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Binding of Lysozyme to Common Pili of *Escherichia coli*

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Common pili from *Escherichia coli* were found to bind hen egg white lysozyme. The binding was highly dependent on ionic strength, and the maximum binding occurred near an ionic strength of 0.02. The pili were aggregated by lysozyme, and this process could be followed by optical turbidity, electron microscopy, and coprecipitation. Near the maximum saturation of binding, one lysozyme molecule was bound by two pilus protein subunits. Electron micrographs of this aggregate indicated that they were paracrystalline structures. Piliated bacteria were more readily agglutinated by lysozyme than were nonpiliated bacteria. Since lysozyme is considered to be an antibacterial humoral factor and since pili are considered to be a colonization factor, the binding of lysozyme may represent an important bacterium-host interaction.

The present research shows the conditions that promote the coaggregation of two different proteins. One protein, pilin, occurs as a large polymer attached to the outer surface of bacteria (2). The other protein, lysozyme, is a humoral factor found in many animals.

Escherichia coli usually comprises a portion of the mutualistic bacteria in animal guts. Nearly all strains possess common pili. The expression of these pili, however, appears to be controlled by a phenomenon known as phase variation (17, 23). Some *E. coli* strains are also pathogens. These *E. coli* also possess pili, and the procedure for isolating some of the pili is the same as that for common pili (6). Thus, pili from pathogenic *E. coli* are chemically similar to common pili. Because pili occur on the surface of bacteria, there has been much speculation that they may be important in bacterial colonization of the host (5, 9, 10, 18, 20, 21).

One of the defenses of an animal host to invasion by bacteria is the secretion of lysozyme. This humoral enzyme is present in many body fluids and is present in normal human blood serum at a concentration of about 11 μg/ml (3). The cells that secrete lysozyme are those with lysosomal activity, particularly polymorphonuclear leukocytes (PMNs). Reports on the interaction of piliated *E. coli* with PMNs have been few, but there have been many reports on the interaction of piliated *Neisseria gonorrhoeae* with PMNs (4, 15, 24, 27). However, these have been confusing. Recently, Naccache et al. (13) have shown that the secretion of lysozyme by PMNs is decreased by low external calcium ion concentrations. Figure 1 shows that common pili aggregate in the presence of divalent cations, and, pending a more extensive study of this reaction, pili would appear to be able to sequester calcium ions. Our present study indicated a propensity for the pili to sequester the secreted lysozyme as well. These results may indicate that piliated bacteria have a complicated interaction with PMNs and point out some of the parameters that should be considered when studying bacterium-PMN interactions.

In the present paper, we characterize the conditions for hen egg white lysozyme aggregation of both common pili and piliated bacteria. The main thrust of the work was physical and chemical characterization of this aggregation, but we also investigated possible enzymatic properties of lysozyme, work which supplements some of the earlier research on lysozyme interaction with *E. coli*.

MATERIALS AND METHODS

Chemicals and solutions. Many of the chemicals and solutions have been described in the companion paper (12). $MgCl_2$, NaCl, and $CaCl_2$ of American Chemical Society standard were obtained from Fisher Scientific Co., Pittsburgh, Pa. *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer was prepared by adjusting a 5 mM solution to pH 7.0 with NaOH. Salt-free hen egg white lysozyme, i.e., lysozyme dialyzed against distilled water before lyophilization, was obtained from the Worthington Biochemicals Corp., Freehold, N.J. Lysozyme was prepared as a stock solution by dissolving 20 mg in 10 ml of distilled water. The concentration of lysozyme was determined from a UV spectrum scan, using an extinction coefficient of 26.4 at 281.5 nm (29). The M9 salt solution used for dilution of bacteria consisted of the following dissolved in 1 liter of distilled water: NH_4Cl, 1 g; KH_2PO_4, 3 g; and $Na_2HPO_4 \cdot 12H_2O$, 15.1 g.

Bacteria. Only *E. coli* K-12 strains were used.

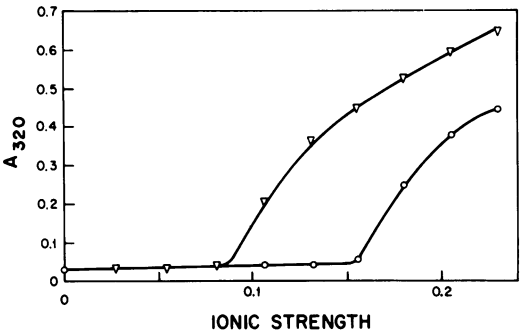

FIG. 1. *Comparison of $CaCl_2$ and $MgCl_2$ on paracrystallization of purified pili. The experiment was performed by adding increments of salt solution to a pilus preparation of 0.8 mg/ml of distilled water and by following turbidity at 320 nm (A_{320}). The added salts were (▽) $CaCl_2$ and (○) $MgCl_2$. (From Fig. 2, note that $MgCl_2$ more readily caused crystallization than did NaCl; thus, the relationship $Ca^{2+} > Mg^{2+} \gg Na^+$ for promoting crystallization.)*

F⁻W1-3 (25) was the bacterial strain that was primarily studied. OU10-31, an intermediately piliated strain, and OU10-1, a nonpiliated, isogenic strain of OU10-31, were used in some studies. Pili were prepared as described in the companion paper (12).

Protein determinations. The method of Sutherland (22), as described in the companion paper (12), was used, with the following modifications. When detection of pili was desired, a 0.1-ml protein sample was mixed with 0.4 ml of 8 M urea adjusted to pH 2.4 with HCl. This mixture was heated at 100°C for 5 min before protein was determined as described in the companion paper. (12). When detection of pili was not desired, distilled water was substituted for the low-pH urea, and the heating step was omitted. Standard curves were obtained by using either bovine serum albumin or hen egg white lysozyme.

Turbidity measurements. Two different procedures were used to measure turbidity (absorbance). To measure lysozyme-pilus aggregation, turbidity readings were done in 1-cm path-length quartz cuvettes, using a Cary 118 spectrophotometer at 320 or 340 nm. To measure lysozyme-bacterium agglutination, a Klett-Summerson colorimeter with a blue filter (420 nm) was used.

Electron microscopy. A Siemens model 101 electron microscope was used for all studies. The grids were prepared by placing a drop of sample on a Formvar or carbon-coated grid and staining with uranyl acetate.

Bacterial survival. The bacteria, strain F⁻W1-3, were grown to late log phase in L-broth in a bubble tube at 37°C. They were harvested by centrifugation at 1,000 × *g* for 5 min. The pellet was suspended in sterile, dilute TES buffer and centrifuged again to wash the cells. The pellet was finally suspended in 10 ml of sterile, dilute TES buffer. A volume of the bacteria was then mixed 1:1 with a sterilely prepared volume of salt solution. The bacteria were incubated in the salt solutions for 30 min at 27 or 37°C, diluted in M9 salt solution, and plated on L-agar.

Experiments were performed in which the above-mentioned technique was used but TES buffer was replaced with Tris buffer, as recommended by Repaske (19).

RESULTS

We present three different kinds of data to support the observation of lysozyme-pilus aggregation: (i) optical turbidity; (ii) coprecipitation; and (iii) electron microscopy. In addition, we present evidence that lysozyme preferentially aggregated piliated bacteria more than nonpiliated bacteria. The following results indicate the ionic and pH sensitivities of the aggregation process and show that the lysozyme may bind stoichiometrically and in a well-oriented manner to the pilus.

Optical turbidity studies. The most dramatic demonstration of lysozyme complexing with pili was observed by using turbidity measurements. The turbidities of mixtures of purified pili and lysozyme were highly dependent on ionic strength (Fig. 2 and 3). The data were obtained with the pili initially in TES buffer which had an ionic strength of 0.0012, assuming that the dipolar ion species contributed zero coulombic charge. The maximum turbidity occurred between ionic strengths of 0.003 and 0.03 and did not depend on the ionic species. NaCl, $MgCl_2$, and $CaCl_2$ were about equal in determining the position and size of this maximum. In some experiments, the maximum appeared as a plateau, whereas in others the maximum appeared to have two distinct peaks (Fig. 3). Since two peaks may have composed this maximum, there may have existed at least two favored orientations for binding of lysozyme to the pilus. In the ionic strength range of 0.04 to 0.16, there was minimal turbidity in the case of $MgCl_2$, but it should be noted that this turbidity never fell to the same level as that of the pilus or lysozyme controls. Above an $MgCl_2$ ionic strength of 0.16, the pili formed paracrystals regardless of the presence or absence of lysozyme. Presumably, these paracrystals were composed of pili alone. (Note that the pilus purification procedure made use of this ionic dependence.) NaCl was less effective than $MgCl_2$ in causing paracrystal formation, and $CaCl_2$ was more effective in promoting paracrystal formation (Fig. 1).

The turbidity of the lysozyme-pilus complex was so dependent on ionic strength that pH dependence was difficult to assess. Figure 4 shows a plot of turbidity versus pH. We considered these data unusual until we plotted turbidity versus ionic strength (Fig. 5). We interpreted

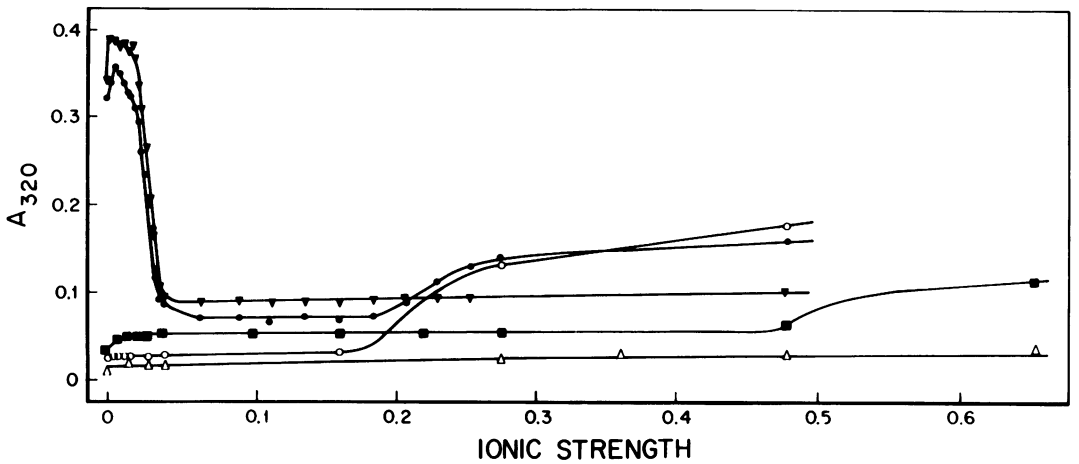

FIG. 2. *Turbidity at 320 nm (A_{320}) of lysozyme-pilus mixtures as a function of ionic strength. Salt solution was added in increments to samples, and then the turbidity was measured and corrected linearly for dilution. The initial concentration of lysozyme was 0.15 mg/ml, and the initial concentration of pili was 0.09 mg/ml. The pili had been dialyzed into 5 mM TES (pH 7.0) before being mixed with lysozyme in distilled water. Symbols: ●, $MgCl_2$ added to mixture of pili and lysozyme; ○, $MgCl_2$ added to pili alone; ▼, NaCl added to mixture of pili and lysozyme; ■, NaCl added to pili alone; △, $MgCl_2$ added to lysozyme alone.*

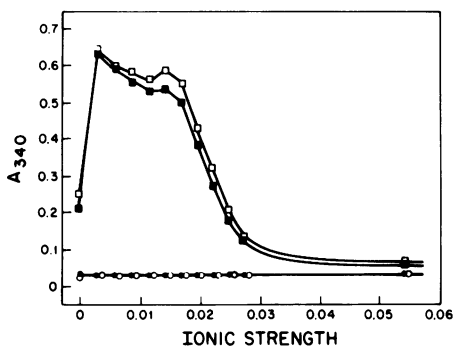

FIG. 3. *Turbidity at 340 nm (A_{340}) of lysozyme-pilus mixtures, showing detail at low ionic strength. The experiment was performed as for Fig. 2. Initial lysozyme concentration, 0.2 mg/ml; initial pili concentration, 0.8 mg/ml. Symbols: ■, $MgCl_2$ added to mixture of pili and lysozyme; ●, $MgCl_2$ added to pili alone; □, $CaCl_2$ added to mixture of pili and lysozyme; ○, $CaCl_2$ added to pili alone.*

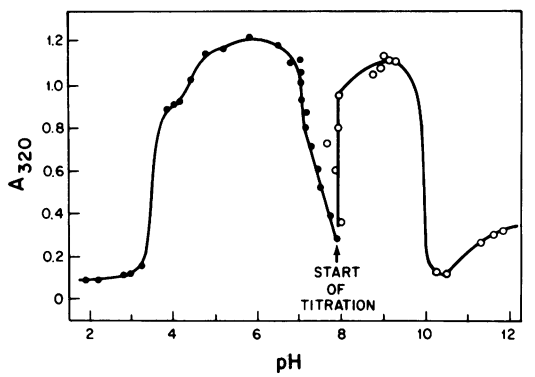

FIG. 4. *Plot of turbidity at 320 nm (A_{320}) of pilus-lysozyme mixture versus pH. NaOH or HCl was added in increments to a solution of pili (0.36 mg/ml) and lysozyme (0.16 mg/ml). At the beginning of the experiment, pili had been dialyzed against distilled water and crystalline lysozyme had been dissolved in distilled water. Data were not corrected for dilution. Symbols: ●, HCl addition; ○, NaOH addition.*

Fig. 5 as indicating that, in the pH range 5.8 to 9.0, the major influence of any addition of acid or base was due to its salt content and that the reaction was relatively independent of pH. The apparent limits to this salt dependence were near the approaches to the respective isoelectric points of pili (near pH 3.9) and lysozyme (near pH 11).

Sutherland determination of lysozyme binding to pili. Because freshly isolated pili were refractory towards the regular Sutherland procedure, it was used to differentiate pili from lysozyme (Fig. 6). Lysozyme at various concentrations was mixed in distilled water with a constant concentration of pili. Samples (1 ml) of lysozyme-pilus mixture were allowed to equilibrate at room temperature for 1 h, and then they were centrifuged at 12,000 × *g* for 20 min. The supernatants, containing free lysozyme or unprecipitated pili, or both, were carefully removed with Pasteur pipettes, and the pellets, containing the lysozyme-pilus complex, were suspended in 1 ml of dilute TES buffer containing 0.01 M

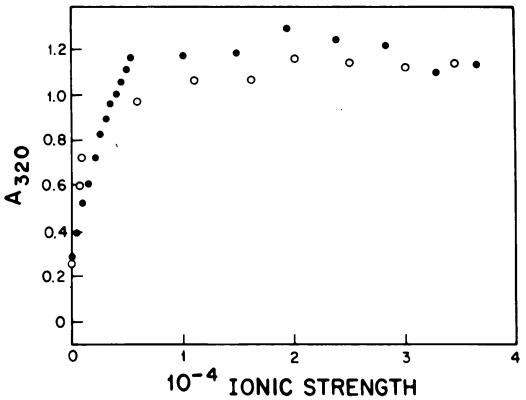

FIG. 5. *Data from Fig. 4 replotted as at 320 nm (A_{320}) versus ionic strength due to acid or base addition. Same symbols as used in Fig. 4.*

$MgCl_2$. ($MgCl_2$ greatly aided suspension of the lysozyme-pilus complex.) Two equal samples were removed from each supernatant and each resuspended pellet. On one sample, acid-urea denaturation was performed; only water was used with the other. The Sutherland determination was then done.

The experiments performed in this way indicated that the sites available to the lysozyme molecule became saturated as the lysozyme concentration increased. When the ionic strength was near 0.02, the lysozyme was nearly totally adsorbed to the pili (Fig. 6). Figure 7 shows some of the data obtained with other ionic strengths. In general, the data of Fig. 7 confirm the ionic dependence observed in Fig. 2 and 3; i.e., the extent of lysozyme uptake by pili was greatest in the ionic strength range of maximum turbidity and was less at very low ionic strengths and at ionic strengths above 0.03.

Figure 7 also indicates that there was an apparent ratio of one lysozyme molecule binding to two pilus subunits. These data were slightly deceptive because the protein concentration for both the pili and lysozyme was measured in terms of a lysozyme standard curve. Generally, the Sutherland protein procedure is dependent on the aromatic amino acid content of the protein (7). Thus, lysozyme, which has three tyrosine residues and six tryptophan residues per molecule, should be detected more easily than pili, which have only two tyrosine residues per subunit. If the Sutherland method were linearly dependent on these aromatic amino acids, then 1 mg of lysozyme should give close to four times the Sutherland intensity of 1 mg of pili. In fact, when the pili were heated in acid, the ratio was about 1.11 mg of pili per mg of lysozyme. This was calculated on the basis of an acid-treated

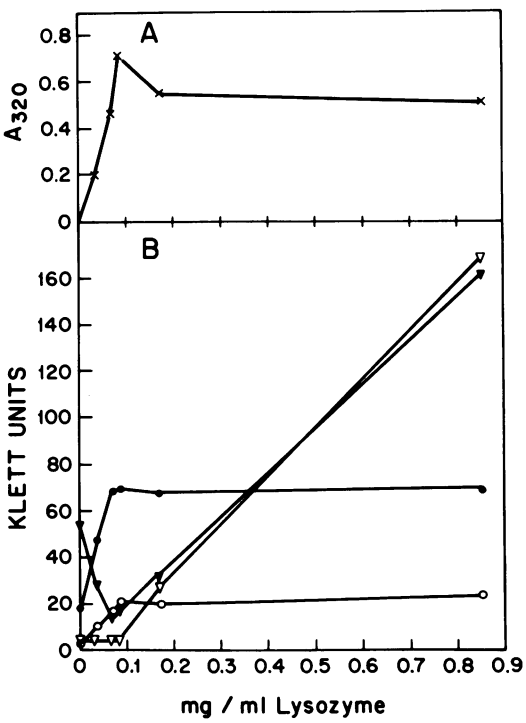

FIG. 6. *Binding of lysozyme to pili as a function of lysozyme concentration at an ionic strength of 0.022 (0.02 M NaCl, 0.002 M TES; pH 7.0). (A) Various concentrations of lysozyme were added to a constant concentration of pili (0.54 mg/ml). The resulting turbidity was then read at 320 nm (A_{320}). (B) The samples from (A) were then centrifuged at 12,000 × g for 20 min. The supernatant was carefully removed, and the pellet was suspended in a volume equal to the initial volume. The two forms of the Sutherland procedure were then performed on each fraction. Symbols: ●, Sutherland method on resuspended pellet; 100°C, pH 2.4, 8 M urea; ○, Sutherland method on resuspended pellet, using distilled water without heating; ▼, Sutherland method on supernatant; 100°C, pH 2.4, 8 M urea; ▽, Sutherland method on supernatant, using distilled water without heating.*

pilus preparation for which a UV spectrum was made in 0.1 M NaOH. (This also assumed a molecular weight of 17,000 for pilin and that the two-pilin tyrosine had an extinction coefficient of 2.33×10^3 at 293.5 nm [1].) Thus, the data in Fig. 7 may be close to the actual ratios.

Electron microscopy. Electron microscopy showed that the lysozyme-pilus aggregates were well-ordered in structure and appeared to be similar to the paracrystals of pure pili that occur in 0.1 M $MgCl_2$ (Fig. 8). This new paracrystalline form occurred only in the limited ionic strength range of 0.001 to 0.03. Measurement (Bausch and Lomb microcomparator) of the diameter of individual pilus rods, both side by side and well

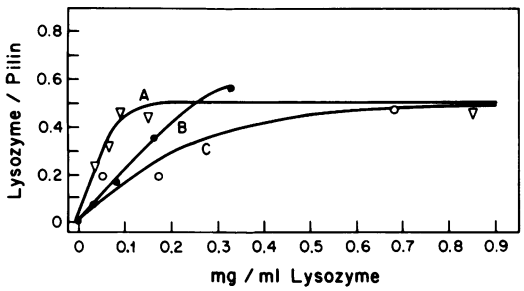

FIG. 7. *Saturation of lysozyme-binding sites on pili at different ionic strengths. It is important to note that this ratio was calculated on the basis of Sutherland protein concentrations, with lysozyme as a standard. The ionic strengths are as follows: (A) 0.022; (B) 0.002; and (C) 0.10.*

separated, indicated that, when lysozyme was bound at saturating concentrations, the pili had a 3 ± 0.5% larger diameter than the pili without lysozyme.

The formation of the paracrystals could also be verified by using a light microscope with dark-field optics.

Aggregation of bacteria by lysozyme. Because the pili were so readily aggregated by lysozyme, we decided to test intact bacteria for aggregation by lysozyme. For these experiments, EDTA was omitted from the solutions. At first, slide agglutinations were attempted, but these only served to demonstrate that nearly all bacteria can be agglutinated by lysozyme regardless of piliation. What we noticed using this technique was the difference in the rate of aggregate formation by piliated and nonpiliated *E. coli*. To overcome the defects of the slide method, we decided to examine the rate of sedimentation of bacterial aggregates in a Klett-Summerson colorimeter. This technique was based on the fact that only a portion of the cuvette is in the light path of the colorimeter. Thus, as aggregates sediment by gravity, they register a drop in turbidity. The results from a typical experiment are shown in Fig. 9.

For these experiments, three strains of *E. coli* with different piliation were used. The most striking feature was that strain F⁻W1-3, which was heavily piliated, cleared the most rapidly. This strain had almost totally precipitated well before the lightly piliated OU10-31 and the nonpiliated OU10-1 strains had begun to sediment. (The degree of piliation was qualitatively determined by two means: direct examination in an electron microscope; and by yield, using the pilus purification procedure.) Also, it should be noted that lysozyme aggregation of the bacteria was strong enough to overcome the motile behavior of these bacteria. Because of this, the controls lacking lysozyme were particularly important. The bacteria sedimented in this way did not lyse and retained complete viability upon plating in normal saline on Lennox agar.

Effect of lysozyme on viability of bacteria. In the generally accepted procedure for lysis of *E. coli* by lysozyme, EDTA is present and a moderately low ionic strength (about the same range in which lysozyme binds to pili) is used (19). We discovered that under these conditions the bacteria grew and divided, as compared with samples of bacteria prepared with the same solutions but without lysozyme (Fig. 10). (Note that the osmotic shock step recommended by Witholt et al. [28] was not done in these experiments.) The bacteria did produce malshaped forms and spheroplasts, as observed in a light microscope. When the samples were maintained at room temperature, bacteria with and without lysozyme had nearly the same survival rate at all ionic strengths.

DISCUSSION

Ionic environment appeared to be the single most important variable affecting the association of lysozyme with pili. The data of Fig. 2, 3, 5, and 7 all confirm this fact. This salt dependence had a maximum near an ionic strength of 0.02 and was independent of the ionic species tested. This dependence correlated with the maximum degree of optical turbidity and coprecipitation. Lysozyme bound at an ionic strength as high as 0.15, which is considered to be the physiological ionic strength, but with less facility than at lower ionic strengths. When maximum binding occurred, electron micrographs of the pili showed that the paracrystals formed were similar to those occurring in the presence of 0.1 M $MgCl_2$ (Fig. 8B and C). Because paracrystals were formed, there was a suggestion that lysozyme had a stereochemically specified binding site on the pilus. Another fact suggesting that there was a specific binding site was that the binding of lysozyme occurred with a ratio of one lysozyme molecule per two pilus subunits.

After characterizing the conditions required by lysozyme to bind to pili, we expanded the scope of our experiments to include intact bacteria. We discovered, in agreement with the data from isolated-pili experiments, that piliated bacteria were more readily aggregated than nonpiliated bacteria by lysozyme at an ionic strength of 0.025. This agglutination may offer another tool for studying piliation. The unexpected result was that bacteria exposed to lysozyme were as viable as the controls lacking lysozyme.

This viability was disconcerting, since Repaske (19) had reported that lysozyme in con-

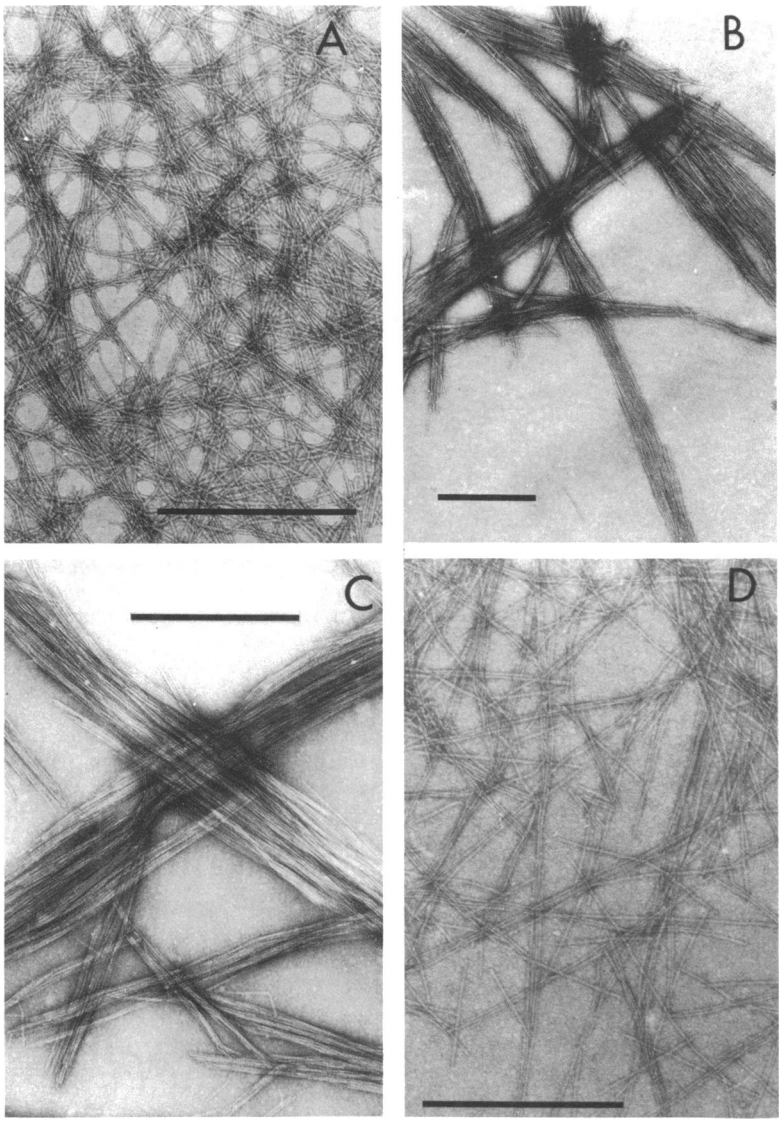

FIG. 8. *Electron micrographs: (A) pili in 0.02 M NaCl; (B) mixture of pili and lysozyme in 0.02 M NaCl; (C) pili in 0.1 M $MgCl_2$; (D) mixture of pili and lysozyme in 0.10 M NaCl. In the mixtures, the pili/lysozyme ratio was 1:1.7 based on Sutherland protein determinations. The bars represent 500 nm.*

junction with EDTA lysed *E. coli*. The ionic conditions found by Repaske for optimal lysis occurred in the same region of ionic strength as that in which lysozyme was bound most avidly to pili. Many groups, including ours, have used the Osborn et al. (16) modification of the Repaske method to lyse *E. coli*, and we were most surprised by this result. Examination of Repaske's experimental method showed that he followed "lysis" by optical turbidity. From the data presented in Fig. 9, we have now demonstrated that this method was a poor choice, since lysozyme also caused the bacteria to aggregate. These aggregates are large, sediment easily at normal gravity, and could have accounted for what Repaske took for disruption of the cells.

Because of the possible disagreement with the lysis results of Repaske, we studied the viability of *E. coli* in the presence of constant EDTA and lysozyme concentrations and various NaCl concentrations. As a result, we discovered that, in the region in which lysozyme was bound most avidly by purified pili, the bacteria exposed to lysozyme apparently survived better than those

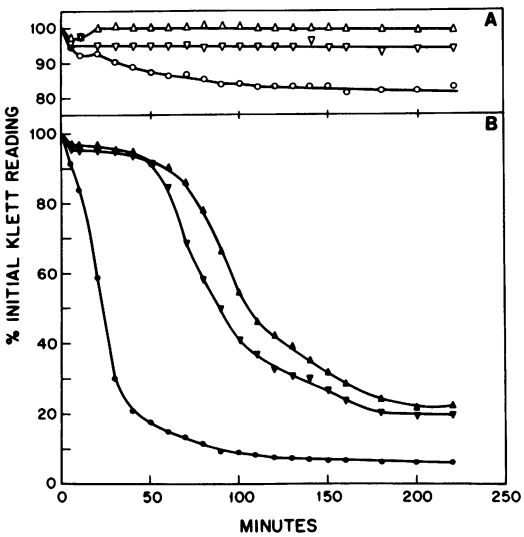

FIG. 9. *Dependence of lysozyme agglutination of intact bacteria on the degree of piliation. Solvent was pH 7.0 and contained 0.075 mg of lysozyme per ml, 0.5 mM TES, and 0.0025 M $MgCl_2$. Initial turbidity of all samples was adjusted to ca. 55 Klett units. (B) ●, strain F⁻W1-3, which is heavily piliated; ▼, strain OU10-31, which is lightly piliated; and ▲, strain OU10-1, which has no pili. (A) Controls in which water was added in place of lysozyme solution: ○, F⁻W1-3; ▽, OU10-31; and △, OU10-1.*

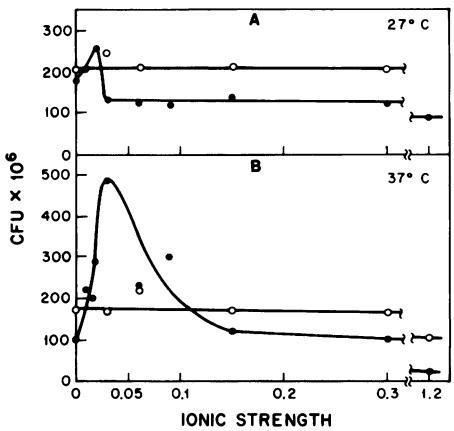

FIG. 10. *Survival of strain F⁻W1-3 in the presence of lysozyme as a function of ionic strength. Pelleted bacteria were suspended in solutions containing 5 mM TES (pH 7.0), 1 mM EDTA, 0.2 mg of lysozyme per ml, and various concentrations of NaCl. Samples were incubated for 30 min, diluted, and plated on L-agar. Symbols: ●, samples containing 0.2 mg of lysozyme per ml; ○, control samples in which lysozyme was replaced with water. (A) Samples incubated at 27°C; (B) samples incubated at 37°C. CFU, Colony-forming units.*

at other ionic strengths and, indeed, may have even grown and undergone division. The controls under these same ionic conditions, surprisingly, yielded fewer colonies upon plating than the lysozyme-treated cells. These results were from samples incubated at 37°C. When the samples were incubated at room temperature, there was very little difference in viability between samples with or without lysozyme. We concluded from our results that lysis of *E. coli* by lysozyme should be reexamined more carefully.

We can only offer some speculation as to why lysozyme has such a binding reaction with pili. Indeed, we can not discern whether the host or the bacterium would benefit by such an in vivo reaction. Presumably, agglutination of the bacterium by lysozyme could be a part of a larger defensive system of the host. Such a system could be phagocytosis for which lysozyme may act as an opsonin (26). Alternatively, lysozyme binding could benefit the bacterium by sequestering lysozyme and, at the same time, promoting a "plaquing" phenomenon. Another speculation is that this reaction is involved in balancing host-bacterium mutualism. In the gut, where the bacterial population would be high, the concentration of pili may provide a mechanism for subverting the action of PMNs. Should a stray bacterium enter host tissue, however, the already high concentration of lysozyme would make the bacterium susceptible to PMNs.

Of course, a final possibility is that this present reaction is only another in the long list of charge-dependent reactions of lysozyme (8).

ACKNOWLEDGMENTS

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