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Loss of Lectin-Like Activity in Aberrant Type 1 Fimbriae of *Escherichia coli*

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Growth of a streptomycin-resistant strain of *Escherichia coli* (VL-2) in the presence of 30 μg of streptomycin per ml resulted in the production by these bacteria of structurally altered, nonfunctional type 1 fimbriae. This strain, when grown in this subinhibitory concentration of streptomycin, became incapable of producing mannose-sensitive hemagglutination (<1% of that of the control). Adhering ability to epithelial cells and human leukocytes was also diminished (42 and 7% of that of the control, respectively). Although these streptomycin-treated bacteria were as heavily fimbriated as untreated bacteria, their fimbriae were significantly longer. Furthermore, in contrast to the fimbriae of the untreated bacteria, those isolated from the drug-treated bacteria were found to lack mannose binding activity as measured by hemagglutination. It appears, therefore, that streptomycin can cause even resistant bacteria to produce an aberrant fimbrial protein, possibly by causing misreading of messenger RNA. These studies indicate that the use of sublethal doses of certain antibiotics whose mode of action is well known may shed light on the genetic and chemical modulation of bacterial factors involved in mucosal colonization.

Colonization of mucosal surfaces depends on the ability of individual bacteria to adhere to individual host epithelial cells (10). In many strains of *Escherichia coli*, type 1 surface fimbriae, which bind to mannose residues on eucaryotic cells, have been implicated as the primary adhesin that mediates adherence of these organisms to mucosal surfaces (16). Recently, we reported that streptomycin in a concentration significantly below that required either to kill or to inhibit completely the growth of a strain of *E. coli* was still capable of altering the ability of the bacteria to agglutinate mannan-containing yeast cells and to adhere to human buccal epithelial cells (5). Type 1 fimbriae were found to be suppressed in number on drug-treated bacteria (4, 5). We were struck, however, by the occasional lack of correlation between degree of surface fimbriation and the adhesive properties of the bacterial population. We thought that the lack of adhesiveness in some of the streptomycin-treated organisms might have been due to the production of aberrant, nonfunctional fimbriae as a result of streptomycin-induced ribosomal misreading (7).

In the course of our studies, we isolated a mutant strain of *E. coli* which, although highly resistant to the lethal effects of streptomycin, consistently produced nonfunctional fimbriae when grown in low concentrations of this drug. We report here that this organism, when grown in the presence of streptomycin, demonstrated diminished adhesiveness for mammalian cells, that the fimbriae on the drug-grown organisms were significantly longer than those on the control organisms, and that the isolated fimbriae from drug-grown organisms lost their lectin-like activity.

MATERIALS AND METHODS

Microorganisms. During our studies on mannose binding activity of clinical isolates of *E. coli* (5), we isolated a spontaneous mutant strain, VL-2, that was streptomycin resistant (minimal inhibitory concentration, >1,000 μg/ml) and was used throughout this study. Strain VL-2 was cloned and purified and found to have maintained resistance to growth inhibition by streptomycin.

Candida albicans organisms were maintained on slants of Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and periodically subcultured. A standard suspension was obtained by subculturing the organisms in nutrient broth for 24 h at 37°C with constant shaking.

Cultural conditions. *E. coli* organisms were subcultured 1:100 from stationary-phase broth cultures and grown in nutrient broth (brain heart infusion; Difco) under static conditions at 37°C for 48 h. Identical portions were subcultured into broth containing various concentrations of antibiotics. Experiments were run in duplicate and included samples with sufficient antibiotic concentrations to determine the min-

imal inhibitory concentration. Growth was monitored by absorbance at 550 nm on a Coleman Junior II spectrophotometer (Coleman Systems, Irvine, Calif.) that had been calibrated against viable counts by standard dilution plating techniques or direct counts on a model Z_F Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). For the *E. coli* strains used in these experiments, 10^9 colony-forming units were equivalent to an optical density of 0.5 at 550 nm.

Yeast cell agglutination tests. *Candida* yeast cells harvested from 2 liters of broth were washed twice with 0.02 M phosphate–0.15 M NaCl (pH 7.4) (PBS) and resuspended in 100 ml of PBS containing 0.1% glutaraldehyde. The mixture was stirred for 1 h at room temperature, sedimented, washed again with PBS, suspended in 100 ml of PBS containing 0.1 M glycine, and stirred for 30 min at 22°C. The cells were then harvested, washed twice, and suspended in PBS–0.2% sodium azide to a concentration of 2×10^7 yeast cells per ml by a hemacytometer count. The yeast cell suspension was stored at 4°C for up to 2 months before use.

Agglutination tests were performed as previously described (5, 12, 14). The yeast agglutinating power of a test suspension was calculated by a modification of the method of Duguid and Gillies (2) as 10^{11} divided by the minimal concentration of bacteria producing yeast agglutination. Minimal concentrations of bacteria were computed by dividing the bacterial concentrations of the original suspension by the highest dilution (titer) capable of giving positive yeast cell agglutination.

Hemagglutination. Anticoagulated guinea pig erythrocytes were washed thrice in PBS and resuspended to 2%. Serial dilutions of test bacteria were prepared in microtiter wells as described previously (5). To each test well was added 20 μl of erythrocyte suspension. To test for mannose sensitivity, 25 μl of a 5% mannose solution was added to a duplicate well containing the undiluted bacteria. The mixtures were gently agitated and incubated at 22°C for 3 h, at which time hemagglutination was recorded. Hemagglutinating power was calculated as for yeast agglutinating power (described above).

Assay of bacterial adherence to human leukocytes. Mannose-sensitive binding of bacteria to leukocytes in a monolayer was performed by the method of Bar-Shavit et al. (1). Human peripheral leukocytes were prepared by sedimentation in dextran of heparinized venous blood from normal donors. Each 10 ml of blood containing 2 mg of heparin was mixed with 5 ml of 6% dextran and allowed to sediment at 22°C for 1 h. The plasma, containing leukocytes and a few erythrocytes, was withdrawn and layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) in a 1:1 ratio and sedimented for 30 min at 600 × *g*. The pellet was suspended in 10 volumes of 0.15 M ammonium chloride–1 mM ethylenediaminetetraacetic acid (EDTA)–10 mM carbonate buffer, pH 7.4, and incubated at 4°C for 5 min, followed by sedimentation for 5 min at 300 × *g* and two subsequent washes in PBS, pH 7.4. The washed pellet was resuspended in 2 ml of PBS, 50 μl of this leukocyte suspension was then carefully applied to glass cover slips (22 by 22 mm) in petri dishes, and the leukocytes were allowed to adhere at 22°C for 5 min. The nonadherent leukocytes were removed by serial gentle washes with 5 ml of PBS at 4°C. On each cover slip, 10^9 colony-forming units of bacteria in 1 ml of PBS was overlayered and incubated with gentle swirling for 30 min at 4°C, followed by aspiration and serial washing of cover slips in beakers containing cold PBS. In control experiments to assay the mannose sensitivity of the binding, 2.5% α-methyl mannose was incorporated into the bacterial suspensions.

Leukocytes were fixed in 95% methanol, and adherent cells were stained with Giemsa. Bacteria adherent to each of 25 leukocytes were counted by bright-field microscopy.

Assays of bacterial adherence to human epithelial cells. The adherence of bacteria to human buccal epithelial cells was performed as previously described (5, 9, 13). Test mixtures consisted of 10^5 epithelial cells per ml mixed with an equal volume of 10^9 streptococcal cells. After the unattached bacteria was rotated at ambient temperature for 30 min, they were separated by differential centrifugation, and the number of adherent bacteria was determined microscopically on stained smears of the epithelial cells.

Ultrastructural studies of fimbriation by electron microscopy. Bacterial suspensions were negatively stained with phosphotungstic acid and examined with an EM6B electron microscope (AEI Scientific Apparatus, Inc., Elmsford, N.Y.) by methods previously described (11). Fimbriae were enumerated by the method of Novotny et al. (8) and as previously described in detail (10). To correlate the degree of fimbriation with mannose-binding activity, fimbriae were enumerated in portions of the culture samples used in the mannose-binding assays.

Preparation of fimbriae and hemagglutination. Fimbriae were isolated from strain VL-2 grown in the presence or absence of streptomycin and partially purified by ammonium sulfate precipitation and sucrose density-gradient centrifugation as described by Silverblatt (17) and Silverblatt and Cohen (18). The ability of the isolated fimbriae to bind mannose residues was determined in assays of the agglutination of guinea pig erythrocytes in the absence and presence of 2.5% α-methyl mannoside as described by Salit and Gotschlich (16).

RESULTS

Characterization of strain VL-2. Strain VL-2 was shown to possess type 1 fimbriae as demonstrated by its ability to cause mannose-sensitive hemagglutination, yeast cell agglutination, mannose-sensitive binding to human leukocytes, and its possession of peritrichous organelles (Table 1). Moreover, it exhibited typical phase variation in its ability to increase its degree of fimbriation and pellicle formation after several cycles of growth in aerobic and static broths. When grown statically and aerobically in brain heart infusion broth, the growth of strain VL-2 was not inhibited by streptomycin up to a concentration of 1,000 μg/ml. In contrast, another aminoglycoside, neomycin, inhibited growth at a concentration of 60 μg/ml.

TABLE 1. *Effects on bacterial fimbriation and mannose-sensitive binding to eucaryotic cells caused by growing a strain of E. coli in streptomycin*

Growth in streptomycin (μg/ml)	% Fimbriation	Yeast agglutinating power	Hemagglutinating power	Hemagglutinating power with mannose	Epithelial cell adherence[a]	Leukocyte adherence[a]	Leukocyte adherence[a] with mannoside
	80	1,000	1,000[b]	<100	23.6 ± 13.7	23.3 ± 12.5[b]	1.9 ± 0.3
30	87	500	<100[c]	<100	10.7 ± 7.8[c]	1.8 ± 2.4[c]	1.4 ± 0

[a] Mean number of bacteria counted per cell ± the standard deviation.
[b] $P < 0.001$ as compared with the assay performed in the presence of mannose or mannoside.
[c] $P < 0.001$ as compared with drug-free bacteria.

Interference with mannose-sensitive adherence by growth in streptomycin. In our previous studies, we showed that subminimal concentrations of streptomycin inhibited both mannose-sensitive adherence and the degree of fimbriation in streptomycin-sensitive strains of *E. coli* (4, 5). All streptomycin-resistant strains of *E. coli*, including spontaneously derived mutants of the clinical strain VL-1 (5), and strains with the known ribosomal mutation, *rpsL* (4), were absolutely resistant to these subinhibitory effects of streptomycin. In contrast, when the streptomycin-resistant strain VL-2 was grown in 30 μg of streptomycin per ml, the strain lost its ability to hemagglutinate guinea pig erythrocytes and to adhere either to human oral epithelial cells or to human leukocytes in a monolayer but retained its ability to agglutinate yeast cells (Table 1). Prior trypsinization of erythrocytes did not affect the results, suggesting that steric interference of the ligand-receptor interaction was not a major factor. The suppression of hemagglutination and of epithelial cell and leukocyte adherence in strain VL-2 was highly significant statistically ($P < 0.001$). The lack of suppression of yeast cell agglutination is unexplained but might indicate a fundamental difference between mannose receptors on human cells and yeast cells in their affinity for the bacterial adhesin.

Aberrant fimbriae on streptomycin-treated bacteria. Because type 1 fimbriae are the known mediators of mannose binding (16), it was of particular interest to examine fimbriation in the streptomycin-resistant strain VL-2. Examination of this strain by electron microscopy revealed that drug-treated organisms had as many fimbriae as the untreated organisms (Table 1), but the fimbriae on the drug-treated organisms appeared longer than those on untreated organisms (Fig. 1). These results suggested that the fimbriae of the drug-treated organisms must have been altered in some way to lose their adhesive properties.

To examine the possibility that the fimbriae of drug-treated *E. coli* VL-2 lacked adhesiveness, fimbriae isolated and purified from *E. coli* VL-2 grown for 48 h in the presence or absence of streptomycin (30 μg/ml) were compared for their ability to agglutinate guinea pig erythrocytes. As can be seen in Table 2, the fimbriae from the streptomycin-treated organisms totally lacked agglutinating activity at a concentration 15-fold higher than the minimal agglutinating concentration of the fimbriae from untreated *E. coli* VL-2. Moreover, ultraphotomicrographs of the purified fimbrial preparations revealed marked morphological differences between the two samples (Fig. 2). Fimbriae isolated from drug-treated bacteria were more homogeneous in size and significantly longer (Table 2). Thus, bacteria grown in the presence of streptomycin synthesized fimbriae which were both structurally distorted and functionally inactive.

TABLE 2. *Loss of lectin-like activity and altered structure of fimbriae isolated from streptomycin-treated E. coli VL-2*

Culture condition[a]	Minimal amount (μg/ml) of protein needed to cause hemagglutination[b]	Mean length (μm) of fimbriae (±SD[c])
Without drug	35	0.15 ± 0.8
With streptomycin, 30 μg/ml	>500	0.39 ± 0.15[d]

[a] Bacteria were cultured with or without antibiotic as described in the text.
[b] No hemagglutination in the presence of α-methyl mannoside.
[c] SD, Standard deviation.
[d] $P < 0.001$ as compared with the control.

Specificity of the streptomycin effect. To assay the specificity of the streptomycin effect on fimbriae, we grew strain VL-2 in subminimal concentrations of neomycin (30 μg/ml). We found that both the degree of fimbriation and hemagglutinating activity were suppressed in the neomycin-treated organisms (<20% of those of the control). Therefore, in the presence of neomycin, strain VL-2 behaves like other neomycin-sensitive strains (4).

Reversal of sublethal effects upon sub-

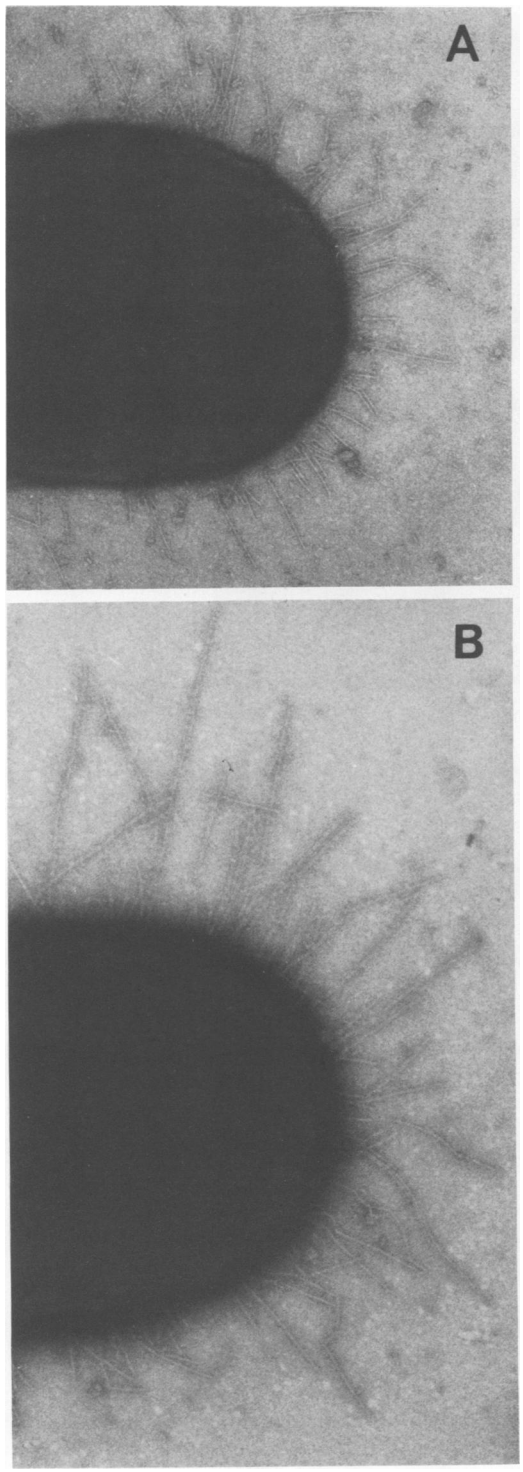

FIG. 1. *Electron micrographs of streptomycin-resistant E. coli strain VL-2 grown for 48 h without (A) and with (B) 30 μg of streptomycin per ml. Note longer fimbriae of the cell grown in the presence of streptomycin. ×30,000.*

culture to antibiotic-free broth. To determine the reversibility of the streptomycin-induced suppression of adherence to mammalian cells, we subcultured serially the antibiotic-treated samples into drug-free medium. All cultures recovered full activity, as measured by the hemagglutination assay, by the second or third subculture. Thus, growth in streptomycin did not promote the selection of a mutant strain defective in adhering ability.

Requirement of growing bacteria for the antibiotic effect. To demonstrate that the antibiotic effect was on the production of fimbriae and not on the fimbriae themselves, various concentrations of streptomycin (up to 500 μg/ml) were added to the microtiter wells containing stationary-phase bacteria that possessed measureable hemagglutinating activity (>500 U). Hemagglutination power was unchanged by the addition of streptomycin. In addition, we found that washing of bacteria previously grown in streptomycin did not restore the suppressed binding activity. Thus, streptomycin needed to be present only during the period of active bacterial growth and fimbrial synthesis to affect binding activity, a result which is consistent with our previous findings (5).

DISCUSSION

Streptomycin inhibits or distorts protein synthesis by binding to the S-12 protein of sensitive ribosomes (15) and thereby interferes with the translation of messenger RNA (7). Subinhibitory concentrations of streptomycin have been known for some time to distort rather than inhibit protein synthesis, with resultant production of aberrant proteins by the treated bacteria (3). The notion that this distorting effect of streptomycin was responsible for diminished mannose binding activity of *E. coli* in our studies is based on the finding that drug-treated *E. coli* VL-2 produced normal amounts of fimbrial protein with abnormal structural and adhesive properties.

In a previous communication (4), we demonstrated that K-12 strains of *E. coli* with the ribosomal mutation (*rpsL*) of the streptomycin receptor protein were resistant to the subinhibitory and lethal effects of streptomycin. We infer that strain VL-2 also possesses the mutation *rpsL* because its high level of resistance to the lethal effects of streptomycin was specific for that antibiotic. Unlike other streptomycin-resistant strains, however, strain VL-2 was sensitive to some of the subinhibitory effects of the antibiotic. Although we have not yet worked out the genetics of streptomycin resistance in strain VL-2, some of its properties are reminiscent of work done by Gorini (7). He defined those bac-

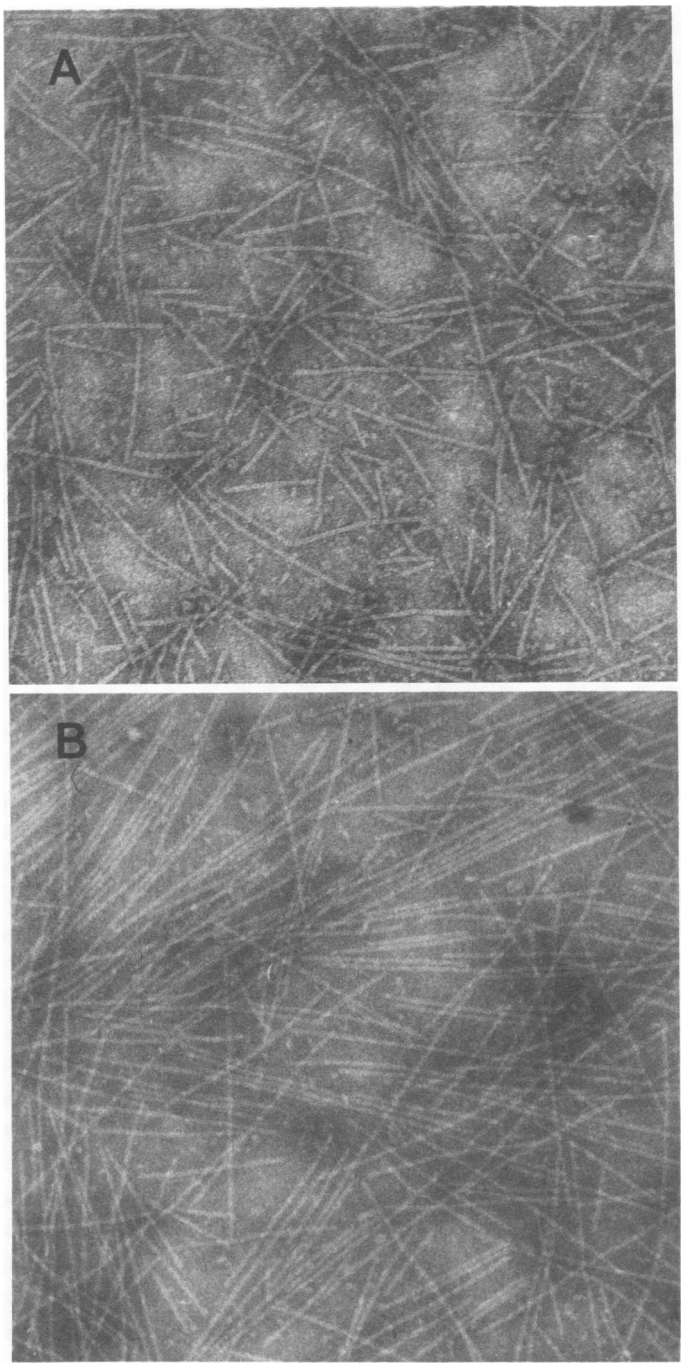

FIG. 2. *Electron micrographs of purified fimbriae from E. coli strain VL-2 grown for 48 h without (A) and with (B) 30 μg of streptomycin per ml. Fimbriae from drug-treated bacteria (B) are significantly ($P < 0.001$) longer than those from controls (A).* ×90,000.

teria that were either fully or partially susceptible to streptomycin as possessing "competent" ribosomes. Full susceptibility results in lethality; partial susceptibility results in protein mistranslation and ribosome readthrough of nonsense mutations (extragenic suppression). A highly resistant organism with the mutation *rpsL* and "incompetent" ribosomes could be-

come competent by acquiring the second ribosomal mutation *ram*. In these doubly mutant strains, streptomycin causes increased protein mistranslation but not killing (7). Careful genetic analysis of strain VL-2 may determine whether or not it possesses the two mutations *rpsL* and *ram*. Though the presence of such double mutants among naturally occurring *E. coli* is, as yet, unrecognized, Eisenstein and Sparling recently demonstrated analogous double mutations in 15% of clinical isolates of gonococci (6).

Fimbriae are proteinaceous filaments that are composed of protomeric subunits (16). Presumably, the lectin-like activity of type 1 fimbriae derives from the primary structure of the repeating subunits. Since all of the known effects of subinhibitory concentrations of streptomycin result from drug-induced mistranslation of messenger RNA (7), we infer that the loss of lectin-like activity of fimbriae from drug-treated bacteria was probably due to mistranslation of fimbrial protein. Alternatively, streptomycin may be indirectly distorting the functional expression of mannose-specific adhesin by affecting another gene product. We are now attempting to discriminate between these two possibilities by comparing the primary structures of subunits isolated from drug-treated and control bacteria. The use of strain VL-2 in these comparative studies may reveal the primary structural requirements of the mannose-specific adhesin.

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