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Escherichia coli Strains with Nonimmune Immunoglobulin-Binding Activity

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We have identified several strains of *Escherichia coli* which contain immunoglobulin-binding activity on the cell surface. Affinity-purified antibodies ordinarily used as secondary antibodies in immunodetection protocols were bound by 6 of 72 strains of the ECOR reference collection of *E. coli*. The Fc fragments of both human and sheep immunoglobulin G (IgG) were also bound, demonstrating the nonimmune nature of the phenomenon. Binding of conjugated IgG Fc directly to unfixed cells was observed by fluorescence microscopy. Western blots showed that the immunoglobulin-binding material occurs in the form of multiple bands, with the apparent molecular masses of the most prominent bands exceeding 100 kDa. No two of the strains have the same pattern of bands. The binding activity in extracts was sensitive to proteinase K. The binding activity of intact cells was reduced preferentially by trypsin digestion, demonstrating exposure at the cell surface. Expression of binding activity in Luria-Bertani broth cultures was favored by a temperature of 37°C and entry into stationary phase of growth.

Nonimmune immunoglobulin (Ig)-binding activity was first observed in *Staphylococcus aureus* with the discovery of protein A (8). This type of binding has since been identified in *Streptococcus* with the discovery of protein G (12) and in numerous other bacteria (briefly summarized in references 2 and 13). A role for Ig-binding proteins in virulence is suspected, and studies which explore and strengthen this suggestion have been reported (5, 19, 23–26, 31). Among gram-negative bacteria, Ig-binding activity has been documented as a surface component of *Brucella abortus* (21), *Taylorella equigenitalis* (33), *Aeromonas salmonicida* (24), *Haemophilus somnus* (32), *Pseudomonas maltophilia* (10), *Actinobacillus actinomycetemcomitans* (18), *Prevotella intermedia* (14), and *Yersinia pestis* (36). We have found that a few *Escherichia coli* strains produce surface-exposed material that binds Igs in a nonimmune manner reminiscent of protein A from *S. aureus* and protein G from *Streptococcus*. This is the first report of nonimmune Ig-binding activity in *E. coli.*

MATERIALS AND METHODS

Strains and culture conditions. The ECOR reference collection of 72 natural strains of *E. coli* (22) was obtained from Robert Selander and Thomas Whittam. The *E. coli* K-12 strain used was CGSC 4401. Expression of antibody-binding material was investigated in cultures grown with agitation at 37°C for 24 h in Luria-Bertani (LB) broth made with 5 g of NaCl per liter, except where otherwise noted.

Preparation and analysis of cell extracts and culture supernatants. Extracts of whole cells used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared from centrifuged cell pellets heated in a boiling water bath for 5 min in sample buffer (10% SDS, 50% glycerol, 60 mM Tris [pH 6.8], 5% 2-mercaptoethanol, 0.005% bromphenol blue). Protein concentrations in extracts were estimated by the Bio-Rad protein assay according to the manufacturer's instructions, with fraction V of bovine serum albumin (BSA) used for standardization. Extracts were fractionated by SDS-PAGE according to the method of Laemmli (15). Electrophoretic blotting of the fractionated material onto Bio-Rad polyvinylidene difluoride (PVDF) was done by the procedure of Towbin et al. (28). Immunodetection of Ig binding was done with the Amersham ECL Western blotting system, using 10% nonfat dry milk as the blocking agent and phosphate-buffered saline (PBS) containing 0.2% Tween 20 (PBS-T) as a buffer for incubations with antibodies and for washes. The blots were washed once for 20 min and twice for 5 min after blocking, incubation with competitors, and incubation with antibodies. For competition studies, a preparative gel with a single wide lane was run and blotted onto PVDF. A 1-h preincubation with sera, Fc fragments of IgG (IgG Fc), or BSA was followed by a 1-h incubation with horseradish peroxidase (HRP)-conjugated antibody. Incubations and washes were done in a Bio-Rad Mini-Protean Multiscreen apparatus, which allowed us to test each reagent in a separate channel. Gels and molecular mass standards on PVDF blots were stained with Coomassie brilliant blue R-250 (0.003% [wt/vol] in methanol-water-glacial acetic acid [45/45/10]) (3). For analysis of culture supernatants, 20-ml cultures were centrifuged at 6,000 × *g* for 10 min. The upper 15 ml was carefully removed, precipitated with 10% trichloroacetic acid on ice for 1 h, and centrifuged at 18,000 × *g* for 15 min. The pellets were washed twice with 80% acetone, dried, resuspended in 30 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) plus 120 μl of sample buffer, and analyzed by SDS-PAGE and Western blotting.

Proteinase K treatment of cellular extracts. Whole cells were suspended (10%, wt/vol) in PBS containing 2% SDS, and extracts were prepared by heating the cells in a boiling water bath for 5 min. The extracts were then digested with various concentrations of proteinase K for 10 min at 25°C. The reactions were stopped by heating in a boiling water bath for 5 min. Control samples lacking proteinase K were similarly treated. Sample buffer was added to a final concentration of 40%, and the extracts were heated again for 5 min prior to SDS-PAGE.

Trypsin treatment of intact cells. Cell pellets from 20-ml cultures were diluted to 10% (wt/vol) in 50 mM potassium phosphate (pH 6.1)–5 mM EDTA–0.02% NaN_3. Volumes of 300 μl were treated with various concentrations of type III bovine pancreatic trypsin (Sigma) at 37°C for 1 h. The reactions were stopped with 2 mM phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim). Control samples lacking trypsin were similarly treated. For fluorescent-antibody binding studies, 10 μl of each cell suspension was removed at this point. In addition, to control for possible residual trypsin activity during subsequent incubation with fluorescent antibody, 5-μl portions of cell suspension from trypsinized and undigested samples were combined before incubation with antibody. These cells were washed three times in 200 μl of PBS containing 0.1% BSA (PBS-BSA) and 2 mM PMSF (PBS-BSA/PMSF) and suspended in 10 μl of PBS-BSA/PMSF containing fluorescein isothiocyanate (FITC)-conjugated Fc fragment of human IgG (human IgG Fc-FITC) (200 μg of antibody per ml). Incubation with antibody and subsequent preparation for microscopic observation are described in the next section. Cells to be analyzed by SDS-PAGE and Western blotting were centrifuged, resuspended in 1,200 μl of sample buffer, and heated in a boiling water bath for 5 min.

Fluorescent-antibody binding microscopy. Cells from 200 μl of LB broth cultures were centrifuged and washed three times in PBS-BSA. The cells were suspended in 10 μl of PBS-BSA containing human IgG Fc-FITC at a concentration of 200 μg of antibody per ml and incubated for 2 h at room temperature with gentle rocking. A 200-μl volume of PBS-BSA was added, and the cells were centrifuged, washed three times with 200 μl of PBS-BSA, and resuspended in 20

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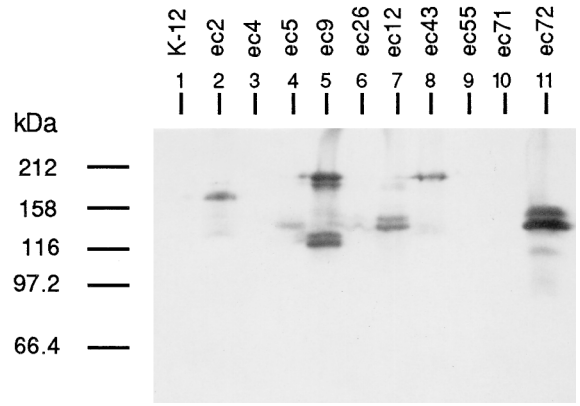

FIG. 1. Ig-binding activity in *E. coli* strains of the ECOR reference collection. Extracts of whole-cell pellets from 24-h cultures were fractionated by SDS–7.5% PAGE, blotted onto PVDF, and probed with donkey anti-rabbit Ig-HRP (50 ng of antibody per ml), as described in Materials and Methods. Each lane contains approximately 20 μg of protein. Molecular mass standards are shown on the left. Strain numbers are shown above the lanes.

μl of PBS-BSA. For the experiment in which the cells were treated with trypsin, PBS-BSA/PMSF was used for antibody incubation and washes. One microliter of the cell suspension was spread in the well of a fluorescent-antibody slide (Bellco Glass), air dried, stained with 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml, washed four times with PBS-BSA, and air dried again. A drop of antiquench mounting medium (*p*-phenylenediamine, 1 mg per ml in 90% glycerol) was added to each well, and a coverslip was sealed in place. The slides were stored at −20°C in the dark. Cells were observed with a 100× 1.4 Na Plan APO objective on a Nikon Optiphot-2 microscope equipped with Nomarski differential interference contrast optics for total cells and an EF-4 B-1E filter cube for cell-bound human IgG Fc-FITC fragments. Cells were photographed with an Olympus PM30 photosystem.

Antibodies, normal sera, and protein A. Affinity-purified antibodies conjugated with HRP were used for Western immunoblot studies. The concentrations of undiluted whole antibodies (expressed as milligrams of antibody per milliliter) and commercial sources were as follows: donkey anti-rabbit Ig, 0.5 (Amersham); sheep anti-rat Ig, 0.5 (Amersham); sheep anti-mouse Ig, 0.5 (Amersham); goat anti-mouse Ig, 0.4 (Sigma); goat anti-rabbit Ig, 0.5 (Sigma); goat anti-chicken Ig, 0.5 (Kierkegaard and Perry Laboratories, Inc.); and rabbit anti-bovine Ig, 4.8 (Sigma). HRP-conjugated $F(ab')_2$ fragment of donkey anti-rabbit Ig (0.5 mg of antibody per ml) was obtained from Amersham. The following purified HRP-conjugated fragments of IgG were obtained from Rockland and reconstituted to the indicated antibody concentrations (in milligrams per milliliter): human IgG Fc, 1; sheep IgG Fc, 1; and $F(ab')_2$ fragment of human IgG, 10. For HRP-conjugated antibodies, the molar ratios of peroxidase to antibody molecule, Fc fragment, or $F(ab')_2$ fragment were 2 for all Rockland products, 2 to 3 for all Amersham products, and 1 for all Sigma products. Normal sera from donkey, sheep, goat, rabbit, and mouse from Sigma and human serum from a local donor were used in competition experiments. Protein concentrations (in milligrams per milliliter) for these sera were as follows: donkey, 60.7; sheep, 67.2; goat, 70.9; rabbit, 46.8; mouse, 51.4; and human, 60. Sera were diluted to 5 mg of protein per ml with PBS for competition studies. For fluorescent-antibody microscopy, purified human IgG Fc-FITC (Rockland) was used. This was reconstituted to 2 mg of antibody per ml; the ratio of fluorochrome to antibody protein was 3.0. Soluble protein A isolated from cell walls of the Cowan strain of *S. aureus* was obtained from Sigma.

RESULTS

Certain strains of *E. coli* express antibody-binding material. Our original observation of Ig-binding material in several *E. coli* strains is illustrated as follows. A Western blot prepared from whole-cell extracts was developed with an affinity-purified commercial secondary antibody, with any source of primary antibody omitted. The secondary antibody was affinity-purified donkey anti-rabbit Ig conjugated with HRP (donkey anti-rabbit Ig-HRP). Figure 1 shows that six strains of the ECOR reference collection (22) (ec2, ec5, ec9, ec12, ec43, and ec72) gave strong signals, while four others and *E. coli* K-12 did not. Signals were not seen when the secondary antibody was omitted. Several points about the Ig-binding material may be noted from Fig. 1. It occurred in multiple forms in each positive strain, except for ec5 and ec43; it was very large, with the apparent molecular masses of the most prominent bands exceeding 100 kDa; and each strain exhibited a unique pattern of Ig-binding material. Overexposure of the same blot showed that positive strains had additional, minor bands that bound Ig (not shown). On the basis of migration in this 7.5% polyacrylamide gel, the approximate apparent molecular masses for the most prominent bands of Ig-binding material were as follows: ec2, 128, 145, and 170 kDa; ec5, 135 kDa; ec9, 120, 128, 185, and 195 kDa; ec12, 138, 145, 185, and 190 kDa; ec43, 195 kDa; and ec72, 110, 130, and 145 kDa. However, size estimates were highly dependent on the acrylamide concentration of the gel. When 10% acrylamide was used instead of 7.5%, the size estimates fell in a higher range, as will be illustrated below. When the remaining 62 strains of the ECOR reference collection were tested for Ig binding, all were negative, indicating that Ig binding was shown by a minority of *E. coli* strains insofar as the ECOR collection is representative (data not shown). The Ig-binding activity was cell associated and not released in significant quantities to the medium. This was shown by precipitating culture supernatants of ec2, ec5, ec9, ec12, ec43, and ec72 and analyzing the precipitates by Western blotting (Materials and Methods). Less than 0.1% of the binding activity was present in culture media (not shown).

To determine whether Ig-binding activity extended to antibodies produced by animal species other than donkey, we tested binding of the following HRP-conjugated affinity-purified antibodies: sheep anti-rat Ig, sheep anti-mouse Ig, goat anti-mouse Ig, goat anti-rabbit Ig, goat anti-chicken Ig, and rabbit anti-bovine Ig, using strain ec9 as the source of whole-cell extract for Western blots. We found that all of the antibodies tested were bound, and all revealed the same pattern of multiple bands.

Competition between secondary antibodies and normal sera. Binding of affinity-purified antibodies from numerous animal sources could be explained in several ways, such as simple nonspecific protein binding, contamination with antibodies directed specifically toward epitopes of the positive strains, antigenic mimicry, or nonimmune binding of Ig molecules in a region distinct from the antigen recognition site. To discriminate among these explanations, competition experiments using ec9 extracts were done. Figure 2 shows parallel Western blots of ec9 total cell extract loaded as a single wide lane for each gel, fractionated by SDS–7.5% PAGE, and blotted onto PVDF. The blot was placed in a multislot apparatus, which allowed treatment of the blot with several different reagents simultaneously, each in a separate channel (lane) of the apparatus. The blot shown in Fig. 2A was preincubated with normal sera from various animals or control reagents (PBS-T or BSA) and then probed with donkey anti-rabbit Ig-HRP. Preincubation with human, sheep, donkey, or goat normal serum (protein concentrations adjusted to 5 mg per ml) greatly reduced the subsequent binding of donkey anti-rabbit Ig-HRP at a concentration of 25 ng of antibody per ml (Fig. 2A, compare lanes 3 to 6 with control lanes 1, 2, and 10). Mouse serum (Fig. 2A, lane 8) and BSA at a concentration of 10 mg per ml (lane 9) failed to block binding. The use of rabbit serum (Fig. 2A, lane 7) was irrelevant, since the donkey antibody recognizes rabbit Ig specifically. Figure 2B shows a parallel Western blot using sheep anti-rat Ig-HRP at a concentration of 50 ng of antibody per ml instead of donkey anti-rabbit Ig-HRP. Sheep, donkey, and goat sera competed with sheep antibody in a manner similar to that observed for donkey antibody (compare lanes 4 to 6 of Fig. 2A and B). Human serum reduced binding (Fig. 2B, lane 1), but its effect was somewhat less pronounced

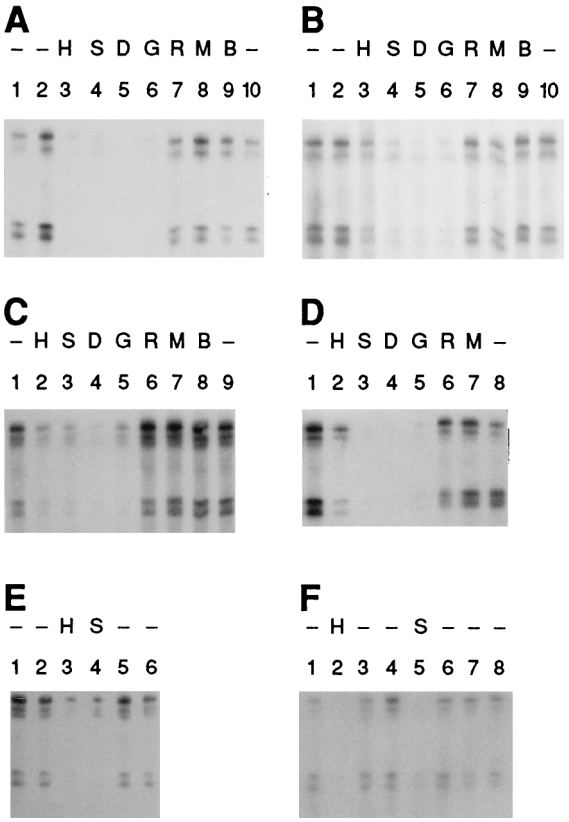

FIG. 2. Competition for binding by normal sera and Fc fragments. Samples of ec9 extract containing 20 μg of protein were loaded as a single wide lane for each gel, fractionated by preparative SDS–7.5% PAGE, and blotted onto PVDF. Each blot was incubated first with competitor and then with HRP-conjugated antibody, as described in Materials and Methods. The conjugated antibodies and amounts used (in nanograms per milliliter) were as follows: donkey anti-rabbit Ig-HRP, 25 (A); sheep anti-rat Ig-HRP, 50 (B); human IgG Fc-HRP, 5 (C and E); and sheep IgG Fc-HRP, 100 (D and F). Normal sera adjusted to 5 mg of protein per ml with PBS-T were used as competitors in panels A to D. The origins of the competing sera are abbreviated as follows: H, human; S, sheep; D, donkey; G, goat; R, rabbit; and M, mouse. B, 10 mg of BSA per ml. Unconjugated IgG Fc fragments (1 mg per ml) were used as competitors in panels E and F (H, human Fc; S, sheep Fc).

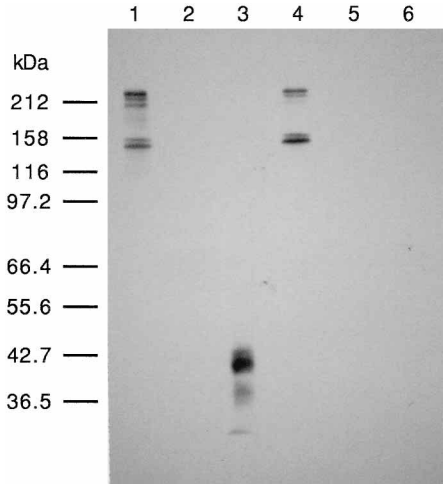

FIG. 3. Fc fragment binding by ec9 extract and protein A. SDS-PAGE fractionation and blotting were as described in Materials and Methods. Lanes 1 and 4, 25 μg of total protein from *E. coli* ec9; lanes 2 and 5, 25 ng of protein A; lanes 3 and 6, 250 ng of protein A. Lanes 1 to 3 were probed with the Fc fragment of human IgG-HRP (5 ng of antibody per ml); lanes 4 to 6 were probed with the Fc fragment of sheep IgG-HRP (5 ng of antibody per ml).

than those of sheep, donkey, and goat sera. Rabbit and mouse sera failed to block binding under these conditions (Fig. 2B, lanes 7 and 8). Since results mentioned above had demonstrated binding of rabbit antibody to the ec9 material, the failure of rabbit serum to compete probably indicated that the binding sites on the sheep and rabbit antibodies did not overlap. Other, more limited surveys showed that binding of donkey anti-rabbit Ig-HRP to ec12 and ec72 material was also reduced by prior incubation with various normal sera (not shown).

These competition experiments argue against antigenic mimicry, since normal sera from donkey and sheep blocked the binding of HRP-conjugated antibody from the same species. In fact, four different species of normal sera blocked the binding of both donkey and sheep secondary antibodies. The ineffectiveness of BSA argues against indiscriminate protein binding. These results are consistent with a nonimmune mechanism of Ig binding. However, an argument that binding was due to specific recognition by a contaminating antibody which copurified with all of the affinity-purified antibodies used in the above studies and which was present in all of the sera used in the competition experiments could still be made. Therefore, we took a different approach to explore the possibility that contaminating antibodies were responsible.

Binding of Fc fragments of human and sheep IgG. Nonimmune binding of Igs by bacterial proteins often involves a region of the molecule contained in the Fc fragment. This fragment lacks the antigen recognition site of the antibody. We tested the ability of the *E. coli* material to bind the purified Fc fragment of human IgG and found that the material from all six positive strains bound the Fc-HRP conjugate in a pattern that was indistinguishable from binding of donkey whole antibody, as was shown in Fig. 1. Even when the human IgG Fc-HRP was diluted to 500 pg of antibody per ml, binding was readily detectable (not shown).

The binding of ec9 extract to Fc fragments from human and sheep was compared to that of protein A. Figure 3 shows a Western blot with duplicate loadings of ec9 extract containing 25 μg of total *E. coli* protein (lanes 1 and 4) and two quantities of protein A (25 ng in lanes 2 and 5 and 250 ng in lanes 3 and 6). The blot was cut in half and probed with the Fc fragment of human IgG (lanes 1 to 3) or sheep IgG (lanes 4 to 6). By Coomassie staining a control gel, we estimated that Ig-binding material represented less than 1% of the total protein of ec9. The left side of the blot shows that binding of the human Fc fragment by 25 μg of ec9 total protein greatly exceeded binding by 25 ng of protein A and was similar to the binding observed for 250 ng of protein A. The right side of the blot shows that while the Fc fragment of sheep IgG bound to ec9 material (lane 4), it failed to bind to protein A (lanes 5 and 6). The high affinity of protein A for the Fc fragment of human IgG and its low affinity for the Fc fragment of sheep IgG have been established previously (summarized in reference 16). The high-affinity binding of the ec9 material to Fc fragments from two animal species demonstrated that this material bound Igs in a nonimmune manner. Its binding capacity for human Fc in Western blots was comparable to that of protein A, and its ability to bind sheep Fc exceeded that of protein A. The binding to Fc fragments of IgG from sources other than human and sheep has not been tested.

Competition experiments similar to those described above for donkey and sheep whole antibodies were extended to hu-

man and sheep IgG Fc fragments. Figure 2C shows a Western blot of ec9 extract preincubated with the same competing normal sera used in the experiments whose results are shown in Fig. 2A and B but with human IgG Fc-HRP (5 ng of antibody per ml) in the second incubation. Human, sheep, donkey, and goat sera reduced the subsequent binding of human IgG Fc-HRP (Fig. 2C, compare lanes 2 to 5 with control lanes 1 and 9). Rabbit and mouse sera failed to inhibit binding (Fig. 2C, lanes 6 and 7). We concluded that the binding sites for Fc overlap those of the competing whole antibodies present in normal sera from human, sheep, donkey, and goat. Figure 2D shows the results of a competition experiment which was identical except that sheep IgG Fc-HRP (100 ng of antibody per ml) was used in the second incubation. Normal sera from sheep, donkey, and goat strongly blocked subsequent binding of sheep IgG Fc-HRP (Fig. 2D, compare lanes 3 to 5 with control lanes 1 and 8), while rabbit and mouse sera failed to do so (lanes 6 and 7). The ability of normal human serum to compete was intermediate. Donkey serum was consistently more effective than other sera in blocking the subsequent binding of whole antibodies and Fc fragments. This finding may indicate a particularly high affinity of the ec9 material for the donkey antibody, but this issue has not been resolved. Finally, we wished to relate the human Fc binding and sheep Fc binding by testing for competition between them. Figure 2E shows a Western blot of ec9 extract probed with human IgG Fc-HRP (5 ng of antibody per ml) after preincubation with human (lane 3) and sheep (lane 4) IgG Fc. Both human and sheep IgG Fc blocked binding of human IgG Fc-HRP. Figure 2F shows results of an experiment which was similar except that sheep IgG Fc-HRP (100 ng of antibody per ml) was used in the second incubation. Preincubation with human (Fig. 2F, lane 2) or sheep (lane 5) IgG Fc (1 mg per ml) blocked the subsequent binding of sheep IgG Fc-HRP.

Binding to the $F(ab')_2$ fragment of donkey IgG. Ig-binding proteins from certain bacteria bind $F(ab')_2$ fragments by a nonimmune mechanism (reviewed in references 2 and 20). We observed that material from ec2, ec5, ec9, ec12, ec43, and ec72 bound the affinity-purified $F(ab')_2$ fragment of donkey anti-rabbit IgG at a concentration of 50 ng per ml in a pattern identical to that observed for the whole donkey antibody, as was shown in Fig. 1. In contrast, we were unable to demonstrate binding of the $F(ab')_2$ fragment of human IgG to the ec9 material even at a concentration of 25 μg of antibody per ml, a condition in which binding of this fragment to protein A was observed (not shown). We conclude that the ec9 material has a relatively high affinity for the $F(ab')_2$ fragment of donkey IgG but no detectable affinity for human $F(ab')_2$. For Ig-binding proteins of other bacteria, the ability to bind $F(ab')_2$ and Fc fragments depends on the animal origin of the IgG (reviewed in references 2 and 20). For some mammalian species, both the Fc and the $F(ab')_2$ fragments of IgG are bound by protein A, whereas for others, one or the other, but not both, is bound (1, 7). For example, for IgG from rabbits, protein A strongly binds the Fc fragment but fails to bind the $F(ab')_2$ fragment (1, 7, 20). Likewise, the 30-kDa Ig-binding protein of *P. maltophilia* binds human IgG Fc but not $F(ab')_2$ (10). In contrast, protein A binds IgG $F(ab')_2$ but not Fc from opossums and dolphins (7, 20). These examples show that the heterogeneity of binding by the *E. coli* material, i.e., to $F(ab')_2$ fragments of donkey but not human IgG, is not unprecedented. The binding to $F(ab')_2$ fragments other than those of donkey and human IgG has not been investigated.

Protease susceptibility. To test whether the binding activity observed for ec9, ec12, and ec72 is proteinaceous, whole-cell extracts prepared in 2% SDS were digested with proteinase K.

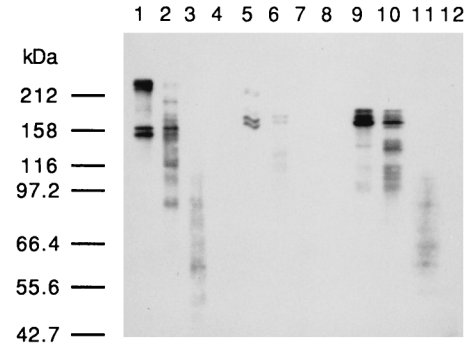

FIG. 4. Proteinase K digestion of whole-cell extracts from ec9, ec12, and ec72. The extracts were digested at 25°C for 10 min, fractionated by SDS–10% PAGE, and blotted onto PVDF, as described in Materials and Methods. All lanes contained extracts from the same quantity of cell material, measured before digestion. Undigested samples in lanes 1, 5, and 9 contained approximately 60 μg of protein. The blot was probed with donkey anti-rabbit Ig-HRP (50 ng of antibody per ml). Lanes 1 to 4, ec9; lanes 5 to 8, ec12; lanes 9 to 12, ec72. No proteinase K (lanes 1, 5, and 9) or proteinase K at 0.04 (lanes 2, 6, and 10), 0.2 (lanes 3, 7, and 11), and 1.0 (lanes 4, 8, and 12) μg per ml was used.

At 1 μg per ml, proteinase K completely eliminated Ig-binding activity from each strain (Fig. 4, lanes 4, 8, and 12). Treatment with 0.04 μg of proteinase K per ml resulted in an intermediate ladder-like banding pattern of Ig-binding material for all three strains (Fig. 4, lanes 2, 6, and 10). The results of proteinase K digestion implicate protein in the Ig-binding material but do not exclude the possibility that other classes of molecules covalently bound to or closely associated with protein might also be involved in its structure.

Cell surface location of binding activity. Ig-binding activity of other bacteria is associated with the cell surface. To test for the presence of the Ig-binding material on the cell surface of the *E. coli* strains, accessibility to trypsin was analyzed. Freshly harvested intact cells were treated with trypsin at concentrations of 1, 10, and 100 μg per ml. Whole-cell extracts were prepared, fractionated by SDS–10% PAGE, blotted, and probed with donkey anti-rabbit Ig-HRP (Fig. 5A). Four major Ig-binding bands were observed in the control lane containing extracts of untreated ec9 (Fig. 5A, lane 1). (Note that the apparent molecular masses of these bands differed substantially from the size estimates derived from 7.5% acrylamide gels; e.g., compare lane 1 of Fig. 5A to lane 5 of Fig. 1. A shift in apparent molecular mass was true for ec12 and ec72 also.) Lanes 2 to 4 of Fig. 5A show that trypsin eliminated the three largest major bands from ec9 in a concentration-dependent manner and produced a new major band of approximately 59 kDa. The smallest of the four major bands in untreated cells was unaffected by trypsin. In the case of ec12, trypsin reduced all major bands to smaller bands with apparent molecular masses of approximately 63, 80, and 97 kDa (Fig. 5A, lanes 6 to 8). At 1 μg per ml, trypsin reduced the size of the major bands of ec72 to approximately 115 and 143 kDa (Fig. 5A, lane 10). Trypsin at concentrations of 10 μg per ml or greater completely eliminated the bands (Fig. 5A, lanes 11 and 12). Sensitivity of whole cells to trypsin implied that the binding activity was exposed on the cell surface. The resistance of the smallest major Ig-binding band of ec9 to trypsin suggested that this form was not exposed at the cell surface. The data shown in Fig. 5B constitute an essential control for this conclusion concerning cell surface location. Figure 5B shows a Coomassie brilliant blue-stained 10% acrylamide gel corresponding to the

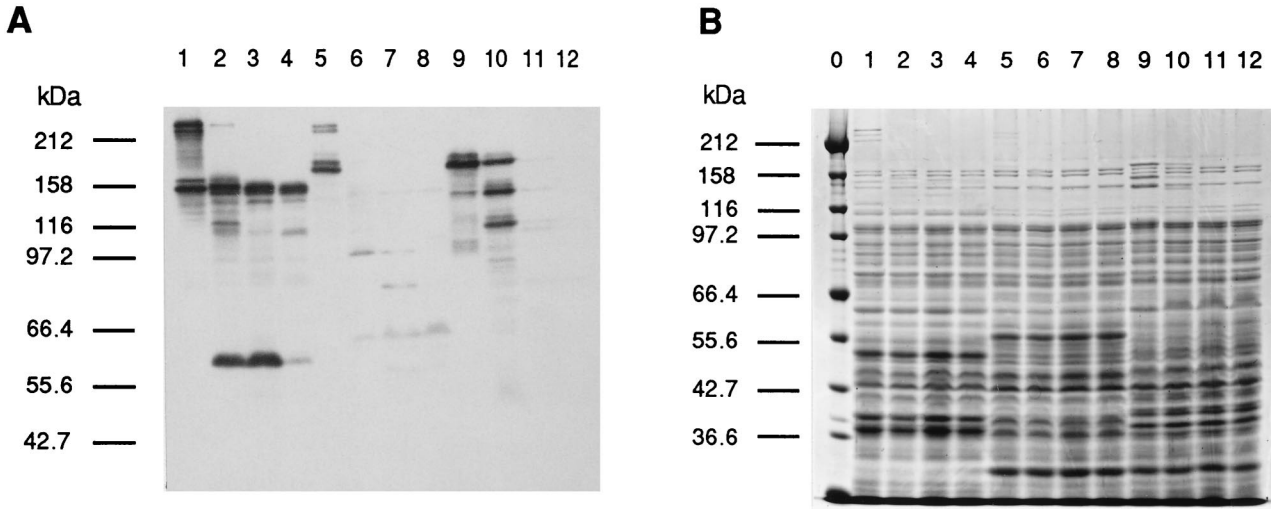

FIG. 5. Trypsin treatment of intact cells of ec9, ec12, and ec72. Whole cells were treated with trypsin at 37°C for 1 h. Extracts prepared by heating the cells in sample buffer were fractionated by duplicate SDS–10% PAGE. All lanes contained extracts from the same quantity of cell material, measured before digestion. Undigested samples in lanes 1, 5, and 9 contained approximately 10 μg of protein. (A) Western blot prepared and probed as described for Fig. 4. (B) Coomassie brilliant blue-stained gel corresponding to the blot shown in panel A. Lane 0, molecular mass standards; lanes 1 to 4, ec9; lanes 5 to 8, ec12; lanes 9 to 12, ec72. No trypsin (lanes 1, 5, and 9) or trypsin at 1 (lanes 2, 6, and 10), 10 (lanes 3, 7, and 11), or 100 (lanes 4, 8, and 12) μg per ml was used.

blot shown in Fig. 5A. This gel showed that protein degradation by trypsin was extremely selective, leaving most proteins intact. In Fig. 5B, comparison of lanes 2 to 4 with lane 1 shows that several slowly migrating bands (>200 kDa) visible in the untreated sample were selectively removed by trypsin treatment of ec9 whole cells. Removal of these bands correlated precisely with the loss of Ig-binding material in the corresponding lanes of the blot shown in Fig. 5A. Comparison of lanes 10 to 12 with lane 9 of Fig. 5B demonstrated trypsin's removal of bands in the range of approximately 150 to 170 kDa from ec72. This too correlated with the size reduction of Ig-binding material in lanes 10 to 12 of the blot. Similar removal of bands from ec12 was observed (Fig. 5B, compare lanes 6 to 8 with lane 5), although the bands were less intensely stained and barely visible. Undigested bands of ec12 were located at a position just slightly above that of the 212-kDa size standard.

Ig binding by intact cells. The results of the previous section indicated the accessibility of the Ig-binding activity of intact cells to trypsin. Consequently, we expected that the binding activity of intact cells would be directly accessible to antibodies as well. Therefore, we tested for the binding of IgG Fc-FITC to intact cells by fluorescence microscopy. Unfixed cells were incubated with antibody and prepared for microscopy. Figure 6 shows representative results of these experiments for ec9, ec72, and a negative-control strain, K-12. The same fields were observed by both fluorescence microscopy (Fig. 6, upper row) and Nomarski differential interference contrast optics (lower row). Figure 6C shows four brightly fluorescing and several less intensely fluorescent ec9 cells. The same field visualized by Nomarski optics contained a total of 64 cells. Figure 6B shows 10 fluorescing ec72 cells in a field of 125 cells. In contrast, the negative-control strain K-12, presented in Fig. 6A at the same exposure during photography and printing, revealed no fluorescing cells in a field of 115 cells. A second negative control, strain ec8, was identical to K-12 in its failure to bind fluorescent antibody (not shown). These experiments showed that unfixed, intact cells of two strains could directly bind the Fc fragment. An unexpected aspect of these experiments which we do not understand at present was the heterogeneity of fluorescent-antibody binding within the population, i.e., a small percentage of cells exhibited strong fluorescence while the majority of cells were not clearly distinguishable from negative controls. This heterogeneity persisted despite repeated single-colony isolations.

We expected that the material responsible for the fluorescent-antibody binding would be sensitive to treatment of the cells with trypsin in a fashion similar to that detected by Western blotting. To test this prediction, cells of ec9 were treated with trypsin before incubation with human IgG Fc-FITC. Figure 6D shows a representative sample of trypsinized ec9 cells visualized by fluorescence microscopy. No FITC-stained cells were visible, even though 458 cells were present in the field. Untreated ec9 cells from this experiment (not shown) were indistinguishable from the cells shown in Fig. 6C in brightness and proportion of fluorescing cells. To ensure that residual trypsin was not directly interfering with Fc-FITC binding, equal parts of treated (Fig. 6D) and untreated cells were mixed before Fc addition. This mixed sample showed an intermediate number of brightly fluorescing cells, as would be expected if binding to the cells from the undigested sample were not affected by residual trypsin activity (not shown). The same samples used in this trypsin digestion experiment were checked both by Western blotting and by SDS-PAGE and Coomassie blue staining exactly as described for Fig. 5, and the results were the same as depicted in Fig. 5. Thus, trypsin-accessible Ig-binding material on the cell surface was responsible for the fluorescence of intact cells, the presence of at least two high-molecular-mass Coomassie blue-staining bands seen after SDS-PAGE fractionation of cell extracts, and the multiple-banded positive signals on Western blots.

Factors affecting expression in strain ec9. Several environmental factors influenced expression of the Ig-binding material in strain ec9. Expression was minimal during log-phase growth in LB broth but increased substantially as the culture reached stationary phase. The same result was obtained when the LB broth was diluted 1/10, indicating that nutrient limitation rather than oxygen depletion was the basis for the effect. Expression in stationary-phase LB broth cultures was influenced by temperature, being greatest at 37°C, less at 34°C, minimal at 31 or 42°C, and undetectable at 27°C.

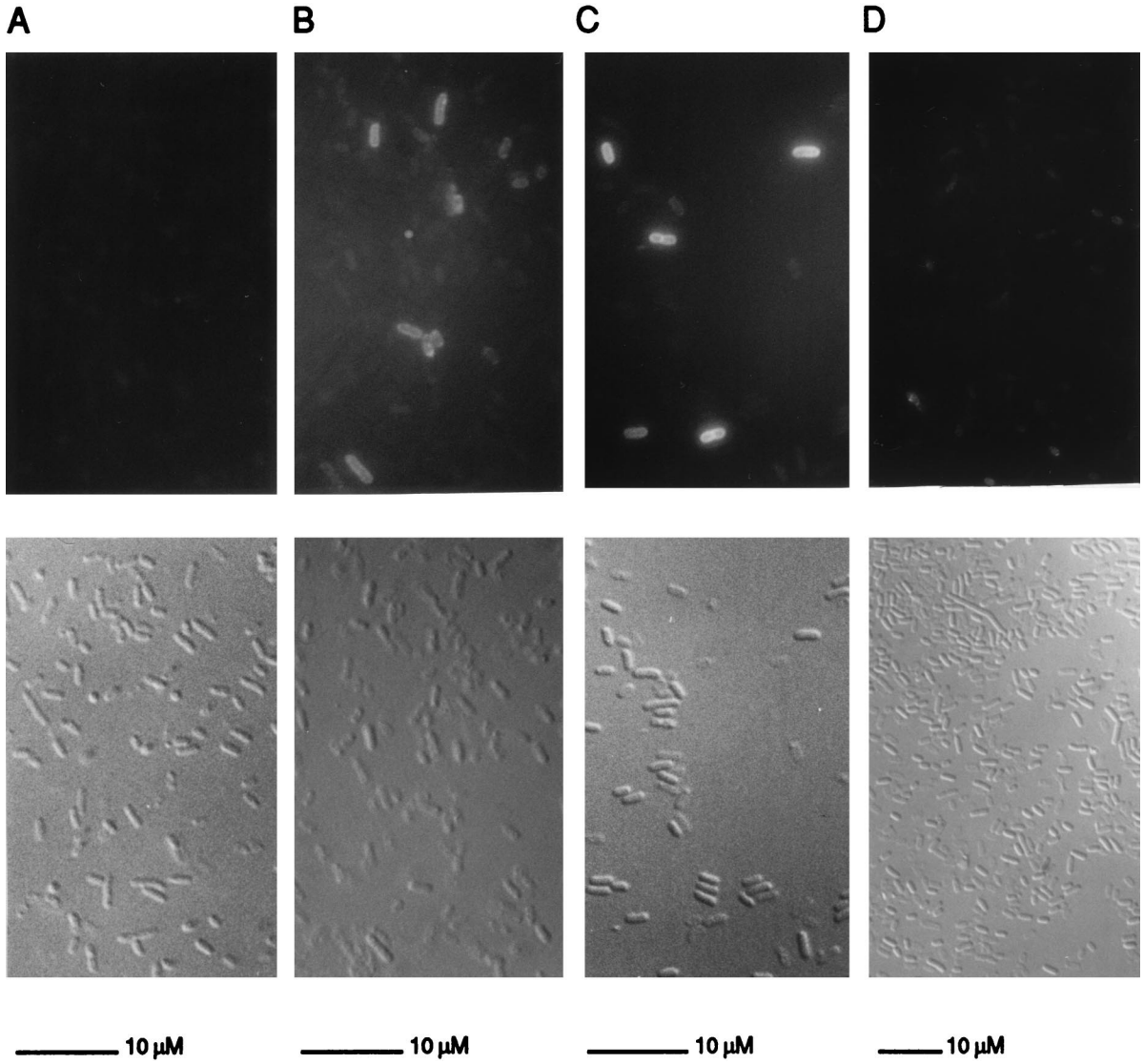

FIG. 6. Binding of human IgG Fc-FITC to intact, unfixed cells and the effect of trypsin on binding. Freshly harvested cells from 18-h cultures of strains ec9, ec72, and K-12 were incubated with human IgG Fc-FITC (200 μg of antibody per ml) and prepared for microscopy as described in Materials and Methods. Fluorescing cells (upper row) and total cells (lower row) of strains K-12 (A), ec72 (B), and ec9 (C) are shown. Freshly harvested cells of strain ec9 were treated with trypsin (100 μg per ml) and then incubated with human IgG Fc-FITC (200 μg of antibody per ml) (D). Fluorescing cells in panels A to C were photographed and printed at identical exposures.

DISCUSSION

We have found proteinaceous Ig-binding material on the cell surface of strains of *E. coli* such as ec9. The cell surface location was defined by its accessibility to trypsin under conditions in which the cells remained morphologically intact (Fig. 6) and in which the great majority of cellular proteins were not digested (Fig. 5). Ig binding occurred whether the material was in its native state, as demonstrated by fluorescence microscopy, or had endured hot-SDS treatment prior to Western blotting. Remarkably, the amounts visible on the cell surface by fluorescence microscopy were not uniform. In overnight stationary-phase cultures, a few cells fluoresced intensely, others fluoresced more moderately, and most were not clearly distinguishable from negative-control cells. An important question is whether the radical difference in appearance represented corresponding differences in amounts of the protein made per cell or accessibility for Ig binding. This issue has not been resolved at present, but the observation that the majority of the material, and all of the highest-molecular-weight forms, can be accessed by trypsin (Fig. 5) leads us to favor the idea that different cells produce radically different amounts. A pattern of Ig-binding heterogeneity has been found for cells in populations of certain strains of group A streptococci (29).

A feature of the Ig-binding activity observed in ec2, ec9, ec12, and ec72 was multiple banding (Fig. 1). Multiple bands could represent products of distinctly different genes, products of different members of a gene family that contain various numbers of repetitive sequences within the genes, differently processed or modified products of a single gene, or components of a high-molecular-mass complex not fully dissociated by being heated in sample buffer containing 10% SDS and 5% 2-mercaptoethanol. Our experiments did not distinguish among these possibilities. However, it is notable that multiple banding of Ig-binding proteins has been observed in hot-SDS

extracts from gram-positive organisms and, in the case of staphylococcal protein A, has been shown to be due to peptidoglycan fragments covalently bound to the Ig-binding protein (27). Multiple banding has also been observed for Ig-binding proteins of gram-negative bacteria (18, 35). For example, *H. somnus* produces Ig-binding forms with apparent molecular masses of 350, 270, 120, and 41 kDa (6, 34, 35). Experiments have suggested that the multiple bands represent different associations of the 41-kDa subunit (34, 35).

Our studies of environmental influences on the expression of Ig-binding activity are provocative. We have identified a temperature of 37°C and the stationary phase of culture as major factors that foster expression. Temperature is known to influence expression of numerous virulence factors in *E. coli* and related bacteria. Among them are fimbrial adhesins or pili required for attachment to host tissues and virulence factors required for invasion, intracellular replication, and spread to adjacent cells (reviewed in reference 17). The possible relationship between Ig-binding proteins and virulence has been discussed in numerous primary reports (5, 19, 23–26, 31) and reviews (4, 9, 30). The ECOR reference collection contains 72 natural strains of *E. coli* derived from animal and human hosts (22). All six of the Ig-binding strains we have identified were originally isolated from human hosts. Strains ec2, ec5, ec9, ec12, and ec43 were isolated from the feces of healthy hosts, and ec72 was isolated from the urine of a woman suffering from a urinary tract infection. We found that all other ECOR strains originally isolated from hosts suffering from urinary tract infections lacked Ig-binding activity. Thus, we found no obvious correlation with this type of virulence.

The ECOR reference collection was assembled to embrace the range of genetic diversity that exists among all *E. coli* strains (22). Genetic relationships among the strains have been established with multilocus enzyme electrophoresis (MLEE), and four clonal groups (A, B1, B2, and D) have been identified (11). Four of the Ig-binding strains (ec2, ec5, ec9, and ec12) belong to the same clade within MLEE group A, yet each strain is more closely related to at least one group A strain which lacks observable Ig-binding activity than it is to other group A strains which have this activity. Furthermore, each strain displayed a unique size range of Ig-binding material as visualized by Western blotting. Understanding this phenomenon will require delineation of the underlying genetics. It will be especially important to know if homologous genes encode the material in different strains and whether the absence of observable material in most strains is due to the absence of the critical structural gene or the lack of its expression. Work is in progress to address these questions.

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