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Specific Absence of Type 2 Fimbriae on a Coaggregation-Defective Mutant of *Actinomyces viscosus* T14V

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The coaggregation-defective (COG^-) mutant *Actinomyces viscosus* T14V(PK455), which is unable to participate in lactose-sensitive adherence, and its COG^+ parent were compared to structurally define the mutational loss of cell-associated lectin activity. Immunoelectrophoretic comparisons of crude extracts or isolated fimbriae from both strains demonstrated type 2 fimbriae (previously designated Ag2) in preparations obtained from the parent but none in those obtained from the mutant. This result was verified by the immunoelectron-microscopic identification of type 1 (previously designated Ag1 or VA1) and type 2 fimbriae on the parent organism but only type 1 fimbriae on the mutant. A comparison of the amounts of extractable fimbriae of each type and the capacity of the cells to bind ^{14}C-labeled monoclonal antibodies specific for each fimbrial component showed that the mutational loss of type 2 fimbriae had no significant quantitative effect on fimbriation of the COG^- mutant with type 1 structures. Cells of *A. viscosus* T14AV, a mutant with various adherence defects that include the COG^- phenotype, displayed fimbriae of both types, but in greatly reduced amounts. Thus, the properties of mutant strain T14V(PK455) associated the lectin activity with type 2 fimbriae, whereas those of strain T14AV provided little insight into the mechanism of lactose-sensitive adherence. In addition, the precise nature of the cell surface modification displayed by strain T14V(PK455) provides clear evidence for the existence of distinct and independent fimbriae on *A. viscosus* T14V.

Microbial adherence, colonization, and the subsequent initiation of oral inflammation are dependent on interactions of surface structures present on bacteria with those on other bacteria or oral tissues. Investigations of the adherence mechanisms of *Actinomyces viscosus* T14V have revealed two antigenically distinct types of fimbriae that differ in their functional properties (2). Type 2 fimbriae (previously designated Ag2) are the sites of a lactose-sensitive lectin (4, 20) and are responsible for both the coaggregation of actinomycete cells with certain plaque streptococci (5, 13, 16, 17) and the hemagglutination of neuraminidase-treated erythrocytes (8, 9). In contrast, type 1 fimbriae (previously designated Ag1 or VA1) may mediate the direct attachment of bacteria to the tooth surface. This possibility is favored by studies of *A. viscosus* T14V adherence to beads of saliva-treated hydroxyapatite (7, 22, 23), an interaction that is not inhibited by various sugars but is blocked by antibody against type 1 fimbriae. Thus, information from studies performed in vitro supports the concept that separate and specific structures on *A. viscosus* T14V are involved in the adherence of this organism to different surfaces within the oral environment.

The study of mutant bacterial strains with altered cell surface properties represents a feasible approach to defining the contributions made by individual components to bacterial adherence and colonization. The application of this approach to the oral actinomycetes has been limited by the lack of mutant strains with cell surface modifications that are structurally precise. For example, the surface of one mutant, *A. viscosus* T14AV, has been found to differ from that of its parent, *A. viscosus* T14V, in several respects, including decreased fimbriation (3), the appearance of a microcapsule (18), and the increased production of a viscous, extracellular polysaccharide (1, 10). Consequently, the various adherence defects displayed by this strain (1, 17, 23) and its decreased ability to colonize germfree rats and initiate periodontal pathology (1, 10) are probably not associated with a distinct structural change of the mutant cell surface.

Recently, a simple enrichment scheme employed the coaggregation of *A. viscosus* T14V with *Streptococcus sanguis* 34 to separate acti-

nomycete cells with cell-associated lectin activity from those that were coaggregation defective (COG^-). This selection resulted in the isolation of a spontaneous COG^- mutant unable to exhibit lactose-sensitive adherence (12). The phenotypic loss of lectin activity presumably can be explained by any of several defects in the structure and biosynthesis of type 2 fimbriae or by more complex changes affecting the accessibility of these structures. Thus, the present investigation, in which the cell surface antigens of the COG^- mutant were compared with those of its parent, was initiated to establish the structural difference between these strains.

MATERIALS AND METHODS

Bacterial strains. *A. viscosus* strains T14V and T14AV have been described previously (5, 8), as have the COG^- mutant *A. viscosus* T14V(PK455) and its antibiotic-resistant parent, *A. viscosus* T14V(PK256) (12). For most purposes of this study, bacteria were cultured in complex medium (5) or in a chemically defined medium, RPMI 1640 medium with L-glutamine (GIBCO Laboratories, Grand Island, N.Y.), as indicated.

Bacterial antigens. Crude preparations of soluble bacterial antigens were prepared by the Lancefield procedure as outlined by Powell et al. (18), by autoclaving cells in saline (10), and by extensive sonication (five 4-min periods) of washed bacteria with a Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) set at maximum power (185 W) and cooled with an ice bath.

The isolation of bacterial fimbriae involved continuous flow sonication at 100 W to remove fimbriae from intact cells and gel filtration chromatography of the soluble extract to separate the removed fimbriae from smaller components (3, 4, 20). In the present study, the fimbriae isolated by this procedure from *A. viscosus* T14V(PK256) and T14V(PK455) were further purified by precipitation in the presence of 40% saturated ammonium sulfate at 4°C. The precipitate was collected by centrifugation, dissolved in coaggregation buffer (0.15 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1 mM Tris-hydrochloride [pH 7.6], 0.02% sodium azide), and dialyzed against this buffer to remove ammonium sulfate.

The separation of type 1 from type 2 fimbriae has been described previously (20) and involved the use of affinity columns prepared by coupling monoclonal antibody 2A (4) against type 2 fimbriae to CNBr-activated Bio-Gel A150m (Bio-Rad Laboratories, Richmond, Calif.). Type 1 fimbriae passed through the column and were recovered, whereas type 2 fimbriae were specifically adsorbed and recovered after elution of the column with 3 M NaSCN. Enzyme-linked immunosorbent inhibition assays (see below) of these preparations in which specific monoclonal antibodies were used revealed 0.5% contamination of the type 1 preparation with type 2 fimbriae and less than 0.2% contamination of the type 2 preparation with type 1 fimbriae.

Rabbit antisera. The repeated intravenous injection of washed bacterial cells was used to prepare antiserum against *A. viscosus* T14V (6).

Monospecific antisera were prepared against type 1 and type 2 fimbriae. The immunization schedule consisted of two 50-μg doses of purified fimbriae administered subcutaneously in multiple sites. Primary injections were given in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), and boosters were given 3 to 4 weeks later in incomplete adjuvant. Immune rabbit immunoglobulin G (IgG) was isolated by DE-52 cellulose (Whatman, Inc., Clifton, N.J.) column chromatography from serum obtained 2 to 4 weeks after the final injection of antigen. To establish specificity, the antibodies against type 1 or type 2 fimbriae were titrated by enzyme-linked immunosorbent assay (see below) on plates coated with each purified component. These titrations showed that the immune IgG against each type of fimbriae was contaminated with detectable antibody against the other component. The contaminating antibody in each preparation was specifically removed by precipitation at equivalence with the corresponding purified fimbriae to produce monospecific reagents.

Monoclonal antibodies. The preparation and characterization of monoclonal antibody 2A(γG1κ) against type 2 fimbriae has been described previously (4). Experiments establishing the specificity of monoclonal antibody 8A(γG2aκ) against type 1 structures will be presented in a subsequent publication (manuscript in preparation). Both proteins were purified from ascites fluid (4).

Binding studies with ^{14}C-labeled monoclonal antibodies. Approximately 1 mg of each monoclonal antibody in 0.2 ml of phosphate-buffered saline (0.9% NaCl, 0.02 M sodium phosphate [pH 7.2], 0.02% sodium azide [PBS]) was labeled by reductive methylation with 1 mM [^{14}C]formaldehyde (57 mCi/mmol, New England Nuclear Corp., Boston, Mass.) in the presence of 20 to 50 mM $NaCNBH_3$ (11). The reaction mixture was incubated for 2 h at room temperature, applied to a small column (0.7 by 28 cm) of Sephadex G50 medium (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and eluted with this buffer. Labeled antibody was recovered in fractions collected at the void volume of the column and stored at 4°C. Measurements of radioactivity by scintillation counting in Ultrafluor (National Diagnostics, Inc., Somerville, N.J.) and measurement of protein (15) with rabbit IgG used as a standard gave specific activities of 1.07×10^4 cpm/μg of protein 2A and 1.03×10^4 cpm/μg of protein 8A. Fractions of the total radioactivity specifically bound by excess antigen were 82% for protein 2A and 85% for protein 8A.

Binding experiments were performed in 500-μl polypropylene Microcentrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.) by mixing constant amounts of labeled antibody (approximately 1 μg) with various quantities of bacteria in 0.2 ml of PBS containing 0.05% Tween 20. After occasional mixing at room temperature for 1 h, the tubes were spun for 7 min at 12,000 rpm in a Beckman model 12 Microfuge, and 100-μl volumes of each cell-free supernatant were carefully withdrawn for scintillation counting. Bound antibody was determined as the difference between total radioactivity added and the amount unbound. The data, plotted as micrograms of bound antibody versus milligrams (dry weight) of added bacteria, was linear (correlation coefficient, >0.99), and the slope, determined by linear regression, was used to estimate

the amount of antibody bound per milligram (dry weight) of bacteria. This simple approach was justified by the high apparent affinity ($K_a \geq 10^9$ M^{-1} [unpublished data]) of each monoclonal antibody.

Immunological methods. Crossimmunoelectrophoresis was performed as described previously (4, 20), and rocket-line immunoelectrophoresis was performed by the method of Krøll (14).

Enzyme-linked immunoassays for the quantitation of specific fimbrial components were performed by an inhibition technique similar to that of Rennard et al. (19). Various quantities of fimbrial antigen were incubated with constant amounts, generally 0.1 μg, of monoclonal antibody against type 1 or type 2 fimbriae in 0.2 ml of PBS–0.05% Tween 20. These mixtures were set up in wells of a microtiter plate, incubated overnight at 4°C, and transferred to antigen-coated wells of a flat-bottomed, polystyrene Microelisa plate (Dynatech Laboratories, Inc., Alexandria, Va.). The antigen-coated plate was prepared by prior incubation of the wells with unfractionated fimbriae at 5 μg/ml. The reaction of soluble monoclonal antibody with adsorbed antigen was allowed to proceed for 30 min at room temperature. The wells were washed with PBS–0.05% Tween 20, and bound antibody was detected with a perioxidase-conjugated, rabbit anti-mouse IgG reagent (Cappel Laboratories, Cochranville, Pa.). Values of optical density at 492 nm for individual wells were determined with a Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.) and were approximately 1.0 in the absence of soluble inhibiting antigen. A standard curve prepared from the inhibition data with each purified component was used to estimate the amounts of specific fimbriae in unknown samples.

The techniques for immunoelectron microscopy were those described previously (4, 6). In the present investigation, peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) was used to localize specific rabbit antibodies bound to the bacterial cell surface. Sections were lead stained (21) to enhance the visibility of unlabeled fimbriae.

RESULTS

Crossimmunoelectrophoresis revealed type 2 fimbrial antigen in crude extracts prepared from the COG⁺ parent strain, *A. viscosus* T14V(PK256), but not in those prepared from the COG⁻ mutant strain, T14V(PK455) (data not shown). Fimbriae isolated from both strains by identical procedures were compared by observing their reactivities with antiserum against *V. viscosus* T14V by rocket-line immunoelectrophoresis (Fig. 1A). The antigenic difference between strains was due to the isolation of type 2 structures from the parent but not from the mutant strain (Fig. 1C). Type 1 fimbriae isolated from both strains were identical, reacting as two separate but related components with the anti-T14V serum (Fig. 1A) and as a single component with monospecific serum (Fig. 1B). These observations, though pertinent to the antigenic structure of type 1 fimbriae, were not related to the difference between parent and mutant. The fimbriae removed by sonic extraction were quanti-

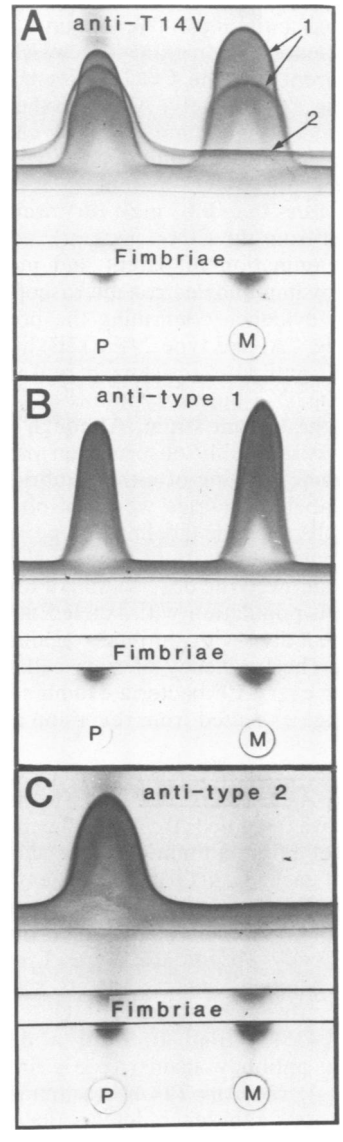

FIG. 1. Comparison of fimbriae isolated from the COG⁺ parent strain, *A. viscosus* T14V(PK256), with those isolated from the COG⁻ mutant strain, *A. viscosus* T14V(PK455), by rocket-line immunoelectrophoresis. The wells contained 5 μg of fimbrial protein from the parent (P) or the mutant (M) strain. The trough, labeled "Fimbriae," was filled with fimbrial antigens of *A. viscosus* T14V at 80 μg/ml in 1% agarose to provide reference lines for the comparison of parent and mutant antigens. The upper portion of agarose on different plates contained antiserum against *A. viscosus* T14V (A), type 1 fimbriae (B), and type 2 fimbriae (C). Arrows in (A) identify type 1 or type 2 fimbrial antigens. Electrophoresis with anode toward the top.

tated by enzyme-linked immunosorbent inhibition assays based on the reactions of specific monoclonal antibodies. The amounts of type 1 fimbriae removed per gram (dry weight) of the COG^+ parent and the COG^- mutant were 1.6 and 1.8 mg, respectively. Although the amounts of type 1 fimbriae removed from each strain were similar, type 2 fimbriae were extracted only from the COG^+ parent (3.6 mg/g [dry weight]). Less than 0.03 mg/g (dry weight) were recovered from the COG^- mutant.

The examination of parent and mutant cell surfaces by immunoelectron microscopy provided visual evidence confirming the presence of type 1 (Fig. 2A) and type 2 (Fig. 2B) fimbriae on the COG^+ antibiotic-resistant parent strain and type 1 (Fig. 2C) but no type 2 (Fig. 2D) structures on the mutant strain. Although unlabeled fimbriae were readily observed on parent cells after specific labeling of either fimbrial component, unlabeled fimbriae were not observed on mutant cells after the labeling of type 1 fimbriae. The extreme sensitivity of immunoelectron microscopy allowed the observation of rare cells in the mutant population with labeled fimbriae after the reaction with antibody against type 2 fimbriae. The frequency of such cells was less than 1 for every 10^6 bacteria examined, a value in the range expected from reversion of a mutation.

The binding of ^{14}C-labeled monoclonal antibodies to *A. viscosus* T14V and to its various mutants was studied to further compare the amounts of different fimbriae exposed on the cell surface of each strain (Table 1). As expected, *A. viscosus* T14V and the drug-resistant COG^+ strain, T14V(PK256), bound similar amounts of each antibody specific for type 1 or type 2 fimbriae. Though unaltered in its reaction for type 1 fimbriae, the COG^- mutant, T14V(PK455), failed to bind a detectable amount of antibody against type 2 fimbriae. In contrast, *A. viscosus* T14AV, a mutant known for its various adherence defects, including the COG^- phenotype (3, 17), bound each monoclonal antibody but in amounts greatly reduced from those of the T14V parent strain. These determinations, performed with cells grown in defined medium, were repeated with cells cultured in complex medium, and the results obtained (data not shown) followed the pattern described. It was found, however, that the amount of each antibody bound by cells grown in complex media was generally only about half that bound by cells grown in defined media.

DISCUSSION

The comparison of a COG^- mutant to its *A. viscosus* T14V parent strain has clearly linked the mutational loss of cell-associated lectin activity with the specific loss of type 2 fimbriae. This finding is in complete agreement with the previous detection of lectin activity in association with isolated type 2 structures (3, 4) and with the blocking of lectin-mediated bacterial adherence by Fab fragments specific for this component (20). Thus, three different lines of supporting evidence firmly establish lactose-sensitive adherence as an exclusive function of a single structure. Of equal significance to the present study was the demonstration that the loss of type 2 fimbriae occurred without an apparent change in the extent of fimbriation by type 1 structures, an observation that strongly indicates a cell surface modification that is limited to a single component. Although the genetic basis of this change remains to be determined, its effect is clearly different from that displayed in the surface architecture of *A. viscosus* T14AV. This mutant, initially distinguished by its altered ability to colonize germfree rats and cause periodontal pathology (1, 10), differs from its parent strain, *A. viscosus* T14V, in a number of surface-related properties (3), including a greatly reduced amount of detectable fimbriae (Table 1). The multiple defects of *A. viscosus* T14AV serve to emphasize the advantage of selection techniques for cell surface mutants that depend on specific properties of individual structures, such as the lectin activity of type 2 fimbriae, over those which involve multiple components, such as virulence.

Studies with mutants that are specifically modified should help to reveal the involvement of individual structures in host-parasite interactions. Thus, comparative studies of the COG^- mutant and its parent performed to define the adherence functions of type 2 fimbriae could be extended with appropriate animal models to provide information on the role of this structure in the complex process of microbial colonization. In vivo studies with the COG^- strain would, of course, have to take into consideration the occurrence of COG^+ revertants, a possibility raised by the observation from immunoelectron microscopy of type 2 fimbriae on rare cells in the mutant population. Although such revertants may be favored under certain conditions, their presence in low numbers should not diminish the usefulness of the existing COG^- strain when selective pressure is not a factor.

A further use of the COG^- strain is in studies of the type 1 fimbrial component. For example, an antiserum produced against this structure purified from the COG^- mutant was found to be monospecific (unpublished observation), whereas antiserum against the same component purified from the parent strain has invariably contained detectable antibody against type 2

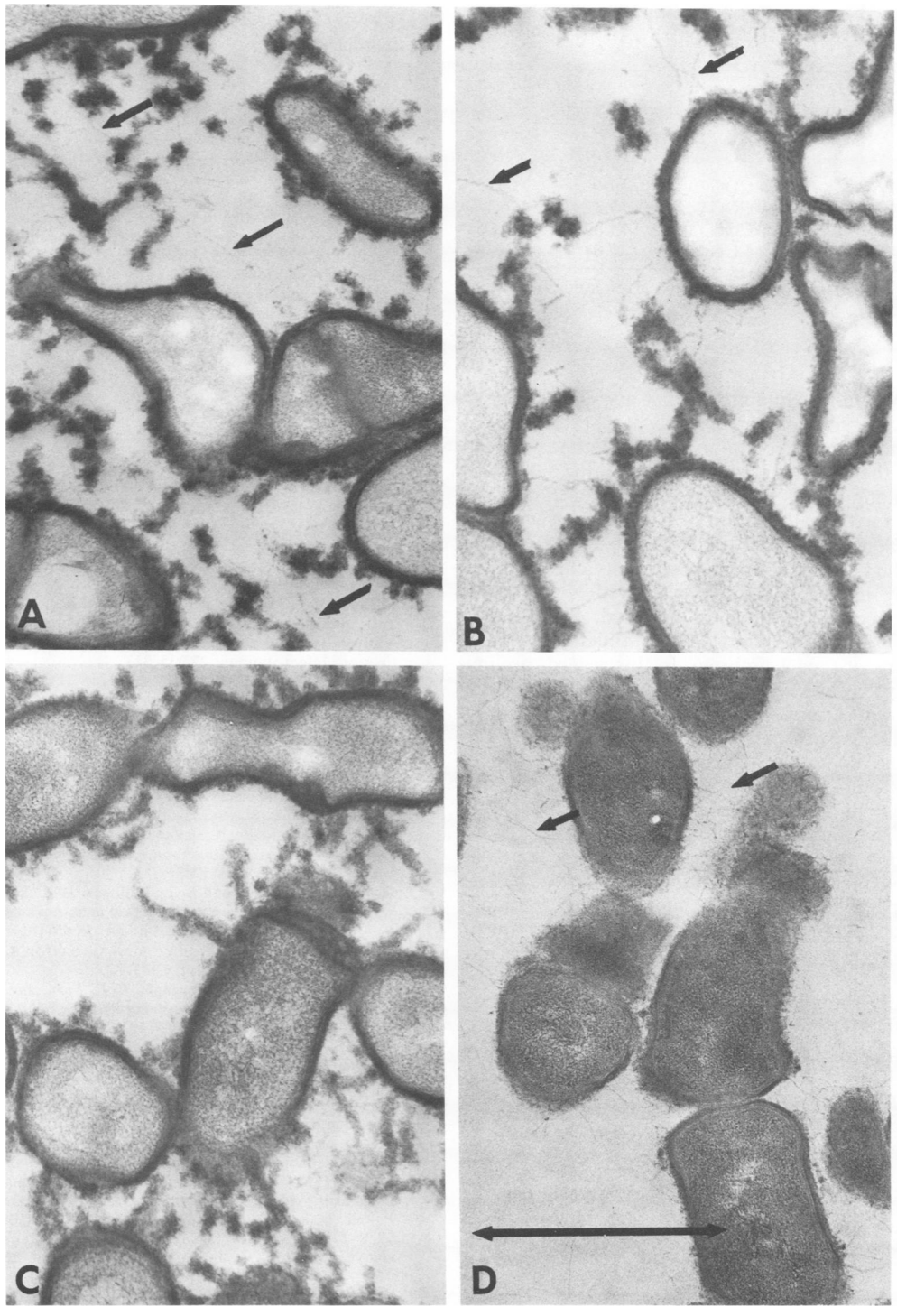

FIG. 2. Electron micrographs of thin sections of the COG$^+$ parent strain, *A. viscosus* T14V(PK256) (A and B), and the COG$^-$ mutant strain, *A. viscosus* T14V(PK455) (C and D). Bacteria were incubated with antibody against type 1 (A and C) or type 2 (B and D) fimbriae, washed, and reacted with peroxidase-conjugated goat anti-rabbit IgG. The presence of precipitate on certain fimbriae depicts sites of immunochemical labeling; the arrows (→) identify fimbriae that remained unlabeled (×35,000). The marker in (D) (↔) represents 1 μm.

TABLE 1. Binding of ^{14}C-labeled monoclonal antibodies to type 1 and type 2 fimbriae on *A. viscosus* cells

Strain[a]	Description	Antibody bound (μg/mg of bacteria [dry wt])	
		Anti-type 1 (8A)	Anti-type 2 (2A)
T14V	COG^+	16.6	14.7
T14V(PK256)	COG^+	15.3	16.1
T14V(PK455)	COG^-	15.8	<0.05
T14AV	COG^-	1.04	0.38

[a] Bacteria were cultured in a chemically defined medium.

fimbriae, a contaminant that accounts for 0.5% of the protein in the fimbriae preparation used as immunogen (see Materials and Methods). Clearly, the elimination of a contaminating antigen by genetic means represents a highly useful strategy for the purification of bacterial surface components and one that complements existing schemes that are based on affinity chromatography in which columns prepared with specific monoclonal antibodies are used. Studies with COG^- cells should also help to define the functions of type 1 fimbriae. In this regard, preliminary results show that cells of the COG^- and parent strains adhere equally well to beads of saliva-treated hydroxyapatite (W. B. Clark, personal communication), a finding that is consistent with the critical involvement of type 1 fimbriae in this adherence process (7, 22, 23).

The results presented here encourage the development of selection techniques for the isolation of a class of mutants specifically lacking type 1 fimbriae. Such mutants would complement the presently characterized COG^- strain in further studies of each fimbrial component. Moreover, a selection for cells lacking type 1 fimbriae applied to those already lacking type 2 structures should yield additional mutants lacking both components. Such strains would be extremely useful in a wide variety of studies, especially those in which the adherence functions of nonfimbrial cell surface structures are examined.

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