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Localization and Enumeration of Fimbria-Associated Adhesins of *Bacteroides loescheii*

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Monoclonal antibodies that specifically inhibit coaggregation between *Bacteroides loescheii* PK1295 and its two gram-positive partners *Streptococcus sanguis* 34 and *Actinomyces israelii* PK14 were used to enumerate and localize two distinct types of fimbria-associated adhesins on the surface of *B. loescheii*. Binding studies with radiolabeled monoclonal antibodies indicated that a maximum (B_{max} calculated from Scatchard plots) of approximately 400 adhesin molecules specific for *S. sanguis* and 310 adhesin molecules specific for *A. israelii* reside on the surface of the cell. Immunoelectron microscopy revealed that the adhesins were not an integral part of the fimbrial subunit; rather, they were usually found on the distal portion of the structures arranged in a random fashion.

Pathogenic bacteria often attach to host tissue prior to initiating an infection. Extensive studies with certain pathogenic strains of *Escherichia coli* have shown that they attach via tissue-specific adhesins located on their cell surfaces. Regardless of the site of infection, fimbriae appear to be implicated in this attachment process (6, 14). Until recently, the adhesin was believed to be an integral part of the fimbrial subunit in a number of both uro- and enteropathogenic strains (2, 5). Detailed genetic studies with representatives of the two types of pathogens, however, have revealed that the adhesin and fimbrial subunit proteins are encoded by noncontiguous genes (3, 11, 14, 15). Results of these studies indicate that the association of the two components may be more complex than previously anticipated, involving additional elements such as processing proteins which may act to modify, export, and affix the adhesin to the cell surface (16). Although elegant genetic analyses and supporting biochemical studies have yielded much information on the structure and composition of the fimbriae, little is known about the properties of the adhesin molecules found on *E. coli* in particular and other gram-negative bacteria in general.

Bacteroides loescheii, a gram-negative resident of the oral cavity, coaggregates with a number of gram-positive oral bacteria including *Streptococcus sanguis* 34 or *Actinomyces israelii* PK14. By using monoclonal antibodies (MAbs) and Fab fragments that specifically block coaggregation, the adherence interactions with *S. sanguis* and *A. israelii* were shown to be mediated by two distinct types of fimbria-associated adhesins on the surface of *B. loescheii* (18). The adhesin specific for *S. sanguis* 34 is composed of a 75-kilodalton monomer, while the adhesin specific for *A. israelii* PK14 is composed of a 45-kilodalton subunit. In this report, we describe how MAb 5BB1-2, which recognizes the adhesin specific for *S. sanguis* 34 and inhibits the coaggregation between *B. loescheii* PK1295 and *S. sanguis* 34, and MAb 4AC3, which recognizes the *A. israelii* PK14 adhesin and prevents coaggregation between *B. loescheii* PK1295 and *A. israelii* PK14, were used to demonstrate that the two adhesins are associated with the fimbriae of this organism and to show how they are arranged there.

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MATERIALS AND METHODS

Cultivation of bacteria. *B. loescheii* PK1295 was cultivated in Schaedler broth (17) under anaerobic conditions at 37°C. Cells used for MAb binding studies and immunoelectron microscopy were harvested from exponentially growing cultures and adjusted to an optical density of 260 Klett units (5 × 10^9 cells per ml [Klett-Summerson colorimeter; 660-nm filter]). Preparations of fimbriae were obtained by mild sonication of frozen cells (18).

Radiolabeling of MAbs. The injection schedules, protocols for the preparation of hybridomas, the harvesting of ascites fluid, and the purification of immunoglobulin G (IgG) have been described in detail elsewhere (18). The two MAbs selected for these studies, 5BB1-2 (a MAb that recognizes the adhesin specific for *S. sanguis*) and 4AC3 (a MAb that recognizes the adhesin specific for *A. israelii*), inhibited coaggregation at levels below 5 μg of protein per ml and failed to agglutinate *B. loescheii* cells (18). One milligram of each of the purified MAbs was incubated with 1.5 mCi of ^{125}I (ISM-300; Amersham Corp., Arlington Heights, Ill.) in the presence of Iodobeads (Pierce Chemical Co., Rockford, Ill.) according to the instructions of the manufacturer. Unreacted ^{125}I was removed by passing the mixture over an AG1-X8 econo-column (Bio-Rad Laboratories, Richmond, Calif.). The specific activity of the radiolabeled MAb was determined after trichloroacetic acid precipitation by gamma counting. Loss of radioactivity was measured weekly by using decay standards prepared shortly after the preparation of ^{125}I-labeled MAb.

IgG binding assay. A suspension containing 5 × 10^7 cells was added to a solution containing phosphate-buffered saline (0.02 M phosphate buffer, 0.15 M NaCl [pH 7.2]) plus 2% bovine serum albumin and increasing amounts of ^{125}I-labeled MAb (in a range of 2.5 to 500 ng of protein) in a final volume of 300 μl. The reactions were performed in 1.5-ml Eppendorf tubes at room temperature for 90 min with continuous mixing. Each MAb concentration was assayed in triplicate. Total radioactivity was determined by counting the entire reaction mixture in a Gamma counter (model 9000; Beckman Instruments, Inc., Fullerton, Calif.). Duplicate 50-μl samples from each of the triplicate reaction mixtures were layered onto 200 μl of an oil mixture [1 part bis(2-ethyl-

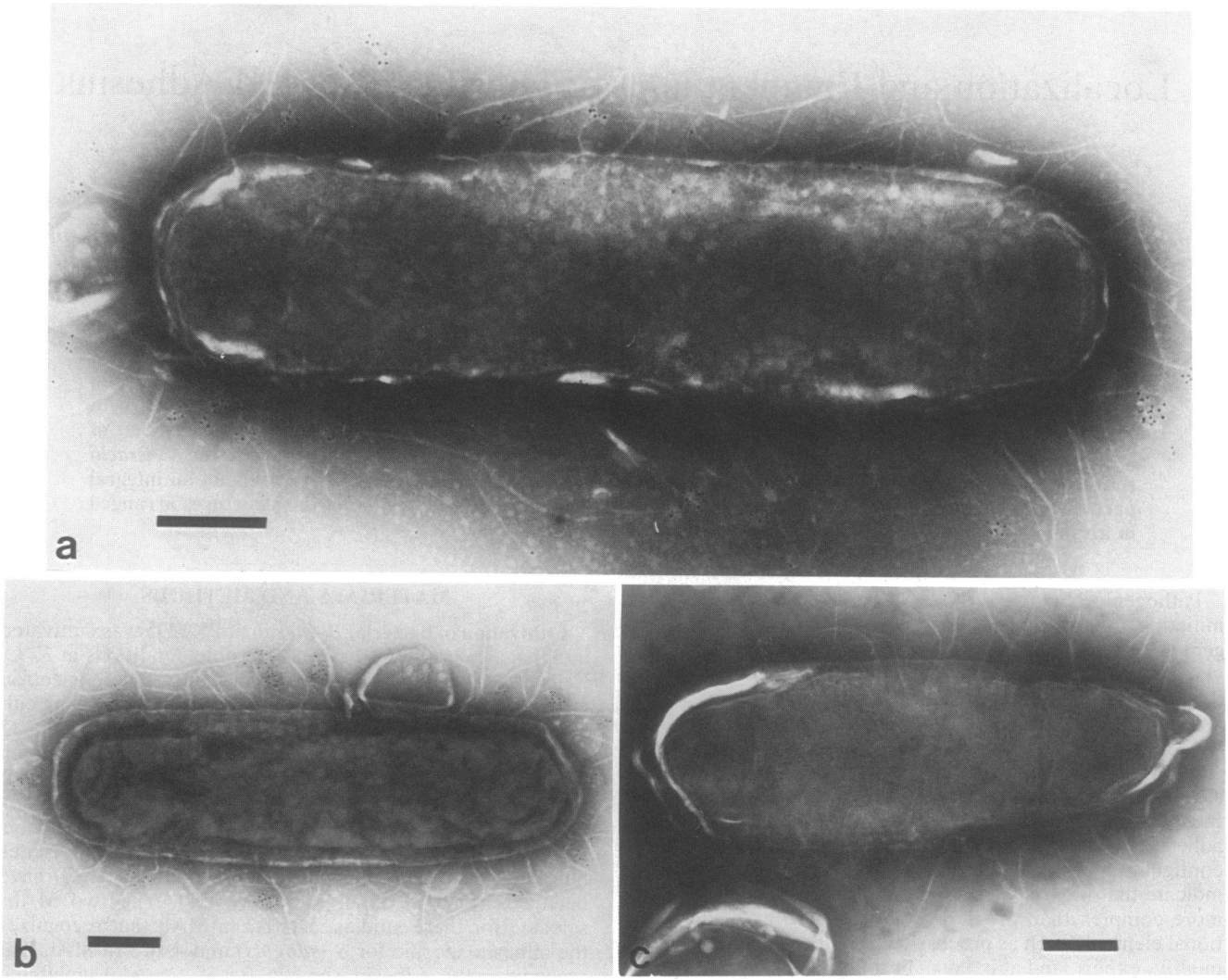

FIG. 1. Localization and arrangement of adhesin specific for *S. sanguis* on the surface of *B. loescheii*. (a) Typical cell showing near maximum amounts of gold-labeled adhesin. Bar, 0.5 μm. (b) Cell exhibiting extensive clustering of adhesins. Bar, 0.5 μm. (c) Control treated with nonimmune mouse IgG. Bar, 0.5 μm.

hexyl)phthalate to 1.5 parts dibutyl phthalate (Eastman Kodak Co., Rochester, N.Y.)] that was placed into sample cups. The samples were centrifuged at 12,400 × *g* for 10 min in a microfuge (model 12; Beckman). The cell pellet was removed and the amount of radioactivity was determined as described above. All experimental determinations were corrected for nonspecific binding and total binding of the MAb.

Immunoelectron microscopy. Five microliters of a suspension containing 5×10^8 bacterial cells per ml was placed onto a Formvar carbon-coated, 200-mesh copper grid. The immobilized cells were treated with a drop of phosphate-buffered saline containing 5% bovine serum albumin. After 15 min the blocking reagent was drawn off the grid and replaced with 10 μg of MAb 5BB1-2 per ml or 20 μg of MAb 4AC3 per ml for a period of 1 h. The grid was then rinsed 3 times with phosphate-buffered saline containing 1% bovine serum albumin and incubated with a 1:10 dilution of colloidal gold-labeled goat anti-mouse IgG (10-nm diameter [Janssen Life Sciences Products, Piscataway, N.J.] or 20-nm diameter [E. Y. Lab, San Mateo, Calif.]) for 30 min. Following removal of the gold-labeled IgG, the grid was washed twice in phosphate-buffered saline and 3 times in distilled water, and then it was dried. Cells on the grid were negatively stained with 2% sodium phosphotungstate.

For thin sectioning of cells, a similar procedure was applied to cells in suspension. After treatment with the primary antibody and the gold-labeled IgG, the cells were fixed with glutaraldehyde, postfixed with OsO_4, dehydrated, and embedded in epoxy resin (13).

RESULTS

Localization of the adhesin by immunogold labeling and electron microscopy. The adhesins specific for *S. sanguis* and *A. israelii* on the surface of *B. loescheii* were visualized by sequentially reacting *B. loescheii* cells with MAb 5BB1-2 (the MAb that inhibited coaggregation between *B. loescheii* and *S. sanguis*) or MAb 4AC3 (the antibody that inhibited coaggregation between *B. loescheii* and *A. israelii*), respectively, and gold-labeled anti-mouse IgG. Electron micrographs of negatively stained preparations revealed that both adhesins are associated with the fimbriae of the bacterium

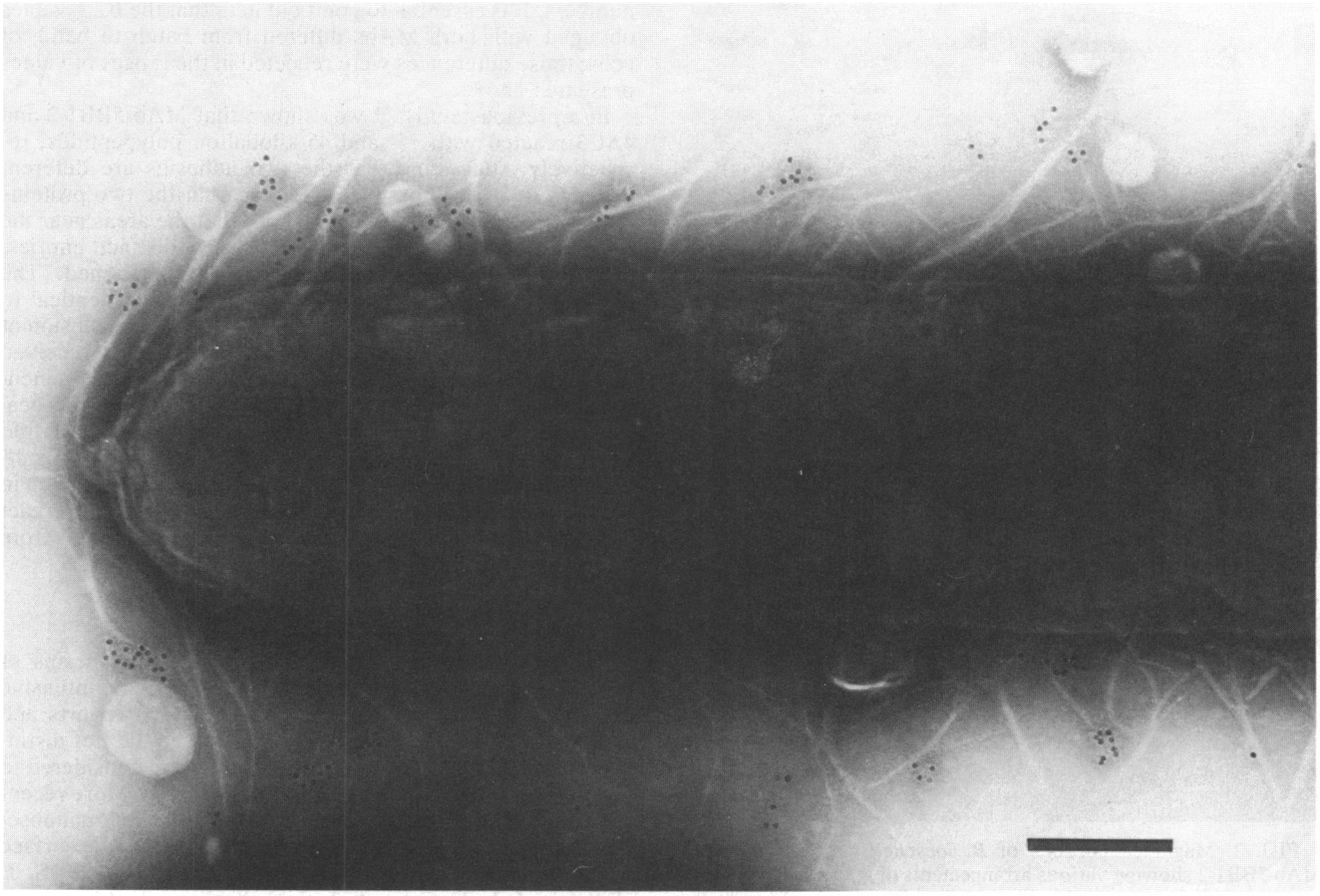

FIG. 2. Arrangement of the adhesin specific for *A. israelii* on the surface of *B. loescheii*, as determined by gold labeling. Bar, 0.5 μm.

(Fig. 1 and 2). Furthermore, they appeared to be located, for the most part, at the distal portion of the organelle (Fig. 3a and b). To verify that the adhesins were found on the fimbriae and were not associated with the outer membrane of the cell, cells labeled with gold particles were fixed, embedded, and sectioned. Essentially all of the gold particles that bound to the *S. sanguis* adhesin–5BB1-2 complex were found roughly 20 to 50 nm away from the cell and none were anchored to the outer membrane of the cell (Fig. 4a). Similar results were obtained with MAb 4AC3. Control cells treated with nonimmune mouse IgG instead of MAb 5BB1-2 were devoid of gold particles (Fig. 4b).

Neither adhesin appeared to be arranged in any consistent fashion on the fimbriae (Fig. 1a and b). Occasionally, they were found in a linear array along a segment of the fimbriae (Fig. 3b, arrow). More often, they appeared in pairs (Fig. 3b), in triads, or as clusters that contained up to 10 gold particles. Some cells tended to have more and larger clusters of gold particles than others (compare Fig. 1a with b and Fig. 3a with b). The larger clusters covered distances of 100 nm or more and appeared to be associated with several fimbriae. To estimate the range over which the adhesins specific for *S. sanguis* and *A. israelii* were found on the surface of *B. loescheii* cells, the number of gold beads associated with 50 randomly selected cells were counted. Roughly 20% carried more than 80 gold beads per cell, 50 to 60% displayed between 30 and 80 gold particles per cell, and 20% had less than 30 beads associated with each cell. The most intensely reactive cells had up to 200 particles per cell. A small number of cells, estimated to be less than 2%, were devoid of gold particles.

Estimation of the number of adhesin molecules per cell. The gold-labeling experiments yielded, at best, very rough approximations of the maximum numbers of adhesin molecules per cell; more accurate estimates were obtained by MAb binding studies. Increasing concentrations of ^{125}I-labeled MAb 5BB1-2 were reacted with a constant number of *B. loescheii* cells to determine the concentration of IgG required to saturate the adhesins on the surface of the bacteria; data from one of three experiments is shown in Fig. 5. Half of the available sites on 5×10^7 cells were saturated at a mean concentration of 1.8×10^{-10} M ($n = 3$). When these data were subjected to a Scatchard analysis (Fig. 5, inset), a mean B_{max} (theoretical number of *S. sanguis*-specific binding sites) of 400 (with a range of 300 to 500; $n = 3$) per cell was obtained (12). A K_d of 2.0×10^{10} liters/mol was calculated from the same Scatchard plot.

Similar experiments were performed with MAb 4AC3, resulting in a saturation curve that extended over a significantly greater range of antibody protein than that of 5BB1-2, with a 50% mean saturation value of 1.7×10^{-9} M ($n = 3$; Fig. 6). The Scatchard plot of these data yielded a mean B_{max} of 310 (with a range of 250 to 450; $n = 3$) and a K_d value of 5×10^9 liters/mol. The B_{max} value was similar to that obtained with the *S. sanguis*-specific adhesin and suggests that the two adhesins may be present in roughly equal

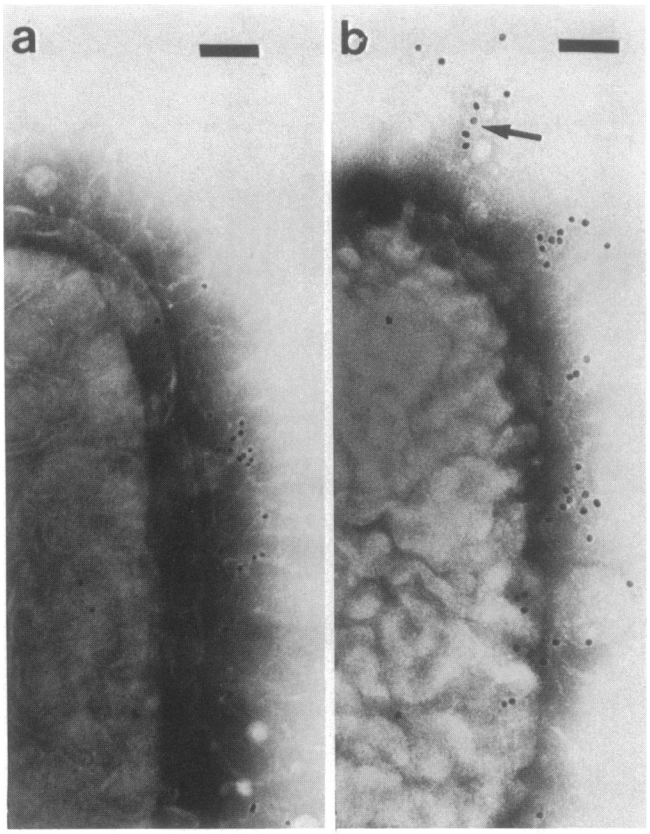

FIG. 3. Magnified portions of *B. loescheii* cells reacted with MAb 5BB1-2 showing various arrangements of gold-labeled adhesin molecules. Cell treated with 10-nm (a) or 20-nm (b) gold particles complexed to anti-mouse IgG. Bar, 0.25 μm.

numbers. It is essential to point out here that the B_{max} values obtained with both MAbs differed from batch to batch of cells; those differences were reflected in the ranges of values presented above.

In a previous study, it was shown that MAb 5BB1-2 and 4AC3 reacted with 75- and 45-kilodalton polypeptides, respectively, suggesting that the two adhesins are different proteins. It is still possible, however, that the two proteins are spatially related. To demonstrate that the areas near the reactive sites of the adhesin were also distinct entities, antibody competition experiments were performed. The protocol for these experiments was essentially identical to that for the binding studies, differing only by the inclusion of an additional step in which the cell suspensions that received increasing concentrations of MAb 5BB1-2 were preincubated with a 100-fold excess of MAb 4AC3. In the reciprocal experiments, the cells were preincubated with a 100-fold excess of 5BB1-2 before MAb 4AC3 was added. The resultant saturation curves were nearly identical to those shown in Fig. 4 and 5 (data not shown), indicating that the MAbs react with different epitopes and do not prevent one another from interacting with their respective adhesins.

DISCUSSION

The relationship between the fimbriae and adhesins of gram-negative bacteria is presently a subject of intensive investigation and increasing debate. Research reports and reviews published as recently as 4 years ago did not distinguish between the two entities; adhesins were considered to be an integral part of the fimbrial subunit (2, 5). More recent studies have demonstrated that functional *E. coli* mannose-sensitive and -resistant adhesins can be found on the surface of the organisms in the absence of fimbriae (3, 9, 15; S. J. Hultgren, J. L. Duncan, A. J. Schaeffer, and A. Amundsen,

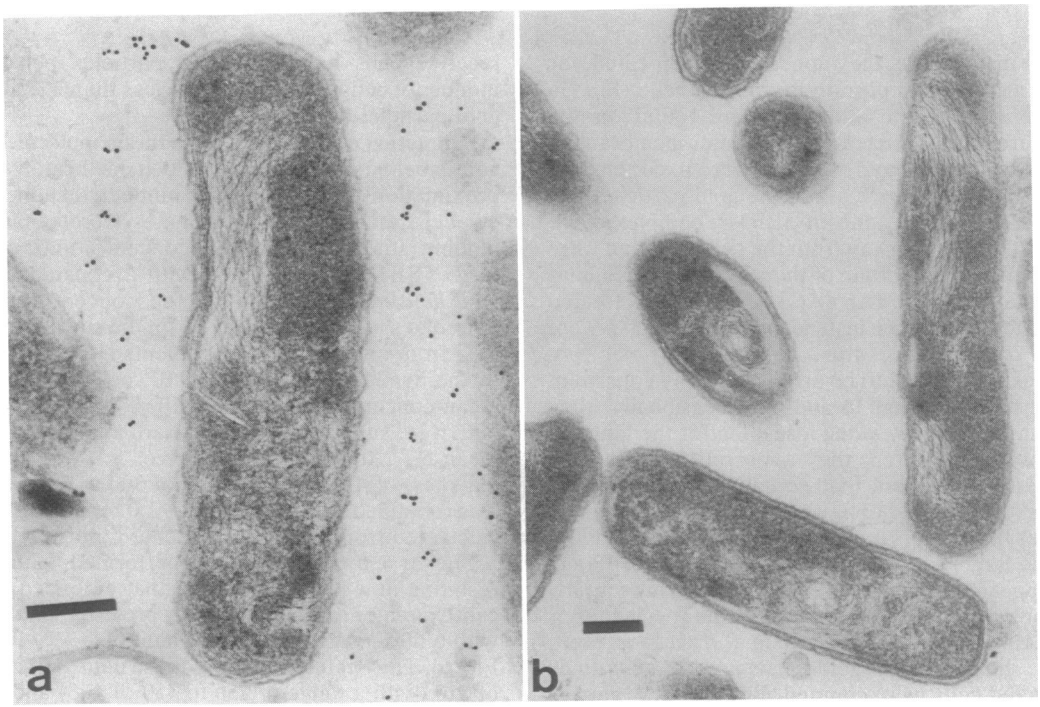

FIG. 4. Thin sections of *B. loescheii* cells fixed and embedded after treatment with MAb and anti-mouse IgG bound to gold particles. (a) Cells treated with 5BB1-2. Bar, 0.5 μm. (b) Control treated with nonimmune mouse monclonal IgG. Bar, 0.5 μm. The fimbriae are poorly visualized in thin-section preparations.

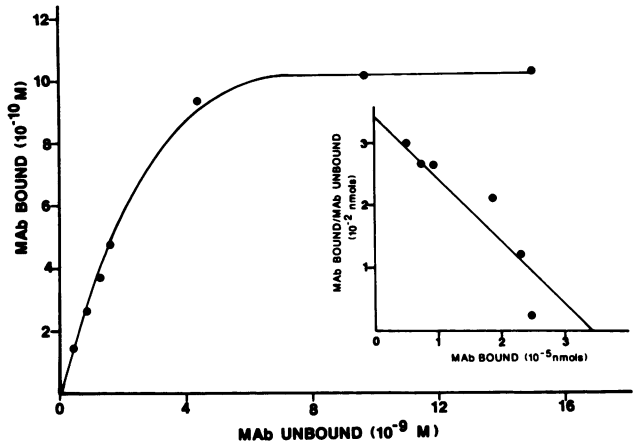

FIG. 5. Saturation of adhesins specific for *S. sanguis* with ^{125}I-labeled MAb 5BB1-2. (Inset) Scatchard plot depicting the theoretical maximum number (B_{max}) of MAb 5BB1-2 binding sites.

Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D55, p. 88). Furthermore, mapping of the cistrons responsible for synthesis of the two components established that the fimbrial gene is distinct from the adhesin gene. However, in one recent report (4) it has been claimed that the flexible fimbrial subunits on several enteropathogenic strains of *E. coli* are composed of the adhesin molecules. Thus, there may be two modes of expression of fimbria-associated adhesins, and at this time, there is no reason to believe that these are mutually exclusive. In the most definitive studies to date, MAbs or polyclonal antibodies prepared against P (PAP) fimbriae (1, 8) or S fimbriae and purified adhesin (10) were used to identify mannose-resistant hemagglutinating adhesins on uropathogenic strains of *E. coli*. In the first two reports (1, 8) the importance of the respective MAbs to inhibit function (hemagglutination) was stressed, although the extent of inhibition and the quantity of MAb required to inhibit the interaction were not reported. Moch et al. (10) actually purified the adhesin and showed that it was distinct from the fimbrial subunit.

Our approach (18) for identifying adhesins differed from those reported previously, in that MAbs were screened specifically for their ability to inhibit adhesin-receptor interactions. Not only were the MAbs isolated by this strategy effective inhibitors of coaggregation, the Fab fragments derived from MAbs inhibited coaggregation completely at levels below 5 μg of protein per ml. By using the most potent inhibitors (those MAbs presumed to be specific for a region near or at the binding sites of the two adhesins), the adhesins were localized on the cell surface and enumerated. Electron micrographs of cells treated with anti-adhesin MAb and reacted with goat anti-mouse IgG bound to colloidal gold particles showed that the lectin-like protein is associated with the fimbriae of the cell. The fact that the gold particles did not cover the entire organelle and that they actually extended away from the structure when arranged in clusters suggests that the adhesins are not an integral part of the fimbrial subunit. Several lines of indirect evidence support this contention. First, electron micrographs of the mutants lacking the adhesins showed that these strains possess fimbriae that are indistinguishable from those on the parent strain (17). Second, a mutant unable to coaggregate with *S. sanguis* (17) did not bind anti-mouse IgG-coated gold particles following treatment with MAb 5BB1-2 (*S. sanguis*-specific adhesin) but did bind gold particles when treated with MAb 4AC3 (*A. israelii*-specific adhesin). Double mutants (mutants incapable of coaggregating with either partner) bound no gold particles when pretreated with either MAb 5BB1-2 or 4AC3 (unpublished data). Third, dot blots with intact cells established that the parent strain possesses antigens that react with the anti-adhesin MAbs, while the mutant that lacks the *S. sanguis*-specific adhesin reacts only with the *A. israelii* adhesin-specific MAb and the double mutants that are missing both adhesin activities fail to react with either MAb (unpublished data). Localization of the adhesins at the distal portion of the fimbriae is probably not accidental. Such placement circumvents the charge repulsion effects that are produced when two different cell types are brought into close proximity to one another (5).

It is curious that the adhesins are not arrayed in any consistent fashion on the fimbriae (Fig. 3a and b), but appear to occur in groups labeled by between 2 to 10 gold particles. These larger arrangements are not likely due to artifacts caused by self-aggregation of the gold particles because the controls showed no such grouping of gold beads. Clustering may be the preferred configuration for these fimbria-associated adhesins since it would serve to enhance the cooperative interactions between the neighboring molecules. Cooperativity would markedly increase the strength of the coaggregation by creating a greater affinity between the two partner cells.

It was previously reported (7) that 90% of *B. loescheii* populations participate in in vitro coaggregation reactions. If the range of gold particles seen on the surfaces of *B. loescheii* cells even approximates the range of adhesin molecules, the number of adhesins required for effective coaggregation may be relatively small. Although no attempts were made to enumerate the number of adhesin sites in the reports describing the P and S fimbriae (8, 10), immunoelectron microscopy revealed that *E. coli* cells are heavily coated with adhesin molecules. In addition, both reports suggest that the adhesins are located on the tips of the fimbriae; this does not appear to be case with *Bacteroides* adhesins. These observations may serve to differentiate the P- and S-fimbriae-associated adhesins from the *Bacteroides* adhesins.

Among the enteric bacteria, the functionally different

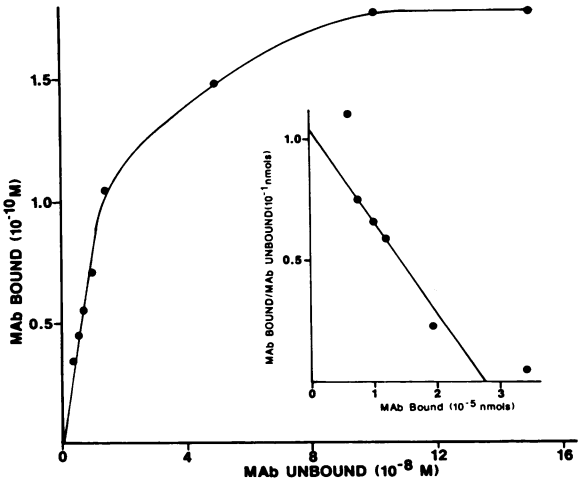

FIG. 6. Saturation of adhesins specific for *A. israelii* with ^{125}I-labeled MAb 4AC3. (Inset) Scatchard plot showing theoretical maximum number of MAb 4AC3 binding sites.

types of adhesins appear to be associated with their own type of fimbriae (6, 14). These fimbriae are readily distinguished from one another by their physical and immunological properties, and a number of fimbriae have been characterized. Little is known about the fimbriae observed on other oral *Bacteroides* species; in fact, only the one type of fimbriae found on *Bacteroides gingivalis* has been adequately described (19, 20); and this organelle is not antigenically related to the *B. loescheii* fimbriae (personal communication, F. Yoshimura). Since the adhesin found on *B. loescheii* is not associated with all, or even most, of the fimbriae, the question arises as to whether this organism possesses more than one type of organelle. In the electron micrographs of *B. loescheii*, it is difficult to discern whether the cells have more than one physically distinct type of fimbria. Some of the fimbriae tend to be entwined with one another, producing a thick 20- to 30-nm-wide bundle, while others occur singly and are less than 10 nm across. However, adhesins are associated with both types of structures. Cloning and expression of *B. loescheii* fimbriae and adhesin genes in *E. coli* will provide a means of characterizing these structures.

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