INFECTION AND IMMUNITY, Apr. 1980, p. 154-159
0019-9567/80/04-0154/06$02.00/0

Vol. 28, No. 1

Influence of Sublethal Concentrations of Antibiotics on the Expression of the Mannose-Specific Ligand of *Escherichia coli*

BARRY I. EISENSTEIN,* EDWIN H. BEACHEY, AND ITZHAK OFEK

Departments of Medicine and Microbiology, University of Tennessee, and the Veterans Administration Hospital, Memphis, Tennessee 38163

The ability of streptomycin, in subinhibitory concentrations, to differentially suppress the acquisition of the mannose-binding activity of *Escherichia coli* was demonstrated in several strains, but not one with a ribosomal mutation to high-level streptomycin resistance, *rpsL*. We also determined that the growth of bacteria in other antibiotics, notably those that interfere with protein synthesis, resulted in diminished mannose-binding activity (as measured by yeast cell agglutination), degree of piliation (as measured by electron microscopy), and adherence to human oral epithelial cells. The aminoglycoside antibiotics streptomycin, gentamicin, and neomycin had the most marked effects relative to their minimum inhibitory concentrations, followed by tetracycline. Both spectinomycin and chloramphenicol had more effect on adherence than on piliation, although spectinomycin had a more pronounced effect on mannose-binding activity than did chloramphenicol. We conclude that antibiotics, at concentrations below their minimum inhibitory concentration, may have profound effects on surface properties of bacteria that may be pertinent for their ability to colonize and infect human mucosal surfaces. The mechanism(s) may vary from one drug to another, but appear to depend on the classic actions of the antibiotics on inhibiting protein synthesis.

Investigations on antibiotic-bacteria interactions have focused upon killing or inhibition of growth of bacteria rather than upon the expression of bacterial virulence factors. One such factor of potential clinical importance is the production of surface ligands which enable the bacteria to adhere to and subsequently colonize host cells (9). Many strains of *Escherichia coli* possess surface ligands that bind the organisms to mannose residues on human epithelial cells (17). The binding activity of these mannose-specific ligands could be quantitated directly on intact organisms by aggregometry with mannan-containing yeast cells (14). Recently, we demonstrated that when a clinical isolate of *E. coli* was grown in varying sublethal concentrations of streptomycin, there was a dose-related interference with the strain's development of mannose-binding activity (7). Concomitant with the drug-induced suppression of mannose binding, as measured by yeast cell agglutination, antigenic and ultrastructural alterations on the surface of the drug-grown organisms were observed. The purpose of the present investigation was to ascertain whether the same type of suppression could be demonstrated in well-characterized laboratory isolates of *E. coli* which develop mannose-binding activity in the stationary phase of growth and whether other antibiotics were capable of producing similar effects. We found that the streptomycin effect could be observed in a variety of strains and that a variety of antibiotics which interfere with protein synthesis caused suppression of mannose-specific ligand.

MATERIALS AND METHODS

Microorganisms and cultivation techniques. The *E. coli* strains studied are listed in Table 1. Selection of single-step mutants of strain M-Str^s was performed by standard techniques (11). *Candida albicans* organisms were maintained on slants of Sabourand dextrose agar (Difco) and periodically subcultured. A standard suspension was obtained by subculturing the organisms in nutrient broth for 24 h at 37°C with constant shaking.

E. coli strains were subcultured 1:100 from stationary phase broth cultures and grown in brain heart infusion broth (BBL Microbiology Systems) under static conditions at 37°C for 48 h. Identical portions were subcultured into broth containing increasing concentrations of antibiotics. Experiments were run in duplicate, and included samples with sufficient antibiotic levels to determine the minimum inhibitory concentration (MIC). Growth was monitored by absorbance on a junior Coleman spectrophotometer (Coleman Systems, Irvine, Calif.) at 550 nm, which had been calibrated against viable counts by standard dilution plating techniques or direct counts in a model

TABLE 1. *Strains of E. coli*

Strain	Characteristics	Origin and reference
M-Str^s	Clinical isolate, antibiotic sensitive	Our laboratory (7, 14)
M-Str^r	Single-step mutant, streptomycin resistant	Our laboratory (7)
M-Spc^r	Single-step mutant, spectinomycin resistant	Our laboratory
Bam P$^+$	B, type 1 pili, antibiotic sensitive	Brinton (4, 19)
CSH 43	K-12, antibiotic sensitive	Cold Spring Harbor Laboratory (12)
CSH 61	K-12, antibiotic sensitive	Cold Spring Harbor Laboratory (12)
CSH 50	K-12, streptomycin resistant (*rpsL*)	Cold Spring Harbor Laboratory (12)

ZF Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). For the *E. coli* used in these experiments, 10^9 colony-forming units were equivalent to an optical density of 0.3 at 550 nm.

Yeast cell agglutination tests. Candida yeast cells harvested from 2 liters of broth were washed twice with phosphate-buffered saline (PBS) and resuspended in 100 ml PBS containing 0.1% gluteraldehyde. The mixture was stirred for 1 h at room temperature, sedimented, washed again with PBS, resuspended in 100 ml PBS containing 0.1 M glycine, and stirred for 30 min at 23°C. The cells were then harvested, washed twice, and resuspended in PBS to a concentration of 2×10^7 yeast cells per ml, with a hemocytometer for counting. After adding sodium azide (0.02%) as preservative, the yeast cell suspension was stored at 4°C for up to 2 months before use.

Agglutination tests were by methods previously described (7). Briefly, equal volumes (10 μl) of standard yeast cell suspension and dilutions of bacterial suspensions were mixed on a glass slide, and the slide was rotated gently. A positive reaction would give course clumping, visible to the naked eye, within 60 s. Dilutions of bacterial suspensions were prepared by pipetting 20 μl of each test suspension, with known bacterial counts, to the first row of wells in U-bottomed clear microtiter trays (Cooke Laboratory Products, Alexandria, Va.), adding 20 μl of PBS in wells in each row, and serially (1:2) diluting and mixing bacterial suspensions. The yeast agglutination power (YAP) of a test suspension was calculated by a modification of the method of Duguid and Gillies (6) as 10^{11} divided by the minimal concentration of bacteria producing yeast cell agglutination. Minimal concentrations of bacteria were computed by dividing the bacterial concentration of the original suspension by the highest dilution (titer) capable of giving positive yeast cell agglutination. No difference in YAP was noted when dilutions were made directly from cultures or from prewashed bacteria.

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 above. To each test well, 20 μl of erythrocyte suspension was added. To test for mannose sensitivity, 25 μl of 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 YAP, described above.

Adherence tests. The experiments were performed by methods described previously (7, 16). Briefly, 0.5 ml of a suspension of epithelial cells (2×10^5 cells per ml in PBS) from the buccal mucosa of one of us was mixed with 0.5 ml of washed *E. coli* adjusted to 1×10^8 to 2×10^8 bacteria per ml. The mixture was rotated end over end for 30 min at room temperature. The epithelial cells were separated from nonadherent bacteria by repeated differential centrifugation, resuspended in 0.05 ml of PBS, and smeared on a glass microscope slide. After drying, the preparation was stained with gentian violet and examined with a bright-field microscope. Slides containing more than four nonadherent bacteria per oil immersion field were omitted from analysis. Adherence was recorded as the average number of bacteria per epithelial cell of 20 counted cells. A control sample of epithelial cells without added bacteria was always counted concurrently. Results of adherence tests were omitted when the background counts exceeded five bacteria per epithelial cell.

Ultrastructural studies of piliation by electron microscopy. Bacterial suspensions were negatively stained with phosphotungstic acid and examined with an AEI, EM6B electron microscope (AEI Scientific Apparatus, Inc., Elensford, N.Y.) by methods previously described (15). Pili were enumerated by the method of Novotny et al. (13) and as previously described in detail (14). To correlate degree of piliation with mannose-binding activity, pili were enumerated in aliquots of the culture samples used in the mannose-binding assays.

RESULTS

Influence of streptomycin on laboratory isolates. To demonstrate the general nature of the streptomycin effect on mannose-binding activity, we measured the influence of streptomycin on selected B and K-12 derivatives of *E. coli*. Samples tested after 48 h of growth demonstrated greater activity than those tested after 24 h of growth (Table 2). We found a dose-

TABLE 2. *Suppression of yeast agglutinating power in E. coli grown in sublethal concentrations of streptomycin*

Concn of streptomycin (μg/ml)	YAP[a] of:					
	Organisms grown 24 h			Organisms grown 48 h		
	Bam P$^+$	CSH 43	CSH 61	Bam P$^+$	CSH 43	CSH 61
0	8,131	7,639	2,814	19,084	18,639	5,525
2	4,109	3,889	953	9,099	7,089	1,700
5	4,300	4,193	<200	4,893	5,775	228
10	2,104	1,625	<200	1,864	3,216	<200

[a] See Materials and Methods for derivation. The results are expressed as geometric means of replicate determinations.

dependent suppression of YAP in all Str^s strains grown in increasing subinhibitory concentrations of streptomycin (Table 2). When guinea pig erythrocytes were used as mammalian target cells, all strains demonstrated mannose-sensitive hemagglutinating activity proportional to YAP and hemagglutinating power was similarly suppressed by streptomycin (data not shown). These results are in agreement with our previous observations concerning the clinical isolate, M-Str^s (7). In contrast, the ribosomal mutant CSH 50 was resistant to the ligand-suppressant effect at concentrations of streptomycin as high as 1,000 μg/ml.

Influence of subinhibitory concentrations of other antibiotics on mannose-binding activity. In experiments designed to test the effects of other antibiotics at subinhibitory concentrations, our clinical isolate, M-Str^s, was grown with or without antibiotics, and mannose binding was measured after 48 h of growth. Antibiotics used with their MICs for M-Str^s are listed in Table 3. To normalize the degree of YAP suppression, mannose binding was expressed as relative YAP (YAP of antibiotic-treated ÷ YAP of untreated × 100%) and amount of antibiotic used was expressed as percentage of MIC (concentration of antibiotic ÷ MIC × 100%). Results (Fig. 1) demonstrated a marked dose-dependent suppression of mannose binding by the aminoglycosides, neomycin, gentamicin, and streptomycin and a reproducible, but less marked suppression by spectinomycin and tetracycline. Chloramphenicol had a minimal effect at 50% MIC, and the remaining antibiotics had no reproducible effect whatsoever. (Analysis of rifampin and naladixic acid was hampered by the frequent and rapid emergence of mutants resistant to those antibiotics.) For those antibiotics that had an effect, the timing

TABLE 3. *MICs of antibiotics studied*

Antibiotic	MIC (μg/ml)[a]
Streptomycin	30–60
Gentamicin	20
Neomycin	30
Chloramphenicol	10
Tetracycline	5
Spectinomycin	100
Rifampin	>128[b]
Clindamycin	>128
Erythromycin	128
Novobiocin	128
Naladixic acid	8–>128[b]

[a] MIC defined as the concentration required to prevent visible growth in brain heart infusion after 48 h of incubation.

[b] Mutants with high-level drug resistance almost invariably selected out after 48 h of incubation.

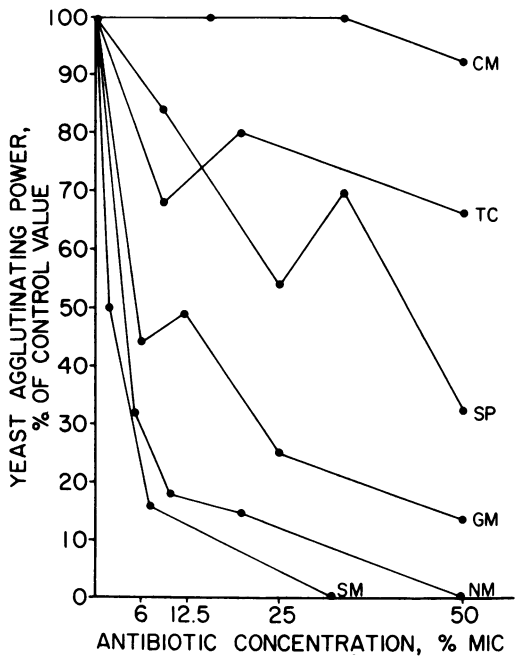

FIG. 1. *Antibiotic-induced suppression of YAP of E. coli M-Str^s. The yeast agglutinating power of cells grown for 48 h in subinhibitory concentrations of various antibiotics was compared to that of cells grown without drug. Results are expressed as the means of at least four separate experiments. Antibiotics studied included: CM, chloramphenicol; TC, tetracycline; SP, spectinomycin; GM, gentamicin; NM, neomycin; and SM, streptomycin.*

of antibiotic addition was critical; similar to our earlier studies (7; I. Ofek et al., *Reviews of Infectious Diseases*, in press), no suppression was seen when antibiotic was added to cultures which had already acquired mannose-binding activity (data not shown). In general suppression of hemagglutinating power closely paralleled suppression of mannose-binding activity.

Cross-sensitivity of single-step mutants to streptomycin and spectinomycin resistance. To verify that single-step mutation to high-level resistance to either streptomycin or spectinomycin, presumably ribosomally mediated (1), would result in specific resistance to the sublethal suppressive effect on mannose-binding activity, we analyzed the cross-sensitivity of mutant strains. In yeast cell agglutination tests, M-Str^r behaved as M-Str^s when grown in spectinomycin but not in streptomycin, and strain M-Spc^r behaved as M-Str^s when grown in streptomycin but not in spectinomycin. (M-Spc^r was resistant to the subinhibitory as well as the inhibitory effects of spectinomycin.)

Reduced ability of antibiotic-grown *E. coli* to adhere to human epithelial cells. We

reported previously that adherence of *E. coli* M-Str^s to epithelial cells was mannose sensitive and that growth of this strain in sublethal concentrations of streptomycin caused marked suppression of its adherence ability concomitant with suppression of mannose-binding activity (7). In the present study we found that similar effects were seen with other antibiotics at concentrations 25 to 50% MIC (Table 4), although the streptomycin effect was the most pronounced. Of note is the reduction of adherence seen with chloramphenicol despite the minimal reduction of YAP of chloramphenicol-grown cells (Fig. 1).

Reduced degree of piliation induced by growth of *E. coli* in subinhibitory concentrations of antibiotics. Growth of *E. coli* M-Str^s in streptomycin, gentamicin or tetracycline, but not chloramphenicol or spectinomycin, resulted in a reduction of the degree of piliation of those cultures (Table 5). Except for spectinomycin, the reduction closely paralleled the reduction of YAP induced by those antibiotics (see Fig. 1).

TABLE 4. *Adherence to human epithelial cells by E. coli M-Str^s grown with or without subinhibitory concentrations of antibiotic*

Antibiotic	Adherence[a]	*P* value[b]
None	27.8 ± 9.8	
Streptomycin	5.0 ± 1.6	<0.001
Gentamicin	13.3 ± 4.9	<0.025
Chloramphenicol	16.7 ± 1.5	0.05
Tetracycline	10.3 ± 5.7	<0.005
Spectinomycin	14.3 ± 3.2	<0.025

[a] Mean number of bacteria per epithelial cell from at least triplicate determinations ± standard deviation.

[b] Derived from two-tailed Student's *t* test comparing adherence of bacterial grown with antibiotic to that without antibiotic.

TABLE 5. *Degree of piliation of E. coli M-Str^s grown with or without subinhibitory concentrations of antibiotic*

Antibiotic	Degree of piliation[a]	*P* value[b]
None	84.4 ± 5.7	
Streptomycin	25.0 ± 11.3	<0.001
Gentamicin	53.0 ± 16.5	<0.001
Chloramphenicol	84.7 ± 6.4	NS[c]
Tetracycline	71.7 ± 13.1	<0.01
Spectinomycin	79.3 ± 21.8	NS

[a] Mean degree of piliation from at least triplicate determinations ± standard deviation. Degree of piliation defined as number of bacteria possessing at least 50 or more type 1 pili attached to the cell surface ÷ total number of bacteria examined by electron microscopy × 100%.

[b] Derived from two-tailed Student's *t* test comparing degree of piliation of bacteria grown with antibiotic to that without antibiotic.

[c] NS, Not significant.

DISCUSSION

We have previously demonstrated that in non-antibiotic-treated cultures, mannose-binding activity correlated with the ability of the organisms to adhere to mannose residues on epithelial cells (14) and that cells grown in sublethal concentrations of streptomycin or penicillin lost their ability to adhere to epithelial cells proportionate to their loss of mannose binding activity (7; Ofek, et al., in press). Salit and Gotschlich have shown that the mannose-sensitive ligand of *E. coli* is a protein and resides on the type 1 pilus (19). Our own studies have provided indirect support for the importance of pili for procaryote-eucaryote binding by showing that heavily piliated cells, whether antibiotic treated or not, adhered better than poorly piliated cells (7, 14; Ofek et al., in press).

Although the present study showed a high degree of association among the properties—piliation, mannose binding, and adherence—some appeared to be more sensitive than others to suppression by different antibiotics. Chloramphenicol and tetracycline affected adherence out of proportion to piliation and YAP, whereas spectinomycin affected both adherence and YAP but not piliation. One explanation for the lack of absolute congruence is that our measurements were insufficiently quantitative to detect important, but small, differences between samples. Another is that the requirements for agglutination and adherence may be different. Such unmeasured parameters as surface charge, steric interference of ligands, and surface hydrophobicity (9) may be more important for one effect than the other.

Specific structures on the bacterial surface are likely to be resonsible for the nonpilus factors which influence binding. Swanson (21) has presented evidence that the attachment of gonococci to human leukocytes is mediated by so-called LA factor which is distinct from pilus and which is differentially destroyed by various chemical treatments. Watt and co-workers have demonstrated the importance of sugar residues of lipopolysaccharide for bacterial adherence (22). Subinhibitory concentrations of various antibiotics have been shown to cause differential inhibition or enhancement of various surface components of bacteria. Streptomycin and neomycin increase phospholipid in *Serratia* (2); kasugamycin, puromycin, and rifampin inhibit cytoplasmic proteins, whereas tetracycline and sparsomycin inhibit outer envelope proteins in *E. coli* (8); spectinomycin alone induces disappearance of membrane protein I-19 in *E. coli* (12). Other studies have shown that antibiotics induce changes in surface morphology (10), increase the organism's sensitivity to the bacteri-

cidal action of serum (20), or interfere with genetic transfer than may involve surface components: chloramphenicol reduces gonococcal competence for transformation (3) and clindamycin inhibits conjugal transfer between *E. coli* strains (5).

Our data suggest that antibiotic suppression of the mannose-specific ligand is a result of classic mechanisms of antibiotic action (18). First, the known ribosomal mutant strain CSH 50 was resistant to the lethal and sublethal effects of streptomycin. Single-step mutants resistant to either streptomycin or spectinomycin growth inhibition demonstrated cross-sensitivity to the subinhibitory effects of the heterologous antibiotic but resistance to those of the homologous antibiotic. We conclude that drug-induced suppression by ribosome-active antibiotics requires sensitive ribosomes. Second, suppression by antibiotics which inhibit protein synthesis required actively growing organisms that had not yet acquired mannose-binding activity; there was no direct effect on preformed ligand. In a previous study (Ofek et al., in press) we demonstrated that penicillin, a surface-active antibiotic, could suppress adhering activity concomitant with the formation of filaments and inhibition of piliation. The present study showed that antibiotics which interfere with protein synthesis at the ribosome have similar effects on adherence and piliation, although presumably through different mechanism(s). Acquisition of functional ligand activity by *E. coli* may require three sequential steps: synthesis of subunits, their assembly, and expression (4). Cell wall-active antibiotics which interfere with carboxypeptidase activity, such as penicillin, may inhibit the terminal steps of ligand expression by preventing the needed anchoring of surface pili (Ofek et al., in press). By analogy, a temperature-sensitive mutant of *E. coli* with diminished carboxypeptidase activity at restrictive temperatures grew as nonseptate filaments lacking pili and mannose-binding activity at the restrictive temperature, but as a fully piliated, adherent rod at permissive temperature (Ofek et al., in press). Ribosome-mediated suppression of mannose binding may be due to either preferential inhibition of one or more of the three independent steps or due to distorted translation of mRNA coding for protein(s) necessary for ligand activity. Irrespective of the mechanism of action, the observation that concentrations of diverse antibiotics which may reach mucosal surfaces during therapy can affect the ability of a common bacterial species to adhere to mucosal cells may have great importance for bacterial colonization and ecology.

ACKNOWLEDGMENTS

We thank V. Long, L. Hatmaker, and S. White for technical assistance. This work was supported by program-directed research funds from the United States Veterans Administration and by Public Health Service research grants AI-13550 and AI-10085 from the National Institute of Allergy and Infectious Diseases. E.H.B. is the recipient of a Medical Investigatorship Award from the U.S. Veterans Administration.

LITERATURE CITED

1. **Benveniste, R., and J. Davies.** 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. **42**:471-506.
2. **Bermingham, M. A. C., D. S. Deol, and J. L. Still.** 1970. Effect of streptomycin on lipid composition with particular reference to cyclic dyssipeptide biosynthesis in *Serratia marcescens* and other organisms. Biochem. J. **119**:861-869.
3. **Biswas, G. D., T. Sox, E. Blackman, and P. F. Sparling.** 1977. Factors affecting genetic transformation of *Neisseria gonorrhoeae*. J. Bacteriol. **129**:983-992.
4. **Brinton, C. C.** 1965. The structure, function, synthesis, and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. **27**:1003-1054.
5. **Coats, J. H., and J. Roeser.** 1977. *In vivo* inhibition of R-factor transfer by clindamycin and its analogues. J. Antimicrob. Chemother. **3**(Suppl. C):53-58.
6. **Duguid, J. P., and R. R. Gillies.** 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. **74**:397-411.
7. **Eisenstein, B. I., I. Ofek, and E. H. Beachey.** 1979. Interference with the mannose binding and epithelial cell adherence of *Escherichia coli* by sublethal concentrations of streptomycin. J. Clin. Invest. **63**:1219-1228.
8. **Hirashima, A., G. Childs, and M. Inouye.** 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. **79**:373-389.
9. **Jones, G. W.** 1977. The attachment of bacteria to the surface of animal cells, p. 139-176. *In* J. L. Reissing (ed.), Receptors and recognition, series B, vol. 3, Microbial interactions. Chapman and Hall, Ltd., London.
10. **Klainer, A. S., and R. R. B. Russell.** 1974. Effect of the inhibition of protein synthesis on the *Escherichia coli* cell envelope. Antimicrob. Agents Chemother. **6**:216-224.
11. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Mizuno, T., H. Yamagata, and S. Mizushima.** 1977. Interaction of cytoplasmic membrane and ribosomes in *Escherichia coli*: spectinomycin-induced disappearance of membrane protein I-19. J. Bacteriol. **129**:326-332.
13. **Novotny, C., J. Caraham, and C. C. Brinton, Jr.** 1969. Mechanical removal of F pili, type 1 pili, and flagella from Hfr and TRF donor cells and the kinetics of their reappearance. J. Bacteriol. **98**:1294-1306.
14. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. Infect. Immun. **22**:247-254.
15. **Ofek, I., E. H. Beachey, and A. L. Bisno.** 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: relationship to colonial morphology and surface pili. J. Infect. Dis. **139**:310-316.
16. **Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell.** 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. **141**:990-1003.
17. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated

by mannose receptors. Nature (London) **265**:623–625.
18. **Peska, S.** 1971. Inhibition of ribosomal functions. Annu. Rev. Microbiol. **25**:487–563.
19. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type I *Escherichia coli* pili. J. Exp. Med. **146**:1169–1181.
20. **Sud, I. J., and D. S. Feingold.** 1975. Detection of agents that alter the bacterial cell surface. Antimicrob. Agents Chemother. **8**:34–37.
21. **Swanson, J.** 1977. Surface components associated with gonococcal-cell interactions, p. 369–401. *In* R. B. Roberts (ed.), The gonococcus. John Wiley & Sons, Inc., N.Y.
22. **Watt, P. F., M. E. Ward, J. E. Heckels, and T. J. Trust.** 1978. Surface properties of *Neisseria gonorrhoeae*: attachment to and invasion of mucosal surfaces, p. 253–257. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.