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Surface-Associated Filamentous Hemagglutinin Induces Autoagglutination of *Bordetella pertussis*

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Filamentous hemagglutinin (FHA) is a major adhesin produced by *Bordetella pertussis*, the etiologic agent of whooping cough. FHA has been shown to be surface associated but is also secreted by virulent bacteria. Microscopic observations of lungs of mice infected with *B. pertussis* showed that the bacteria grow as clusters within the alveolar lumen. When *B. pertussis* was cultivated in vitro with chemically defined medium, bacteria grew as aggregates, mimicking growth observed in vivo. This aggregation was abolished by the addition of cyclodextrin (CDX) to the growth medium and depended on the production of FHA, because a mutant lacking the FHA structural gene failed to form aggregates in a CDX-free medium. Western blot (immunoblot) analyses revealed that, in the absence of CDX, FHA was attached to the bacterial surface and was not efficiently released into the growth medium. Hydrophobic chromatography of FHA showed that CDX drastically reduced the hydrophobicity of FHA, suggesting a direct binding of CDX to FHA, which was further supported by the partial protection of FHA from trypsin digestion in the presence of CDX. In addition, free FHA can interact in a CDX-inhibitable manner with solid phase-immobilized FHA. It can therefore be postulated that the *B. pertussis* aggregates are most likely due to direct FHA-FHA interaction.

Bordetella pertussis, the etiologic agent of whooping cough, produces several virulence-associated adhesion factors such as filamentous hemagglutinin (FHA), fimbriae, and pertactin and also different toxins such as pertussis toxin (PTX), cyclolysin (adenylate cyclase toxin), and dermonecrotic toxin, which largely contribute to the pulmonary infectious process leading to the development of the illness (18, 46). PTX and FHA are known to be important protective antigens in pertussis vaccines and are now included in several new acellular vaccine candidates (1, 16, 35).

FHA is a 220-kDa surface-associated protein and plays a major role in bacterial attachment to the epithelium of the upper respiratory tract (16, 34, 42). So far, three different binding activities have been described for FHA (for a recent review, see reference 19). FHA possesses an arginine-glycine-aspartate (RGD) sequence which mediates its binding to pulmonary macrophage integrins (33), it displays a lectinlike activity for galactose-containing molecules (41), and it is also able to bind to sulfated polysaccharides (25, 26).

When grown in vitro with chemically defined medium, virulent *B. pertussis* appears as a slow-growing bacterium. This slow growth was attributed to the presence in the nutritional medium of growth inhibitors such as peptone, peroxide, or fatty acids (31, 32) and could be overcome by the addition of heptakis (2,6-*O*-dimethyl)β-cyclodextrin (CDX) (13). This effect was explained by the capacity of CDX to neutralize the inhibitory effects of fatty acids such as oleic or palmitic acid (13). Imaizumi and coworkers also observed that CDX addition to the culture medium stimulated both PTX (14) and FHA (12) production in the supernatant of shake cultures. In addition, these authors demonstrated that PTX and FHA produced in the presence of CDX were immunochemically and biologically identical to those produced in CDX-free culture. Furthermore, CDX addition to growth medium facilitates the isolation of *B. pertussis* from clinical specimens significantly (47).

By growing *B. pertussis* in CDX-free liquid medium, we observed a pronounced agglutination of the cells that became macroscopically visible in less than 24 h after the inoculation of shake cultures. This phenomenon did not occur with an FHA-deficient strain and appeared to be partially reversible after growth to mid-log phase by the addition of CDX to CDX-free medium. In addition, we provide evidence indicating that, in the absence of CDX, FHA remains mainly associated with the bacterial outer surface. We also show that purified FHA molecules can directly interact with each other and therefore postulate that surface-exposed FHA is responsible for cell agglutination, probably via hydrophobic interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Streptomycin-resistant (Sm^r) *B. pertussis* BPSM (26), *B. pertussis* BPRA lacking the PTX structural gene (2), *B. pertussis* BPGR4 lacking the FHA structural gene (20), *B. bronchiseptica* BB1015 (24), and a clinical isolate of *B. parapertussis* (29) were grown on Bordet-Gengou agar (4) supplemented with 20% defibrinated sheep blood (BG agar) and containing 100 μg of streptomycin (Sigma) per ml. Liquid cultures were performed as described previously (24), using Stainer-Scholte (SS) medium (37). CDX (Teijin Ltd., Tokyo, Japan) was used in SS medium at concentrations ranging from 0 to 1 mg/ml (13) as indicated. When indicated, 5 mM nicotinic acid (Sigma) was added to the culture medium to induce phenotypic modulation (22).

Immunochemical detection of *B. pertussis* in the lungs of infected mice. Six-week-old female outbred mice (OF1; Institut Pasteur) were first anesthetized with pentobarbital and then received intranasally an inoculum of *B. pertussis* BPSM con-

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taining approximately 5×10^6 bacteria in a volume of 50 μl of saline. After 1 or 7 days, mice were sacrificed and lungs were harvested. Lungs were fixed in a 4% (vol/vol) solution of paraformaldehyde (Sigma) and then dehydrated by incubation in increasing concentrations of absolute ethanol (Merck) (70, 90, 95, and 100%). After 24 h of immersion in 1-butanol (Merck), lungs were embedded in paraffin for 5 h at 56°C. Sections were prepared and treated with absolute methanol containing hydrogen peroxide (3%) to inhibit endogeneous peroxidase. Then, samples were incubated with a rat polyclonal serum raised against purified *B. pertussis* FHA (34a). After being washed and treated with anti-rat immunoglobulins labelled with peroxidase, samples were incubated with diamine benzyline (Dako) as a substrate for staining.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (17) with a 4% stacking gel and a 10% separating gel. Prior to electrophoresis, samples were mixed with one-third volume of solubilization buffer (6% SDS, 15% β-mercaptoethanol, 30% glycerol, 0.005% bromophenol blue in 0.18 M Tris-HCl, pH 6.8) and heated at 95°C for 5 min. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Merck).

Immunoblot analysis. Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose sheets (BA 85; Schleicher & Schuell) was performed according to the method of Towbin et al. (39). Immobilized FHA and PTX were then probed with murine monoclonal antibodies as described previously (7, 9) and developed with alkaline phosphatase-linked goat anti-mouse immunoglobulin G (ProtoBlot System; Promega, Madison, Wis.).

FHA purification. FHA used for covalent coupling to Sepharose beads was purified from culture supernatants containing 1 g of CDX per liter, whereas free FHA used in the FHA-FHA interaction assay was purified from supernatants containing 0.2 g of CDX per liter. All supernatants were obtained from shaken liquid cultures incubated at 37°C until the optical density at 600 nm (OD_{600}) reached 5.5. FHA was purified on heparin-Sepharose CL-6B (Pharmacia) according to the procedure previously described (25). Briefly, 500 ml of *B. pertussis* BPRA culture supernatant was chromatographed at room temperature on 1 g of swollen gel equilibrated with sulfate-free phosphate-buffered saline (PBS; pH 7.4) and packed in a 1-cm-diameter glass column. FHA was eluted with a linear 0 to 500 mM NaCl gradient prepared in 50 ml of PBS and finally dialyzed overnight at 4°C against 20 volumes of PBS. All chromatographic steps were performed at a flow rate of 2 ml/min.

Covalent coupling of FHA to Sepharose. Purified FHA was covalently immobilized to CNBr-activated Sepharose 4B (Pharmacia) according to the recommendations of the manufacturer. Briefly, 6 ml of swollen CNBr-activated Sepharose was first sequentially washed with 20 ml of 1 mM HCl and 200 ml of bidistilled water and then incubated overnight at 4°C under gentle agitation with 50 ml of FHA at 100 μg/ml. The remaining active groups were finally blocked by incubating the gel for 3 h more at room temperature in 100 ml of 1 M Tris-HCl (pH 8.0) containing 0.2 M glycine. After being washed with 100 ml of PBS, the gel was stored at 4°C in PBS plus 20% ethanol until further use. The FHA coupling efficiency was greater than 98%. A control Sepharose matrix was concomitantly prepared according to the procedure described above, except that FHA was replaced by glycine. The glycine-Sepharose matrix was used to quantify the nonspecific binding of FHA to Sepharose beads.

Phenyl-Sepharose chromatography. Two hundred milliliters of *B. pertussis* BPRA culture supernatant containing 1 mg of CDX per ml was analyzed by hydrophobic interaction chromatography, using 10 ml of phenyl-Sepharose 6 Fast Flow (Pharmacia) packed in a 1-cm-diameter glass column and subsequently equilibrated with 100 ml of PBS plus 4 M NaCl. Prior to chromatography at room temperature, NaCl was added to the culture supernatant to a final concentration of 4 M. The sample was then passed through the column at a flow rate of 1 ml/min, and after extensive washing with PBS plus 4 M NaCl, the gel was eluted with PBS, PBS plus 1 mg of CDX per ml, and finally PBS plus 6 M urea. Eluted material was collected and analyzed by SDS-PAGE and immunoblotting after overnight dialysis against PBS for fractions containing 4 M NaCl or 6 M urea.

Hemagglutination assay. The hemagglutination activity of the *B. pertussis* culture supernatants was assayed using a 3% (vol/vol) fresh rabbit erythrocyte suspension prepared in PBS, as described previously (25).

Trypsin digestion of FHA. Purified FHA was dialyzed overnight at 4°C against PBS and then diluted with the same buffer to obtain a final protein concentration of 100 μg/ml. Five hundred microliters of this FHA preparation was digested at 37°C in a final volume of 1 ml, using 2 μl of a trypsin solution (1 mg/ml; Sigma) in PBS. The digestions were also performed in the presence of CDX or heparin at a concentration of 1 or 4 mg/ml, as indicated.

FHA-FHA interaction assay. The FHA-FHA interaction assay was performed in 2.2-ml Eppendorf tubes containing 200 μl of swollen matrix (FHA-Sepharose, glycine-Sepharose, or heparin-Sepharose) suspended in 700 μl of PBS. CDX from a fresh stock solution at 20 mg/ml in PBS was then added at final concentrations ranging from 0 to 1 mg/ml. The tubes were carefully mixed and incubated for 30 min at room temperature. One milliliter of FHA solution at 160 μg/ml was then added to the tubes, and the tubes were incubated under gentle agitation for 30 min at room temperature. The beads were then sedimented by gravity, and the supernatant was collected for SDS-PAGE analysis and protein assay to quantify the remaining free FHA.

Protein assay. Protein concentrations were determined by the method of Bradford (5), using bovine serum albumin (Sigma) as a standard.

RESULTS

***B. pertussis* grows as aggregates within the lungs of experimentally infected mice.** As shown in Fig. 1A, 24 h after intranasal inoculation of *B. pertussis* BPSM, bacteria were found in the alveolar lumen of the lung. We have previously found that the BPSM derivative is as virulent as the Tohama I parent strain (unpublished observations). The bacterial cells were only loosely attached to the host tissues but were tightly clustered in aggregates. Seven days after inoculation, the lungs contained approximately 10-fold more bacteria (data not shown). They were found intimately attached to the alveolar epithelium and remained clustered (Fig. 1B), suggesting that within the infected host, *B. pertussis* surface-exposed components mediate cell-to-cell interactions during the formation of bacterial microcolonies at the site of infection. We cannot rule out that some bacteria detach from the microcolonies, although they would have been detected as dark brown dots by the staining technique used.

In vitro autoagglutination of *B. pertussis*. *B. pertussis* BPSM was grown under agitation in 200 ml of SS medium in the absence or in the presence of CDX at a final concentration of 1 mg/ml. After 24 h of growth at 37°C, corresponding to an

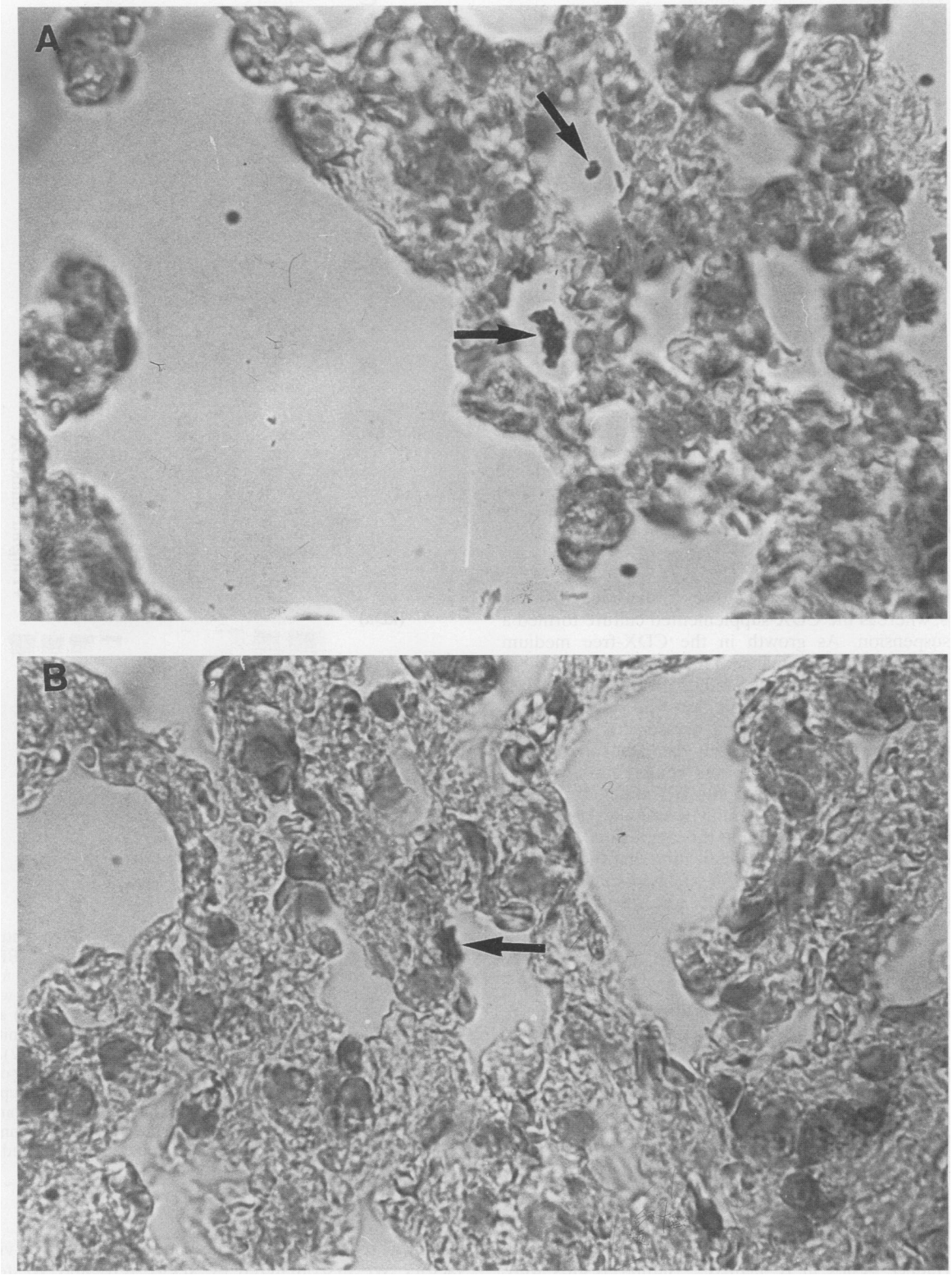

FIG. 1. *B. pertussis* aggregates in the lungs of infected mice. *B. pertussis* BPSM was immunoperoxidase stained with anti-FHA antibodies in mouse lung tissues 24 h (A) and 7 days (B) after nasal inoculation of the bacteria grown on BG agar prior to inoculation. The bacterial aggregates are indicated by the arrows.

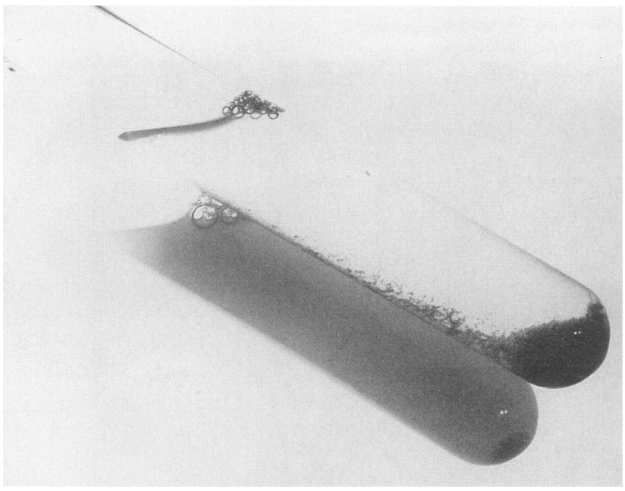

FIG. 2. Autoagglutination of *B. pertussis* grown in CDX-free culture medium. *B. pertussis* BPSM was grown under agitation in 200 ml of SS medium in the absence of CDX or in the presence of 1 mg of CDX per ml. After 24 h of growth at 37°C, 10 ml of each culture was transferred into 20-ml glass tubes which were kept in static conditions for 15 min at room temperature. The upper tube, corresponding to the culture performed in the absence of CDX, shows sedimented bacterial aggregates, whereas the lower tube, containing bacteria grown in the presence of CDX, shows a uniform bacterial suspension.

OD_{600} of 0.8, the CDX-free culture formed macroscopic aggregates, whereas the CDX-supplemented culture formed a uniform suspension. As growth in the CDX-free medium continued and the density reached values above 1.0 OD_{600} unit, the aggregates became larger and finally stuck to the glass of the culture flask. When the agitation was stopped, these aggregates sedimented rapidly. This was clearly seen after transfer of 10 ml of culture into 20-ml glass test tubes, which were then kept for 15 min at room temperature without agitation (Fig. 2). When streaked onto BG plates, the aggregates formed small, brilliant, hemolytic colonies typical of virulent *B. pertussis*, showing that the aggregated material contained living bacteria and thus indicating that the flocculation was not caused by aggregation of lysed material. The colonies grown in the presence of CDX were the same size as those grown in the absence of CDX. This observation suggests that the presence of CDX in the culture medium prevents cellular agglutination.

To determine if addition of CDX to a culture initiated in the absence of CDX could reverse this clotting behavior, 1 mg of CDX per ml was added 30 h after inoculation. Twelve hours after the addition of CDX, the aggregates disappeared almost completely. This reversible effect of CDX on cell clotting was less pronounced when CDX was added 40 h after culture inoculation in the absence of CDX.

When CDX-free *B. pertussis* culture was performed in the presence of 5 mM nicotinic acid, the cell clotting was no longer observed, indicating that flocculation is modulated by nicotinic acid and thereby suggesting that it is most likely under the control of the central *vir-bvg* virulence regulatory locus (23). This locus is responsible for the genetic transactivation of all known virulence genes in *B. pertussis*. Similar results were obtained with 25 mM magnesium sulfate as the modulating agent.

CDX stimulates specific release of FHA into the culture medium. Since the *B. pertussis* agglutination appeared to be controlled by *vir-bvg*, we wanted to know whether this phenomenon was related to any of the known *bvg*-regulated virulence factors. We first analyzed by SDS-PAGE and Western blot (immunoblot) for the presence of two major virulence factors, FHA and PTX, in whole-cell lysates (cell associated) and in the culture supernatants (secreted) from stationary-phase shake cultures grown in the absence or presence of increasing CDX concentrations, up to 1 mg/ml (Fig. 3). Total PTX production

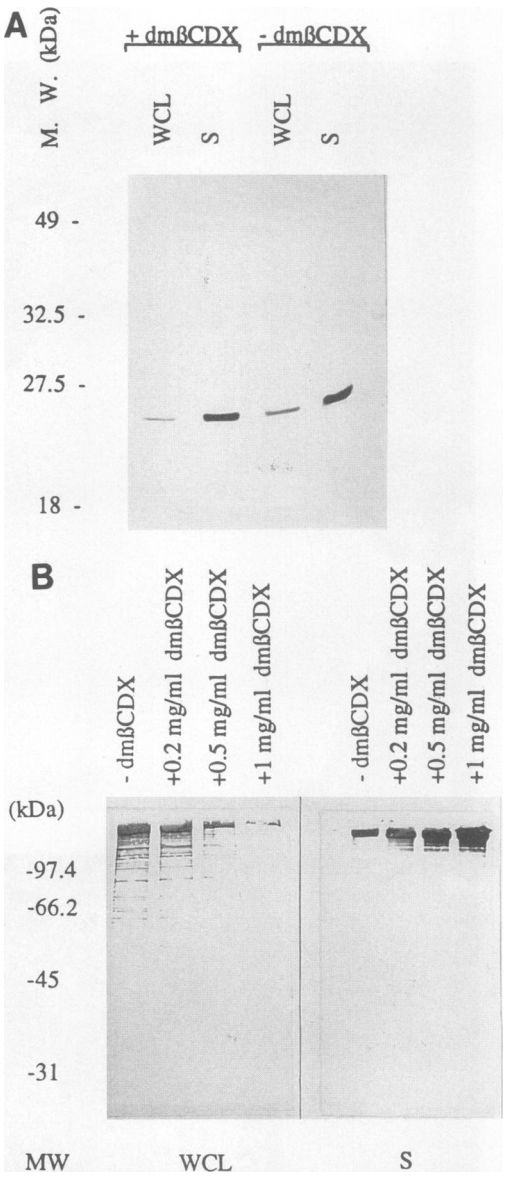

FIG. 3. Western blot analysis of *B. pertussis* BPSM cultures performed in the presence or absence of CDX. *B. pertussis* BPSM was grown in the absence (−dmβCDX) or in the presence of up to 1 mg of CDX per ml (+dmβCDX). Culture supernatants (S) and whole-cell lysates (WCL) were analyzed by immunoblotting with (A) anti-PTX monoclonal antibody 11B10, which reacts with the S2 subunit of PTX (9), or with (B) anti-FHA monoclonal antibody 12.2B11 (7). In both panels A and B, lanes corresponding to WCL contain 100 μg of protein and lanes corresponding to S were loaded with volumes of supernatant standardized on the basis of the culture OD_{600}. In order to standardize, special care had to be taken to measure the OD_{600} values immediately from shake cultures, before the microorganisms flocculated.

as well as its distribution into the cell-associated and secreted forms did not appear to be drastically modified when CDX was added to the culture medium. This is somewhat different from earlier reports in which the addition of CDX was found to increase PTX production (14). At present, we have no definite explanation for this difference, but it could be due to different initial culture conditions, e.g., treatment of *B. pertussis* prior to inoculation in SS medium and/or size of the inoculum. We noted occasionally a significant CDX-mediated PTX increase when the size of the inoculum was decreased, but this was due to the lower amounts of PTX detected in the CDX-free medium when the size of the inoculum was lowered.

In contrast to the effect on PTX, Western blot examination revealed that the distribution of FHA was dramatically affected by the presence of CDX, whereas total FHA production was not significantly modified. Reduction of CDX concentration in the culture medium lowered the amount of FHA detectable in the culture supernatant. Concomitantly, the amount of cell-associated FHA increased drastically, suggesting that CDX does not modify FHA gene expression but facilitates FHA release into the culture medium. Densitometric quantitation of the Western blot shown in Fig. 3 indicated that the addition of CDX resulted in a more than fourfold increase in extracellular FHA and, concomitantly, a more than sixfold decrease in cell-associated FHA. SDS-PAGE analyses of whole-cell lysates and supernatants from *B. pertussis* grown in either culture condition revealed no other significant modification of protein production or distribution, suggesting that FHA is the main *B. pertussis* component significantly influenced by CDX. From this observation, we postulated that the surface-exposed FHA accumulation may be the cause of cell agglutination observed in CDX-free liquid cultures.

FHA accumulation on the cell surface is required for *B. pertussis* agglutination. To investigate the specific role played by FHA in the cell aggregation observed when the bacteria were grown in the absence of CDX, *B. pertussis* BPSM-derived mutants BPGR4, lacking the FHA structural gene but producing normal amounts of fimbriae (20), and BPRA, lacking the PTX operon (2), were cultivated in CDX-free or CDX-supplemented SS medium. In CDX-free medium, BPRA formed aggregates identical to those observed with the parental BPSM strain, whereas in the presence of CDX, this strain formed homogeneous suspensions. In contrast, BPGR4 failed to form aggregates even in the absence of CDX, confirming the primary role of FHA in this phenomenon, although we cannot completely rule out that other factors are involved in aggregation. When *B. pertussis* was grown on BG agar and then suspended in PBS, strain BPGR4 was much easier to mix into a homogeneous suspension than strain BPSM, suggesting that clumping also occurs on BG agar.

CDX partially protects FHA from trypsin digestion. At the molecular level, the CDX-induced release of FHA into the culture medium could be due to a direct interaction between CDX and FHA. In order to detect direct CDX-FHA interaction, purified FHA was digested with trypsin in the presence or absence of CDX or heparin, a sulfated polysaccharide known to bind directly to FHA (25). As shown in Fig. 4, the 220-kDa form of FHA, which bears a trypsin-sensitive cleavage site (8), is rapidly (less than 30 s) cleaved into major fragments with molecular weights ranging from ca. 70,000 to 125,000. The presence of heparin in the digestion solution at a concentration of 1 or 4 mg/ml (Fig. 4, lanes D and E, respectively) did not modify the FHA cleavage as revealed by SDS-PAGE analysis. The digestion patterns obtained in up to 3 h of trypsin digestion were identical for the control reaction (FHA-trypsin, lane A) and the heparin-containing samples. These data

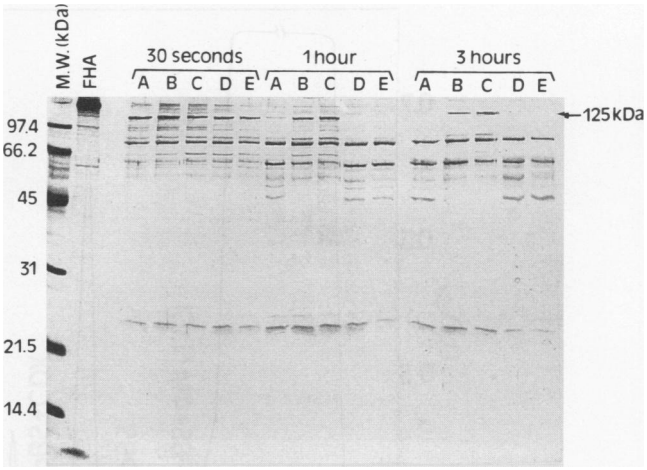

FIG. 4. SDS-PAGE analysis of purified *B. pertussis* FHA digested with trypsin in the presence or in the absence of CDX. Purified FHA was digested for 30 s, 1 h, or 3 h with trypsin in the absence (lanes A) or in the presence of 1 or 4 mg of CDX (lanes B and C, respectively) or heparin (lanes D and E, respectively) per ml. Undigested FHA is shown in the lane labeled FHA, the M_r markers are shown in the first lane, and the sizes of the M_r markers are indicated in the margin. The arrow points to the 125-kDa FHA polypeptide protected from trypsin digestion by CDX.

indicate that the binding of heparin to FHA did not mask trypsin cleavage sites. In contrast, when CDX was used in this digestion assay at the same concentrations (lanes B and C), the 125-kDa fragment of FHA appeared to be partially protected from further degradation. No significantly better protection was observed when CDX was used at 4 mg/ml (lane C) instead of 1 mg/ml (lane B). To rule out an inhibitory effect of CDX on trypsin activity, we also cleaved bovine serum albumin with trypsin in the presence of CDX at a concentration of 1 or 4 mg/ml, essentially as described for FHA. No significant inhibitory effect was detected (data not shown), suggesting that the partial protection of FHA from trypsin digestion is likely due to a direct interaction between FHA and CDX.

CDX alters the hydrophobic properties of FHA. The ease with which FHA is released into the culture medium suggests that it is presumably loosely attached to the outer membrane of *B. pertussis*. It is therefore possible that hydrophobic domains present in FHA are involved in its association with the outer surface. Hydrophobic interactions of FHA with membrane components have already been documented by Irons and MacLennan (15), who showed that FHA displays affinity for membrane lipids, including cholesterol. CDX is a crown-shaped molecule with a hydrophobic core and a hydrophilic outline (38) and could therefore potentially trap surface-exposed hydrophobic regions of FHA, thereby decreasing its hydrophobicity. CDX has previously been shown to reduce the hydrophobicity of immobilized proteins when added to elution buffers during chromatography (27). We applied *B. pertussis* BPSM culture supernatants onto phenyl-Sepharose at a high ionic strength (4 M NaCl) to favor hydrophobic interactions. The gel was then eluted with PBS, PBS plus 1 mg of CDX per ml, and finally, PBS plus 6 M urea (Fig. 5). Analysis of the flowthrough and the eluted fractions by SDS-PAGE and Western blot, using anti-FHA monoclonal antibodies, revealed that most of the proteins retained in the gel in the presence of 4 M NaCl were eluted with PBS, except FHA, which was eluted when PBS was supplemented with 1 mg of CDX per ml (data

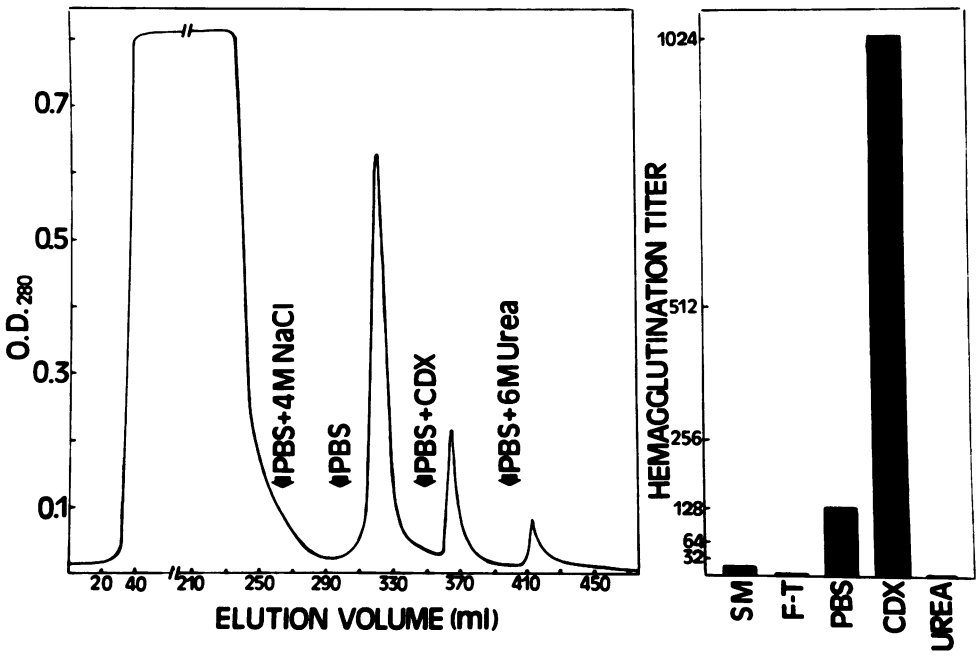

FIG. 5. Hydrophobic interaction chromatography of FHA. Two hundred milliliters of *B. pertussis* BPRA culture supernatant containing 4 M NaCl was applied to 10 ml of phenyl-Sepharose previously equilibrated with PBS plus 4 M NaCl. The flowthrough was collected, and after being washed with PBS plus 4 M NaCl, the gel was eluted stepwise with PBS, PBS plus 1 mg of CDX per ml, and finally, PBS plus 6 M urea. The OD_{280} of the chromatography fractions is shown in the left panel. After dialysis against PBS, the hemagglutination titers (right panel) of the starting material (SM), column flowthrough (F-T), and elution peaks obtained wtih PBS (PBS), PBS plus 1 mg of CDX per ml (CDX), and PBS plus 6 M urea (UREA) were determined with a fresh rabbit erythrocyte suspension. Equal volumes (50 μl) of sample were used, and the concentrations of protein were not adjusted for volume changes. No effect of CDX on hemagglutination was observed.

not shown). No detectable protein eluted with PBS plus 6 M urea, indicating that all FHA had been eluted by PBS plus CDX. This was confirmed by determination of the hemagglutination titers of the flowthrough and the eluted fractions showing that most of the hemagglutination activity was recovered by elution with PBS containing CDX (Fig. 5). These results show that the addition of CDX at a concentration usually used for growth of *B. pertussis* in modified SS medium drastically alters the interaction between FHA and a hydrophobic substrate such as phenyl-Sepharose and therefore suggest that the interaction of CDX with FHA reduces its hydrophobicity.

Immobilized FHA binds soluble FHA. Since surface-associated FHA appeared to mediate *B. pertussis* cell agglutination, we reasoned that surface-exposed FHA molecules may be responsible for bridging the bacterial cells by direct FHA-FHA interactions. To test this hypothesis, we investigated the potential capacity of free FHA to interact with solid-phase immobilized FHA. Therefore, we studied the capability of free FHA molecules to interact directly with FHA molecules covalently bound to Sepharose beads. For this purpose, free FHA (160 μg in aqueous solution) was gently mixed with 200 μl of Sepharose beads bearing 220 μg of immobilized FHA. As controls, similar experiments were performed with glycine-Sepharose or heparin-Sepharose beads instead of FHA-coupled Sepharose. After 30 min of incubation and sedimentation of the beads, the remaining free FHA present in the liquid phase was quantified by protein assay (Fig. 6). In the absence of CDX, 78% of the free FHA cosedimented with the FHA-coupled Sepharose beads, indicating that free FHA molecules can interact with immobilized FHA molecules. Very little FHA bound to glycine-Sepharose and, similar to previous experiments, all FHA bound to heparin-Sepharose. Addition of CDX in the assay reduced the FHA-FHA interaction in a dose-dependent manner, and this interaction reached background levels when CDX was added at a concentration of

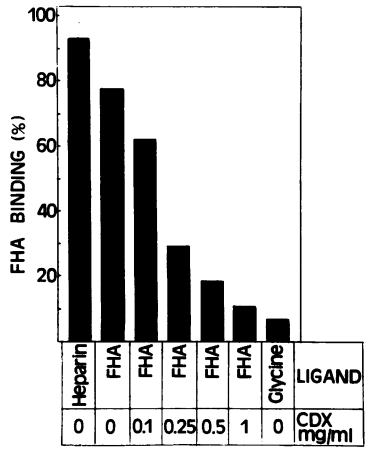

FIG. 6. Influence of CDX on FHA-FHA interaction. One milliliter of FHA solution at 160 μg/ml was added to 200 μl of heparin-Sepharose (heparin), FHA-Sepharose (FHA), or glycine-Sepharose (glycine) in 2.2-ml Eppendorf tubes. CDX was then added at concentrations ranging from 0 to 1 mg/ml, as indicated, and the tubes were incubated for 30 min at room temperature. After sedimentation of the beads, FHA present in the supernatant was quantified by protein assay. The results are expressed as percent FHA not found in the supernatant fraction compared with the amount of FHA in the input sample.

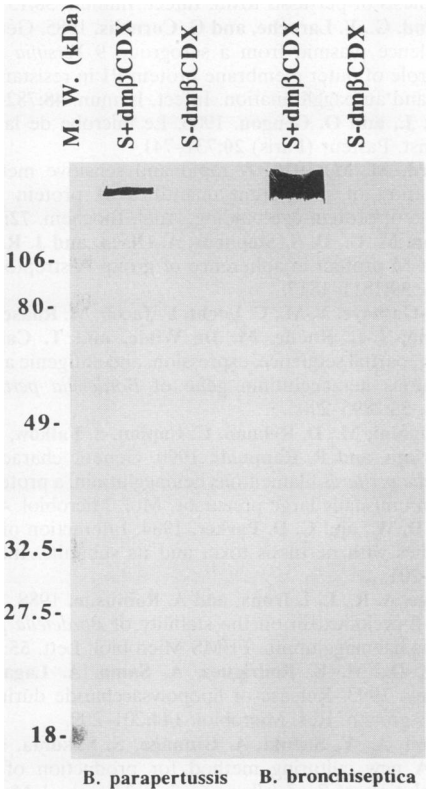

FIG. 7. Western blot analysis of *B. parapertussis* and *B. bronchiseptica* supernatants from cultures performed in SS medium in the absence (S−dmβCDX) or in the presence of 1 mg of CDX per ml (S+dmβCDX). The immunoblot was probed with anti-*B. pertussis* FHA monoclonal antibody 12.1D3 (7). The sizes of the M_r markers are indicated in the margin.

1 mg/ml. CDX has no effect on the FHA-heparin interaction (26).

***B. parapertussis* but not *B. bronchiseptica* aggregates in CDX-free medium.** *B. parapertussis* and *B. bronchiseptica* also produce FHA molecules which are in part immunogenically and functionally related to the *B. pertussis* FHA (19, 26). To investigate the influence of CDX on FHA release and aggregation of these two species, we grew them in CDX-free or CDX-containing SS medium. After 30 h of shake culture in CDX-free medium, *B. parapertussis* cells began to agglutinate, whereas no cellular aggregation was observed in the *B. bronchiseptica* CDX-free cultures, even after prolonged incubation. SDS-PAGE and Western blot analyses with monoclonal antibodies directed against the *B. pertussis* FHA showed that CDX addition to SS medium enhanced FHA release from both species (Fig. 7). FHA was also found in a cell-associated form in both species; however, the relative amounts of this material were difficult to assess because of the presence of high levels of proteolytic cleavage products (not shown).

DISCUSSION

Very soon after the entry of an infectious agent into its specific host, the pathogen has to adhere to target tissues. This adherence is usually mediated by adhesins, which are surface-exposed molecules that interact specifically with host receptors. The rapid adherence of the pathogen to the site of infection that represents an optimal ecological niche for its multiplication will facilitate its survival and subsequent spread in the infected host.

B. pertussis, the etiologic agent of whooping cough, produces FHA as a major adhesin. In liquid cultures of *B. pertussis*, FHA is associated with the outer membrane but is also released from the bacterial surface into the culture medium. Analysis of the lungs of mice infected with *B. pertussis* revealed that the bacteria were clustered in small aggregates and grew as such in the infected tissues. Similarly, *B. pertussis* grown in modified SS medium lacking CDX also formed cell aggregates. This aggregation was not observed when *B. pertussis* was grown in the presence of modulating agents such as nicotinic acid or magnesium sulfate, suggesting that *vir-bvg*-regulated virulence factors were involved in this phenomenon. Using a strain that lacks the FHA structural gene, we found that FHA is involved in cell agglutination. SDS-PAGE and Western blot analyses provided evidence that, in the absence of CDX, FHA accumulates on the surface of *B. pertussis* and is not efficiently released into the culture medium.

Direct interaction between FHA and CDX was documented by the fact that CDX partially protects FHA from trypsin digestion. Direct interaction of CDX with FHA most likely results in alterations of the hydrophobic properties of FHA, since CDX allowed elution of FHA from a phenyl-Sepharose matrix. This observation is in agreement with a previous report by Gorringe et al. (10), who showed that CDX increased the stability of FHA in the supernatant from shaken cultures and that CDX was also able to bind to purified FHA.

CDX molecules are cyclic oligosaccharides consisting of 6, 7, or 8 glycopyranose units, referred to as α-, β-, or γ-CDX (38). These naturally occurring molecules have a rigid doughnut-shaped structure with an internal hydrophobic cavity in which hydrophobic substances can be complexed by displacing water (38). CDX molecules are also highly water soluble due to the presence of many surface-exposed hydroxyl groups. Therefore, they permit the solubilization of many hydrophobic molecules in aqueous solutions. The CDX form usually used as a growth stimulant for *B. pertussis* phase I is β-CDX, which displays an internal cavity diameter of 6.4 Å (0.64 nm). Such a cavity can trap fatty acids such as oleic acid or palmitic acid known to be inhibitors of *B. pertussis* growth (13). FHA is a hydrophobic adhesin (36) loosely attached to the bacterial outer membrane. It can therefore be postulated that hydrophobic regions of FHA involved in the interaction with the outer membrane may also interact with the hydrophobic cavity of CDX. As a consequence, these FHA regions surrounded by CDX molecules may exhibit a drastically reduced hydrophobicity which may alter the topology of FHA in the outer membrane and facilitate its release into the culture medium. This release process can be regarded as a physical extraction of FHA from the bacterial surface, probably facilitated by the agitation of the culture.

CDX not only is able to extract FHA from the bacterial surface but also apparently influences FHA-FHA interaction. That bacterial cell agglutination is probably caused by direct surface-associated FHA-FHA interactions is evidenced by the ability of free FHA to bind to immobilized FHA in the absence of CDX. In the presence of CDX, the bound FHA is eluted, suggesting that CDX abolishes the FHA-FHA interaction. Since the hydrophobic character of FHA can be altered by the addition of CDX, it is tempting to speculate that the FHA-FHA interaction important for the agglutination of the bacterial cells involves hydrophobic domains of FHA.

The direct FHA-FHA interaction described in this study may partially explain the observation made by Tuomanen (40), who showed that pretreatment of mammalian ciliated cells

with purified FHA and PTX enhanced the adherence of virulent *B. pertussis* to the ciliated cells, suggesting that these adhesins can bridge the target cells and the bacteria. Such a mechanism requires the existence of receptors for FHA and/or PTX on both the host cell and the bacterial surfaces. It is possible that surface-associated FHA could play the role of a bacterial receptor for exogenous FHA. During natural infection by *B. pertussis*, FHA may be released from the bacterial surface and attach as free molecules to the neighboring epithelial cells via one or several of its multiple adhesive activities. Such FHA coating of epithelial cells may then favor the spread of *B. pertussis* from the initial infectious focus by FHA-FHA interaction.

B. pertussis infection shares some striking features with group A streptococcal infection. Like *B. pertussis*, group A streptococci produce an adhesin, M protein. M^+ but not M^- streptococci attach as autoaggregates to human tonsillar epithelial cells (6, 45). Because an isogenic pair of M^+ and M^- strains showed no difference in adherence per se (6), it has been suggested that the selective advantage conferred by the presence of M protein (43) may reside in the formation of microcolonies formed in vivo by aggregation. Aggregative adherence of bacteria is not restricted to respiratory pathogens. With other organisms, such as *Staphylococcus aureus*, clumping is induced by a specific factor, the fibrinogen receptor (21). In addition, enteroaggregative strains of *Escherichia coli* adhere to target cells as "stacked brick" aggregates (28). It has been shown that in vivo these organisms attach strongly to each other, as well as to mucosal surfaces, which probably contributes to the persistence of the pathogen (44). The aggregative behavior of these *E. coli* strains is mediated by plasmid-encoded, bundle-forming fimbriae. In the case of *Yersinia enterocolitica*, autoagglutination is mediated, like in *B. pertussis*, by a multifunctional adhesin, named YadA (3).

It is interesting to note that CDX is now widely used as a growth stimulant for both eukaryotic (48) and prokaryotic (30) cells and may have specific and potentially nonphysiological effects on membrane components. A recent study by Hozbor et al. (11) showed that CDX was able to bind to *B. pertussis* cells and induce release of lipopolysaccharide into the growth medium. It is not clear whether the lipopolysaccharide release is related to the release of FHA, but these observations clearly indicate that the addition of CDX to the *B. pertussis* growth medium modifies the molecular organization of the bacterial outer membrane. Therefore, special care should be taken when subcellular locations of antigens are studied with CDX. On the other hand, the addition of CDX to the growth medium of *B. pertussis* represents an interesting method to induce FHA release, which facilitates the industrial-scale purification of this important antigen for vaccine preparation (25).

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REFERENCES

1. **Ad Hoc Group for the Study of Pertussis Vaccines.** 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. Lancet **i**:955–960.
2. **Antoine, R., and C. Locht.** 1990. Roles of the disulfide bond and the carboxy-terminal region of the S1 subunit in the assembly and biosynthesis of pertussis toxin. Infect. Immun. **58**:1518–1526.
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. **Bordet, J., and O. Gengou.** 1906. Le microbe de la coqueluche. Ann. Inst. Pasteur (Paris) **20**:731–741.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Caparon, M. G., D. S. Stephens, A. Olsen, and J. R. Scott.** 1991. Role of M protein in adherence of group A streptococci. Infect. Immun. **59**:1811–1817.
7. **Delisse-Gathoye, A.-M., C. Locht, F. Jacob, M. Raaschou-Nielsen, I. Heron, J.-L. Ruelle, M. De Wilde, and T. Cabezon.** 1990. Cloning, partial sequence, expression, and antigenic analysis of the filamentous hemagglutinin gene of *Bordetella pertussis*. Infect. Immun. **58**:2895–2905.
8. **Domenighini, M., D. Relman, C. Capiau, S. Falkow, A. Prugnola, V. Scarlato, and R. Rappuoli.** 1990. Genetic characterization of *Bordetella pertussis* filamentous hemagglutinin: a protein processed from an unusually large precursor. Mol. Microbiol. **4**:787–800.
9. **Frank, D. W., and C. D. Parker.** 1984. Interaction of monoclonal antibodies with pertussis toxin and its subunits. Infect. Immun. **46**:195–201.
10. **Gorringe, A. R., L. I. Irons, and A. Robinson.** 1988. The effect of methyl-β-cyclodextrin on the stability of *Bordetella pertussis* filamentous haemagglutinin. FEMS Microbiol. Lett. **55**:315–320.
11. **Hozbor, D., M. E. Rodriguez, A. Samo, A. Lagares, and O. Yantorno.** 1993. Release of lipopolysaccharide during *Bordetella pertussis* growth. Res. Microbiol. **144**:201–209.
12. **Imaizumi, A., Y. Suzuki, A. Ginnaga, S. Sakuma, and Y. Sato.** 1984. A new culturing method for production of filamentous hemagglutinin of *Bordetella pertussis*. J. Microbiol. Methods **2**:339–347.
13. **Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato.** 1983. Heptakis(2,6-*O*-dimethyl)β-cyclodextrin: a novel growth stimulant for *Bordetella pertussis* phase I. J. Clin. Microbiol. **17**:781–786.
14. **Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato.** 1983. Effect of heptakis(2,6-*O*-dimethyl)β-cyclodextrin on the production of pertussis toxin by *Bordetella pertussis*. Infect. Immun. **41**:1138–1143.
15. **Irons, L. I., and A. P. MacLennan.** 1979. Substrate specificity and the purification by affinity combination methods of the two *Bordetella pertussis* hemagglutinins, p. 338–349. *In* C. R. Manclark and J. C. Hill (ed.), International Symposium on Pertussis. Department of Health, Education, and Welfare Publication 79–1830. U.S. Government Printing Office, Washington, D.C.
16. **Kimura, A., K. T. Mountzouros, D. A. Relman, S. Falkow, and J. L. Cowell.** 1990. *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect. Immun. **58**:7–16.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Leininger, E., M. Roberts, J. G. Kenimer, I. G. Charles, N. Fairweather, P. Novotny, and M. J. Brennan.** 1991. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. Proc. Natl. Acad. Sci. USA **88**:345–349.
19. **Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld.** 1993. The filamentous haemagglutinin, a multifaceted adhesin produced by virulent *Bordetella* spp. Mol. Microbiol. **9**:653–660.
20. **Locht, C., M.-C. Geoffroy, and G. Renauld.** 1992. Common accessory genes for the *Bordetella pertussis* filamentous hemagglutinin and fimbriae share sequence similarities with *papC* and *papD* gene families. EMBO J. **11**:3175–3183.
21. **McDevitt, D., P. François, P. Vaudaux, and T. J. Foster.** 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. **11**:237–248.
22. **McPheat, W. L., A. C. Wardlaw, and P. Novotny.** 1983. Modulation of *Bordetella pertussis* by nicotinic acid. Infect. Immun. **41**:516–522.
23. **Melton, A. R., and A. A. Weiss.** 1993. Characterization of environ-

mental regulators of *Bordetella pertussis*. Infect. Immun. **61:**807–815.

24. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. Infect. Immun. **59:**3982–3988.
25. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Interaction of the *Bordetella pertussis* filamentous hemagglutinin with heparin. FEMS Microbiol. Lett. **78:**59–64.
26. **Menozzi, F. D., R. Mutombo, G. Renauld, C. Gantiez, J. H. Hannah, E. Leininger, M. J. Brennan, and C. Locht.** 1994. Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. Infect. Immun. **62:**769–778.
27. **Mohseni, R. M., and R. J. Hurtubise.** 1990. Retention characteristics of several compound classes in reversed-phase liquid chromatography with β-cyclodextrin as a mobile phase modifier. J. Chromatogr. **499:**395–410.
28. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. **60:**2297–2304.
29. **Nordmann, P., B. François, F. D. Menozzi, M. C. Commare, and A. Barois.** 1992. Whooping cough associated with *Bordetella parapertussis* in an HIV-infected child. Pediatr. Infect. Dis. J. **11:**248.
30. **Olivieri, R., M. Bugnoli, D. Armellini, S. Bianciardi, R. Rappuoli, P. F. Bayeli, L. Abate, E. Esposito, L. De Gregorio, J. Aziz, C. Basagni, and N. Figura.** 1993. Growth of *Helicobacter pylori* in media containing cyclodextrins. J. Clin. Microbiol. **31:**160–162.
31. **Pollock, M. R.** 1949. The effects of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. Symp. Soc. Exp. Biol. **3:**193–216.
32. **Proom, H.** 1955. The minimal nutritional requirements of the organisms of the genus *Bordetella lopez*. J. Gen. Microbiol. **12:**63–75.
33. **Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright.** 1990. Recognition of a bacterial adhesin by an integrin: macrophage CR3 (αMβ2,CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. Cell **61:**1375–1382.
34. **Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86:**2637–2641.

34a. **Renauld, G., et al.** Unpublished data.

35. **Sato, Y., M. Kimura, and H. Fukumi.** 1984. Development of a pertussis component vaccine in Japan. Lancet **i:**122–126.
36. **Skelton, S. K., and K. H. Wong.** 1990. Simple, efficient purification of filamentous hemagglutinin and pertussis toxin from *Bordetella pertussis* by hydrophobic and affinity interaction. J. Clin. Microbiol. **28:**1062–1065.
37. **Stainer, D. W., and M. J. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
38. **Szejtli, J.** 1990. The cyclodextrins and their applications in biotechnology. Carbohydr. Polymers **12:**375–392.
39. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
40. **Tuomanen, E.** 1986. Piracy of adhesins: attachment of superinfecting pathogens to respiratory cilia by secreted adhesins of *Bordetella pertussis*. Infect. Immun. **54:**905–908.
41. **Tuomanen, E., H. Towbin, G. Rosenfelder, D. Braun, G. Larson, G. Hansson, and R. Hill.** 1988. Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. J. Exp. Med. **168:**267–277.
42. **Tuomanen, E., and A. A. Weiss.** 1985. Characterization of two adhesins of *Bordetella pertussis* for human ciliated respiratory epithelial cells. J. Infect. Dis. **152:**118–125.
43. **Tyewska, S. K., V. A. Fischetti, and R. J. Gibbons.** 1988. Binding selectivity of *Streptococcus pyogenes* and M-protein to epithelial cells differs from that of lipoteichoic acid. Curr. Microbiol. **16:**209–216.
44. **Vial, P. A., R. Robins-Browne, H. Lior, V. Prado, J. B. Kaper, J. P. Nataro, D. Maneval, A. Elsayed, and M. M. Levine.** 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. J. Infect. Dis. **158:**70–79.
45. **Wang, J. R., and M. W. Stinson.** 1994. M protein mediates streptococcal adhesion to HEp-2 cells. Infect. Immun. **62:**442–448.
46. **Weiss, A. A., and E. L. Hewlett.** 1986. Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. **40:**661–686.
47. **Wirsing von Koenig, C. H., A. Tacken, and H. Finger.** 1988. Use of supplemented Stainer-Scholte broth for the isolation of *Bordetella pertussis* from clinical material. J. Clin. Microbiol. **26:**2558–2560.
48. **Yamane, I., M. Kan, Y. Minamoto, and Y. Amatsuji.** 1981. α-Cyclodextrin, a novel substitute for bovine albumin in serum-free culture of mammalian cells. Proc. Jpn. Acad. **57:**385–389.