae were incubated with immobilized heparan sulfate, chondroitin sulfate, or dextran sulfate. Unbound proteins were removed by washing, and adherent proteins were detected with antibodies. The assay was carried out at least three times, and a representative result is shown. Abbreviation: B.p., *B. pertussis*.

sulfated sugars (Fig. 1). Binding to heparan sulfate was most pronounced. The wild-type strain did not bind to dextran (data not shown), indicating that sulfate groups were important in binding to dextran sulfate. The *bvg* mutant bound substantially less to sulfated sugars than did the wild-type strain. Further, the low level of binding of the *bvg* mutant was independent of the cell concentration used. The *fha* (BPGR4) and *fim* (B172) mutants showed approximately the same level of binding as the *bvg* mutant. The B52 *fim* mutant occupied an intermediate position between the wild-type strain and the *bvg* mutant; binding levels were consistently higher than those of the *bvg* mutant but did not reach wild-type levels. The higher level of binding of strain B52 than of strain B172 could be due to the presence of FimD at the cell surface of strain B52, suggesting that the minor subunit binds to sulfated sugars. The difference observed between the two *fim* mutants could not be attributed to different levels of FHA produced by these strains, since the two strains produced equal amounts of this protein (data not shown).

Binding of *B. pertussis* fimbriae to sulfated sugars. The binding experiments with whole cells indicated that production of both FHA and fimbriae is required for adherence of *B. pertussis* to sulfated sugars, suggesting that both molecules attach to sulfated sugars. Indeed, Menozzi et al. (28, 29) have shown that FHA is able to bind heparan sulfate, and the binding site was mapped to the N terminus of the FHA molecule (13). However, the role of fimbriae in adherence to sulfated sugars was not studied. To determine whether fimbriae also were able to bind sulfated sugars, they were incubated with immobilized heparan sulfate, chondroitin sulfate, or dextran sulfate, and the amount of bound fimbriae was determined with monoclonal antibodies. It was found that *B. pertussis* fimbriae bound to sulfated sugars, and saturation of binding was observed within the concentration range used (Fig. 2). Further, *B. pertussis* fimbriae did not bind to dextran (results not shown), indicating that sulfate groups were important for binding. Differences were observed between whole cells and fimbriae in relative binding efficiencies. In contrast to whole cells, no large difference in binding of fimbriae to chondroitin sulfate and heparan sulfate was observed. These differences may be attributed to the fact that binding of whole cells is determined not only by fimbriae, but also by FHA, and possibly other adhesins. No binding to sulfated sugars was observed when K88 fimbriae, which are known to recognize mucus glycoproteins, were used (52). The experiments performed with wild-type and mutant strains suggested that the minor subunit FimD may bind to

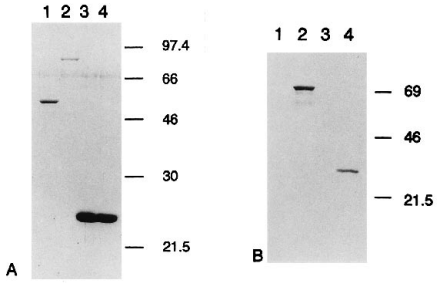

FIG. 3. Analysis of fimbriae devoid of the minor fimbrial subunit FimD. Protein samples were subjected to SDS-PAGE and stained with Coomassie brilliant blue (A) or blotted onto nitrocellulose and incubated with a monoclonal antibody directed against FimD (B). Coomassie brilliant blue staining is not sensitive enough to detect the small amount of FimD present in the fimbrial preparation analyzed in lane 4 of panel A. Lanes: 1, MBP; 2, MBP-FimD; 3, fimbriae after removal of FimD by SDS treatment; 4, fimbriae before SDS treatment. Numbers on the right refer to the sizes of molecular mass markers (in kilodaltons).

sulfated sugars; to determine the extent to which FimD contributed to the binding of fimbriae to sulfated sugars, fimbriae devoid of FimD were used in the binding assay. FimD was detached from fimbriae by mild treatment with SDS, and subsequently FimD and the fimbrial rods were separated by ultracentrifugation. This procedure leaves FimD in the supernatant, while fimbrial rods are found in the pellet. The resuspended fimbriae were analyzed for the presence of FimD by using Western blotting (immunoblotting) and a monoclonal antibody specific for FimD. No FimD was detected in the fimbria preparation (Fig. 3). When FimD$^-$ fimbriae were tested for their ability to bind to sulfated sugars, it appeared that they adhered slightly better than to native fimbriae (Fig. 2). The increased binding of the FimD$^-$ fimbriae may be due to disaggregation or fragmentation of the fimbrial rods as a result of the detergent treatment. These results show that the major subunits are sufficient for binding of fimbriae to sulfated sugars but do not exclude a role for FimD.

To further investigate the role of FimD in the adhesion of *B. pertussis* to sulfated sugars, FimD was overproduced as a purified MBP fusion protein (Fig. 3) and the ability of the fusion protein to bind to sulfated sugars was determined. No significant binding of the MBP-FimD protein to sulfated sugars was observed (data not shown). However, we cannot exclude the possibility that the absence of binding is because the FimD receptor binding domain does not have its natural conformation in the fusion protein.

Inhibitors of fimbria-heparan sulfate binding. Of the three sulfated sugars tested, fimbriae showed the highest degree of binding to heparan sulfate, and the specificity of this interaction was characterized by determining the ability of various compounds to inhibit binding. Fimbriae (10 μg/ml) were preincubated with a fixed concentration of the tested compound (0.04 mg/ml) and subsequently added to immobilized heparan sulfate. Inhibition was expressed as the percent reduction in binding observed relative to untreated controls. To increase the sensitivity of the assay, the fimbria concentration was chosen so that saturation of binding to heparan sulfate was not yet reached (Fig. 2).

Constituents of heparan sulfate were tested in the inhibition assay. Heparan sulfate consists of glucuronic acid linked to sulfated or acetylated D-glucosamine (24, 46a, 54). The monosaccharide building blocks *N*-acetylglucosamine and glucuronic acid were ineffective inhibitors, showing no inhibition at a concentration of 0.04 mg/ml (Table 2). At a higher concentration (50 mg/ml) the monosaccharides did reveal inhibitory activity, reducing binding of fimbriae to heparan sulfate by 34 and 44%, respectively (not shown).

Heparan sulfate can be completely degraded to six unsaturated disaccharides (I-S to IV-S, II-A, and IV-A) with heparinase II (27, 33) (Fig. 4), and the ability of these disaccharides and two additional ones (I-A and III-A [Fig. 4]) to inhibit the binding of fimbriae to heparan sulfate was investigated. Significant inhibition (24 and 16%) was found with only two disaccharides (I-S and II-S, respectively) (Table 2). At the same concentration (0.04 mg/ml), heparan sulfate showed an inhibitory effect of 36%, indicating that the disaccharides were highly effective inhibitors. I-S showed a larger inhibitory activity than did II-S; it contains more sulfate groups, suggesting that more highly sulfated disaccharides have a higher affinity for fimbriae. However, not only the degree of sulfation but also the position of the sulfate groups is important for binding to fimbriae. Disaccharides which showed the same number of sulfate groups but in different positions (i.e., II-S, III-S, and I-A) showed different inhibitory activities. Furthermore, the difference in inhibitory activity of I-S and I-A ($P < 0.0001$) indicated that N sulfation of the disaccharides is especially important for binding to fimbriae; replacement of the *N*-sulfate group by an *N*-acetyl group completely abrogates the inhibitory activity. Sulfation of C-6 of glucosamine is also important, since its removal leads to a significant reduction in inhibitory activity from 24 to 6% ($P = 0.004$) (compare I-S with III-S). In contrast, sulfation of the C-2 of glucuronic acid results in a small, insignificant ($P = 0.17$) increase in inhibitory activity from 16 to 24%, indicating that sulfation of this site is less important for binding to fimbriae (compare I-S with II-S).

Adherence to HEp-2 cells. Sulfated sugars are present as glycosaminoglycans on the surface of mammalian cells. We investigated whether fimbriae were able to bind HEp-2 cells, a cell line derived from human larynx epithelium. Fimbriae devoid of FimD adhered slightly better than did native fimbriae (Fig. 5A), indicating that the major subunit is sufficient for the observed binding. No binding of the MBP-FimD fusion protein was observed.

To determine if sulfate groups were involved in the binding of fimbriae to HEp-2 cells, the HEp-2 cells were treated with chlorate, a potent inhibitor of ATP-sulfurylase (5, 18). The

TABLE 2. Inhibition of fimbria binding to immobilized heparan sulfate by saccharides[a]

Disaccharide[b]	Mean inhibition[c] ± SD	P[d]	% Inhibition[e]
Control	0.75 ± 0.06		
N-Acetylglucosamine	0.75 ± 0.09	NS	0
Glucuronic acid	0.75 ± 0.11	NS	0
I-A	0.79 ± 0.11	NS	0
II-A	0.80 ± 0.14	NS	0
III-A	0.75 ± 0.18	NS	0
IV-A	0.73 ± 0.16	NS	3
I-S	0.57 ± 0.10	0.0003	24
II-S	0.63 ± 0.12	0.0010	16
III-S	0.71 ± 0.18	NS	6
IV-S	0.72 ± 0.18	NS	4
Heparan sulfate	0.48 ± 0.12	<0.0001	36

[a] Fimbriae (10 μg/ml) were preincubated with disaccharides (0.04 mg/ml) or heparan sulfate (0.04 mg/ml) for 30 min at room temperature. Subsequently, the suspension was added to immobilized heparan sulfate, and binding of fimbriae was assayed as described in Materials and Methods. The control consisted of fimbriae without added saccharides.
[b] See Fig. 4 for formulas.
[c] Mean ELISA reading of 20 independent experiments.
[d] P value relative to the control. NS, not significant ($P > 0.05$).
[e] Percent inhibition relative to the control.

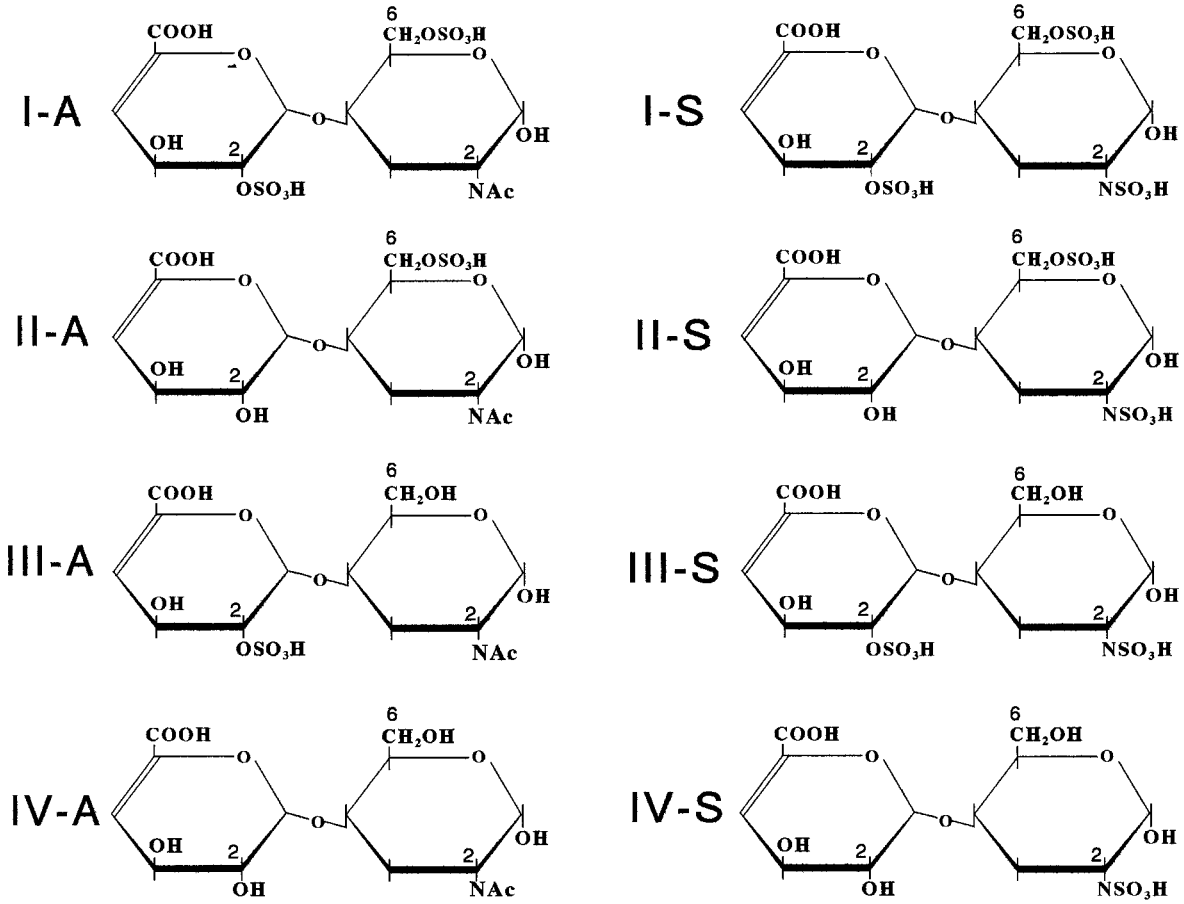

FIG. 4. Structures of heparan sulfate-derived disaccharides used in the inhibition assays.

chlorate concentration used inhibits sulfation of glycosaminoglycans without affecting cell growth (5, 18). Treatment of HEp-2 cells with chlorate decreased the binding 20% relative to that of controls (Fig. 5B). These results show that the adherence of fimbriae to HEp-2 cells is at least partially dependent on the degree of sulfation of the glycosaminoglycans.

When binding of wild-type and mutant *B. pertussis* bacteria to HEp-2 cells, immobilized to microtiter plates, was determined, we observed that the wild-type strain bound well, whereas the *bvg* mutant showed a very low level of binding, which was independent of the concentration of bacteria used (results not shown). Further, the *fha* and *fim* mutants showed the same level of binding as the *bvg* mutant. The latter finding was unexpected since Roberts et al. (41) found that *fha* mutants adhered significantly better to HEp-2 cells than *bvg* mutants. We surmised that the washings used in our assay invoked shear forces which only allowed the detection of strong adherence, such as expressed by the wild-type strain, and not the weaker adherence expected in the mutants. Therefore, we modified the binding assay, so as to minimize shear forces. Essentially, HEp-2 cells were incubated with bacteria, and attached bacteria were spun down by low-speed centrifugation. The amount of nonadherent bacteria in the supernatant was determined by plating. In this assay, the *fha* and *fim* mutants clearly adhered better than the *bvg* strain (Table 3), but less well than the wild-type strain. Of the mutant strains tested, strain B52 ($Fm2^-$ $Fim3^-$ $FimD^+$) showed the highest degree of binding, followed by strain B172 ($Fim2^-$ $Fim3^-$ $FimD^-$) and strain BPGR4 (Fh_a^-). Thus, in this assay, both FHA and fimbriae contributed to adherence to Hep-2 cells.

DISCUSSION

Sulfated sugars are ubiquitous in mammalian tissues and are found in the extracellular matrix, in mucus, and on the surface of epithelial cells (46a). Therefore, it is not surprising that microbes have taken advantage of these sugars to colonize host tissues. Examples include bacteria (*Borrelia burgdorferi* [17], *Helicobacter pylori* [1], and *Staphylococcus aureus* [23]), viruses (herpes simplex virus [53], cytomegalovirus [9], and bovine herpesvirus [34]), and protozoa (trypanosomes [35] and malaria sporozoites [36]). *B. pertussis* also binds to sulfated sugars (28, 29), and the FHA molecule has been implicated in this binding process. FHA binds specifically to the epithelial cell surface molecule heparan sulfate and to the closely related molecule heparin through its N-terminal domain (13). From these observations, it was suggested that FHA was the major adhesin involved in binding of *B. pertussis* to sulfated sugars on host tissues (29). Here we refine this conclusion and show that *B. pertussis* fimbriae also bind to sulfated sugars.

Purified fimbriae were able to bind to heparan sulfate, chondroitin sulfate, and dextran sulfate (Fig. 2). Binding to dextran was not observed, underlining the importance of sulfate groups. *B. pertussis* cells were also able to bind to immobilized sulfated sugars. Further, mutations in either *fha* or *fim* genes decreased the binding of *B. pertussis* bacteria to heparan sul-

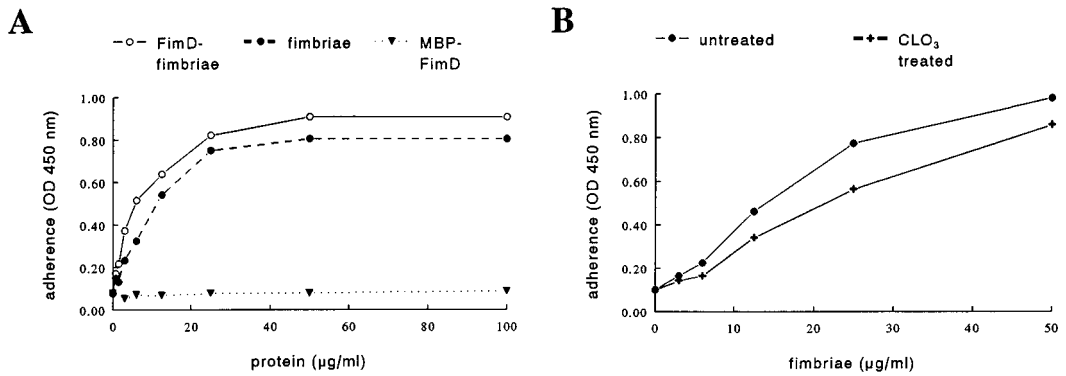

FIG. 5. (A) Adherence of fimbriae (●), fimbriae devoid of FimD (FimD⁻ fimbriae) (○), and an MBP-FimD fusion protein to HEp-2 cells (▼). (B) Effect of chlorate treatment on the binding of fimbriae. The assay was carried out at least three times, and a representative result is shown. Symbols in panel B: +, chlorate treated; ●, untreated.

fate to levels shown by *bvg* mutants (Fig. 1). The finding that strains lacking FHA, or fimbriae, showed the same level of binding as *bvg* mutants, which are devoid of both adhesins, was unexpected. Possibly, the presence of both adhesins is required for stable binding of bacteria to sulfated sugars in our microtiter assay due to the strong shear forces generated during the (washing) procedure. Fimbriae are able to remain bound in this assay, presumably because they are less affected by shear forces than bacteria due to their smaller size.

Binding of fimbriae was also investigated in the less artificial context of cell surfaces. When fimbriae were incubated with HEp-2 cells, binding of fimbriae was observed (Fig. 5). Adherence of fimbriae was dependent on the sulfation state of the cell surface polysaccharides, since pretreatment of HEp-2 cells with chlorate, a potent inhibitor of carbohydrate sulfation, resulted in a significant decrease of binding. Chlorate treatment did not completely block the fimbrial adherence and the observed residual binding could be due to the fact that the inhibition of sulfation was incomplete, or to the presence of other fimbrial receptors on HEp-2 cells. *B. pertussis* bacteria also bound to HEp-2 cells, and both fimbriae and FHA contributed to this binding (Table 3).

B. pertussis fimbriae are comprised of a major and minor subunit (30). The minor subunit, designated FimD, is able to bind to the integrin Vla-5, which is located on monocytes (15). The presence of major subunits in the fimbrial rods was sufficient for binding to Hep-2 cells and immobilized heparan sulfate, since fimbriae without FimD were still able to bind (Fig. 2). However, a mutant strain which expressed FimD but not the major subunits at its cell surface showed higher levels of adherence to heparan sulfate than did a mutant which was devoid of both minor and major subunits. This suggests that FimD may also have affinity for heparan sulfate. Although an MBP-FimD fusion protein was unable to bind to heparan sulfate, this lack of binding may have been due to an unnatural conformation of the receptor-binding domain in the fusion protein. Therefore, the exact role of FimD in the adhesion of fimbriae to sulfated sugars remains to be elucidated.

The binding of fimbriae to heparan sulfate was further characterized by inhibition studies with mono- and disaccharides derived from heparan sulfate were not very effective inhibitors; however, two disaccharides, I-S and II-S (Fig. 4), were found to be very potent inhibitors, being nearly as effective as heparan sulfate (Table 2). The studies with disaccharides indicated that both the degree of sulfation and the arrangement of the sulfate groups on the disaccharides determine the affinity of the interaction with fimbriae. Sulfation of C-2 and C-6 of glucosamine was particularly important for binding to fimbriae. In contrast, sulfation of C-2 of glucuronic acid was less important.

Our work shows that *B. pertussis* fimbriae are able to bind to sulfated sugars and Vla-5 through the major and minor subunit, respectively. It is interesting that these binding activities are also found in fibronectin, a host protein found in the extracellular matrix. Fibronectin is able to inhibit binding of fimbriae to sulfated sugars (results not shown). Proteins which bind to heparan sulfate contain a consensus sequence based upon regions of high positive charge density (7), and two such regions are observed in the major fimbrial subunits (Fig. 6). These regions are very similar in their distribution of negatively and positively charged amino acid residues and also show some sequence identity. Further, the two Fim regions show similarity with two regions from fibronectin, which have been implicated in binding to heparin (Fig. 6) (4). Thus *B. pertussis* fimbriae may mimic natural host ligand-receptor interactions, as has been suggested for FHA (42a).

One may ask why *B. pertussis* expresses two distinct adhesins, FHA and fimbriae, both of which recognize sulfated sugars. Sulfated sugars like heparan sulfate can occur in many different compositions, which show different tissue distributions (24, 46a, 54), and it is possible that FHA and fimbriae recognize distinct sulfated sugars in vivo, thereby conferring binding to different host cells or tissues. In fact, the inhibition experiments with disaccharides show that fimbriae have different affinities for different configurations of heparan sulfate. The ability to express several adhesins which recognize different receptors may play a role in tissue tropism. Since sulfated

TABLE 3. Binding of *B. pertussis* strains to HEp-2 cells[a]

Strain	% Binding	SD	% Binding of wild type
B536 (wild type)	89	8	
B347 (*bvg*)	18	6	20
B52 (*fim2,3*)	78	6	88
B172 (*fimB*)	59	9	65
BPGR4 (*fhaB*)	45	13	50

[a] HEp-2 cells were incubated with *B. pertussis*, and subsequently the number of bound bacteria was determined. Percentage of binding was determined as follows: [(total CFU added − CFU remaining in supernatant)/total CFU added] × 100.

FIG. 6. Regions of Fim2 and fibronectin which show sequence similarity. Two regions of the Fim2 major subunit FM2N (N-terminal region) and FM2C (C-terminal region) are compared with two regions of the fibronectin protein known to be involved in adhesion to heparan sulfate. Dashes have been introduced to increase the number of matches; similarities in sequence have been indicated by shading. Amino acids were numbered according to the published sequences (4, 24a).

sugars are ubiquitous, low-affinity and reversible binding to these receptors may be important for the invading pathogen in the initial stage of infection. High-affinity, stable binding may occur only at sites where the optimal receptors for both fimbriae and FHA are colocalized. Thus, by using several adhesins with distinct binding specificities, bacteria may experience an affinity gradient which facilitates tropism to particular niches. Therefore, it is conceivable that FHA and fimbriae act synergistically during infection. Cooperation between FHA and fimbriae is also suggested by the work of Hazenbos et al. (15), who have shown that binding of fimbriae to monocytes results in activation of CR3, the monocyte receptor of FHA. The cooperation between FHA and fimbriae is reflected in their genetic organization; although most *B. pertussis* virulence genes are dispersed in the chromosome (43), the *fha* and *fim* genes are clustered. In fact, the *fha* and *fim* genes are not only linked but also intermingled; one *fha* gene is located within the *fim* operon and FHA production is coupled to fimbrial expression (30, 49).

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