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Mechanism of Enhancement of Microbial Cell Hydrophobicity by Cationic Polymers

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Polycationic polymers have been noted for their effects in promoting cell adhesion to various surfaces, but previous studies have failed to describe a mechanism dealing with this type of adhesion. In the present study, three polycationic polymers (chitosan, poly-L-lysine, and lysozyme) were tested for their effects on microbial hydrophobicity, as determined by adhesion to hydrocarbon and polystyrene. Test strains (*Escherichia coli*, *Candida albicans*, and a nonhydrophobic mutant, MR-481, derived from *Acinetobacter calcoaceticus* RAG-1) were vortexed with hexadecane in the presence of the various polycations, and the extent of adhesion was measured turbidimetrically. Adhesion of all three test strains rose from near zero values to over 90% in the presence of low concentrations of chitosan (125 to 250 μg/ml). Adhesion occurred by adsorption of chitosan directly to the cell surface, since *E. coli* cells preincubated in the presence of the polymer were highly adherent, whereas hexadecane droplets pretreated with chitosan were subsequently unable to bind untreated cells. Inorganic cations (Na^+, Mg^{2+}) inhibited the chitosan-mediated adhesion of *E. coli* to hexadecane, presumably by interfering with the electrostatic interactions responsible for adsorption of the polymer to the bacterial surface. Chitosan similarly promoted *E. coli* adhesion to polystyrene at concentrations slightly higher than those which mediated adhesion to hexadecane. Poly-L-lysine also promoted microbial adhesion to hexadecane, although at concentrations somewhat higher than those observed for chitosan. In order to study the effect of the cationic protein lysozyme, adhesion was studied at 0°C (to prevent enzymatic activity), using *n*-octane as the test hydrocarbon. Adhesion of *E. coli* increased by 70% in the presence of 80 μg of lysozyme per ml. When the negatively charged carboxylate residues on the *E. coli* cell surface were substituted for positively charged ammonium groups, the resulting cells became highly hydrophobic, even in the absence of polycations. The observed "hydrophobicity" of the microbial cells in the presence of polycations is thus probably due to a loss of surface electronegativity. The data suggest that enhancement of hydrophobicity by polycationic polymers is a general phenomenon.

Hydrophobic interactions are considered to play a major role in a variety of microbiological phenomena, including adhesion (1, 8, 10–14, 17), phagocytosis (19), growth on insoluble substrates (16), and gliding (20). Whereas earlier studies suggested that each microbial strain possesses a fixed degree of surface hydrophobicity (19), more recent investigations have shown that physiological conditions (e.g., growth phase, medium, and presence of antibacterial agents) may often modulate hydrophobic surface properties (1, 2, 17). Cell surface components which promote (hydrophobins) or reduce (hydrophilins) hydrophobic properties may coexist on the cell surface (1, 14).

The hydrophobic interactions which mediate adhesion are often influenced by the composition of the aqueous medium. A wide range of components added to the aqueous phase can inhibit attachment to hydrophobic surfaces. These include amphipathic polymers (e.g., emulsan [12], surfactants [8, 10, 14, 16], and chaotropes [10]). However, few components have been found to promote attachment to hydrophobic surfaces. Ammonium sulfate, a salting-out agent, has been shown to enhance adhesion to hydrocarbons and polystyrene (13). Recently, E. Rosenberg and co-workers showed that the cationic antibiotic gramicidin S enabled *Bacillus brevis* to adhere to hexadecane (17). Certain cationic antibacterial agents (cetylpyridinium chloride and chlorhexidine) can also mediate adhesion to oil droplets (4a).

Cationic polymers, such as chitosan (2), lysozyme (18), and poly-L-lysine (7, 15), have been shown to promote microbial adhesion to various surfaces, although the mechanism has not been determined. The present report demonstrates that low concentrations of these polymers can confer microbial hydrophobicity, as determined by adhesion to hydrocarbons and polystyrene (12). This finding raises the possibility that microorganisms may acquire hydrophobic surface properties in the open environment by adsorbing cationic polymers.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* CSH57 was obtained from E. Z. Ron, Tel-Aviv University; *Candida albicans* 792.1 was obtained from Y. Koltin, Tel-Aviv University. *Acinetobacter calcoaceticus* MR-481, a nonhydrophobic mutant of strain RAG-1, was isolated as described previously (16). Microorganisms were maintained on brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.) at 4°C and transferred every month.

Polycations. Chitosan (practical grade; Sigma Chemical Co., St. Louis, Mo.) was prepared as previously described (6) by heating 0.5 g in 90 ml of 48% acetic acid until a solution

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TABLE 1. Effect of chitosan on microbial adhesion to hexadecane[a]

Strain	Initial turbidity (OD)[b]	Maximal adhesion (%)		Concn (μg/ml) of chitosan required for maximal adhesion
		Without chitosan	With chitosan	
E. coli CSH57	20	0	97	125
C. albicans 792.1	20	0	95	25
A. calcoaceticus MR-481	17	0	92	19

[a] Adhesion of washed microbial suspensions to hexadecane in the presence of various chitosan concentrations was carried out as described in Materials and Methods.

[b] Of washed cell suspensions before mixing in the presence of hexadecane.

TABLE 2. Effect of poly-L-lysine on microbial adhesion to hexadecane[a]

Strain	Initial turbidity (OD)	Maximal adhesion (%)		Concn (μg/ml) of poly-L-lysine required for maximal adhesion
		Without poly-L-lysine	With poly-L-lysine	
E. coli CSH57	22	0	59	625
C. albicans 792.1	18	0	96	200
A. calcoaceticus MR-481	13	0	98	125

[a] Conditions are described in the footnotes to Table 1.

was obtained and then dialyzing it extensively against distilled water. Poly-L-lysine hydrobromide (molecular weight, ca. 9,000) and chicken egg white lysozyme were obtained from Sigma.

Adhesion to hydrocarbon. Adhesion to hydrocarbon has been previously described (1, 11–14, 16, 17). In brief, microorganisms were grown from liquid inocula for 18 h with shaking at 30°C. Cells were washed twice and suspended in distilled water or distilled water containing 34 mM NaCl unless otherwise indicated. Turbidity of cell suspensions was measured at 400 nm in a Uvikon (Kontron, Zurich, Switzerland) 710 spectrophotometer. To semimicro, 1-cm^2, 4-ml disposable polystyrene cuvettes (Rudolf Brand, Wertheim, Federal Republic of Germany) containing 1.2 ml of cell suspension was added 0.4 ml of distilled water containing various polycation concentrations and other salts as indicated. *n*-Hexadecane or *n*-octane (0.2 ml) was added, and the cuvettes were vortexed for 60 s (sufficient for maximal adhesion) on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuque, Iowa). Following phase separation, the turbidity of the lower aqueous phase (A_t) was measured directly in the cuvettes at 400 nm. For turbidity readings of over 1.0, a correction curve relating the observed reading to those of appropriately diluted suspensions was employed. Adhesion of cells to hexadecane was verified by microscopic observation. Results are expressed as the percent decrease in turbidity of the lower aqueous phase (A_t) compared with that of the original suspension (A_0), i.e., $100 - [100(A_t/A_0)]$.

Adhesion to polystyrene. Adhesion to polystyrene microdilution wells has been described elsewhere (13). Bacteria (*E. coli* CSH57) were washed and suspended in 34 mM NaCl to an optical density (OD) of 22. Different concentrations of chitosan were added to the bacterial suspensions, and 200-μl samples of the mixtures were added in quadruplicate to untreated flat-bottom polystyrene microdilution plates (Sterilin, Feltham, Middlesex, England) and incubated for 24 h at room temperature. Following incubation, the plates were rinsed thoroughly in running water. Adhering bacteria were then stained with gentian violet for 2 h at room temperature. To remove excess dye, plates were rinsed again in running water and allowed to dry. Adhesion was measured as the A_{610}, using a Dynatech Microplate Reader (Dynatech MR-600; Synatech Laboratories, Inc., Alexandria, Va.). As a control, 34 mM sodium chloride was substituted for bacterial cell suspension.

Chitosan pretreatment. In order to study the effect of chitosan on the cells and on the oil phase separately, *E. coli* CSH57 cells were washed and suspended to an OD of 20 in 34 mM NaCl. Following 60 s of agitation in the presence of chitosan (125 μg/ml, final concentration), the cells were washed twice and resuspended in 34 mM NaCl. Hexadecane (1.0 ml) was pretreated by vortexing (60 s) in the presence of 0.2 ml of aqueous solution containing 5 mg of chitosan per ml. The hexadecane droplets were then washed four times in distilled water. Adhesion to hexadecane was carried out as described above, employing pretreated versus untreated microbial cells and pretreated versus untreated hexadecane. As a control, adhesion of untreated cells to untreated hexadecane was brought about in the presence of 125 μg of chitosan per ml.

Chemical modification of cell surface. *E. coli* CSH57 was washed and suspended in 34 mM NaCl to an OD of approximately 20. The pH of the suspension was adjusted to 4.75 with dilute HCl. To 7 ml of bacterial suspension were added 15 ml of a 1 M aqueous solution of ethylenediamine (pH 4.75) and 9 ml of a 0.3 M aqueous solution of dicyclohexylcarbodiimide (pH 4.75) (3). Following 30 min of incubation at room temperature (pH was kept constant throughout), cells were washed three times and suspended in 34 mM sodium chloride to an initial OD of 17.5. Adhesion to hexadecane at various initial turbidities was carried out as described above.

RESULTS

The effects of chitosan and poly-L-lysine on microbial adhesion to hexadecane are summarized in Tables 1 and 2. Figure 1 compares the effect of the two polymers on adhesion of *E. coli* CSH57. In all cases, low chitosan concentrations enhanced adhesion to the hydrocarbon from 0 to over 90%; poly-L-lysine increased adhesion levels to 59 to 98%,

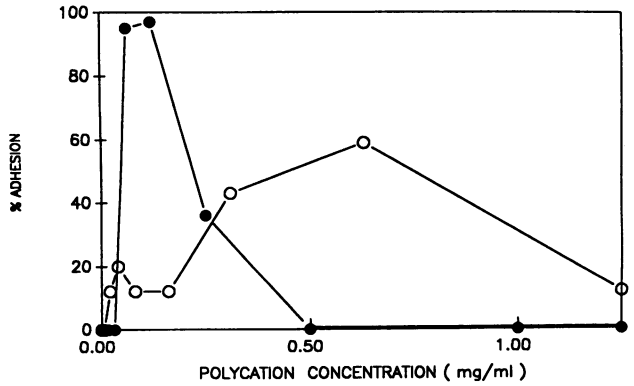

FIG. 1. Adhesion of *E. coli* (initial OD of 20) to hexadecane in the presence of chitosan (●) or poly-L-lysine (○). Adhesion of *E. coli* to hexadecane occurred in the presence of different polycation concentrations as described in Materials and Methods. Adhesion is expressed as the percent decrease in turbidity of the aqueous phase compared with the turbidity prior to mixing.

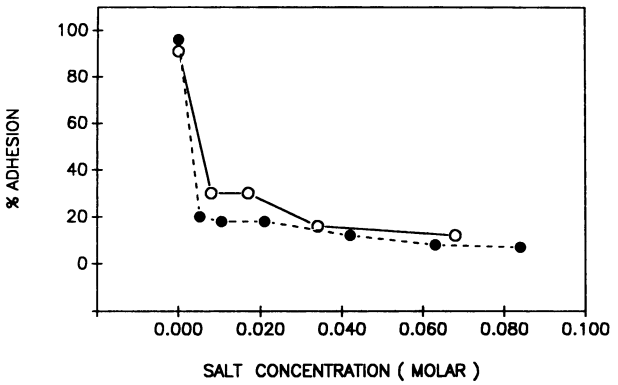

FIG. 2. Effect of salts on chitosan-mediated adhesion of *E. coli* to hexadecane. To 1.2 ml of cell suspension in distilled water at an OD of 20 was added 0.4 ml of distilled water containing chitosan to give a final concentration of 125 μg/ml and containing NaCl (○) or $MgCl_2$ (●) at various final concentrations as indicated.

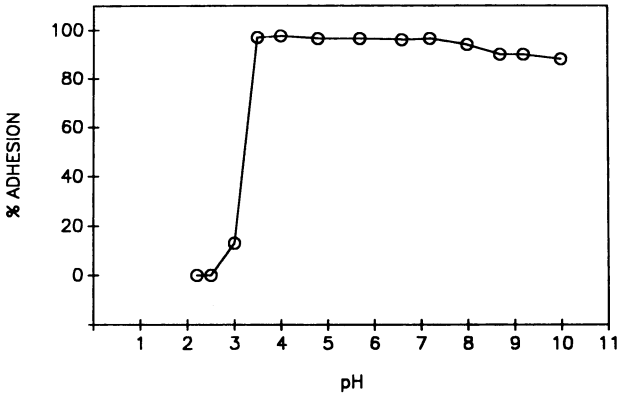

FIG. 3. Dependence of hydrogen ion on chitosan-mediated adhesion of *E. coli* to hexadecane. To 1.2 ml of washed cell suspension in 34 mM NaCl at an OD of 23 was added 0.4 ml distilled water containing HCl or NaOH to achieve the desired pH and chitosan to yield a final concentration of 125 μg/ml.

depending on the microbial strain. However, poly-L-lysine levels required for maximal adhesion (Table 2) were much higher than those observed for chitosan. For example, whereas 25 μg of chitosan per ml sufficed to increase the adhesion of *C. albicans* from 0 to 95% (Table 1), an eight-fold-higher concentration of poly-L-lysine was required to attain a similar adhesion level (Table 2). Moreover, whereas chitosan-mediated adhesion occurred over a very narrow range of polymer concentrations, the range of poly-L-lysine concentrations which promoted adhesion was much broader. Compared with poly-L-lysine, the corresponding monomer, L-lysine, was a poor inducer of hydrophobicity. Adhesion of *E. coli* cells was not enhanced by more than 15% over a wide range of L-lysine concentration (data not shown).

The strength of adhesion was assessed by measuring desorption of cells following centrifugation. Fewer than 10% of the *E. coli* cells bound to hexadecane in the presence of chitosan could be desorbed following 10 min of centrifugation at 1,100 × *g*.

The effects of salts on chitosan-mediated adhesion of *E. coli* to hexadecane is shown in Fig. 2. Both NaCl and $MgCl_2$ were highly inhibitory. For example, adhesion decreased by 70% in the presence of 140 mM NaCl and by 89% in the presence of 80 mM $MgCl_2$. The effect of pH on chitosan-mediated adhesion of *E. coli* to hexadecane is shown in Fig. 3. Values below pH 3.5 inhibited adhesion by over 80%.

In order to determine whether the adhesion-promoting effect occurred by partitioning of the polycation at the cell surface or at the hexadecane-water interface, *E. coli* cells and hexadecane droplets were independently pretreated with chitosan. Chitosan-pretreated cells adhered to hexadecane whether the hexadecane droplets were pretreated or not (89 and 91%, respectively). Similarly, adhesion in the control cuvette, in which untreated cells and hexadecane were mixed in the presence of chitosan, was high (98%). However, chitosan-pretreated hexadecane droplets were completely unable to bind untreated cells (0% adhesion). Thus, chitosan appears to promote adhesion by adsorbing directly to the microbial cell surface.

The effect of lysozyme on *E. coli* adhesion is shown in Fig. 4. In order to avoid enzymatic activity, adhesion experiments were performed at 0°C, using *n*-octane as the test hydrocarbon (hexadecane solidifies at approximately 16°C). Low concentrations of lysozyme (80 to 800 μg/ml) effectively promoted the adhesion of *E. coli* cells to octane. Lysozyme was also highly effective in promoting the adhesion of cells to hexadecane at room temperature (data not shown).

The adhesion of *E. coli* CSH57 to polystyrene as a function of chitosan concentration is shown in Fig. 5. Maximal adhesion was observed at 500 μg/ml. As with adhesion to hexadecane, high polycation concentrations resulted in a decrease in adhesion.

The foregoing data suggest that the microorganisms may be rendered hydrophobic by the complexing of electronegative sites by the polycations. One way to test this premise is to reduce the cell surface electronegativity independently of polycations. *E. coli* CSH57 was mixed with solutions of dicyclohexylcarbodiimide and ethylenediamine. Carbodiimide-activated carboxylate groups were substituted with positively charged ammonium groups from the ethylenediamine. When the derivatized cells were assayed directly for adhesion to hexadecane, it was found that there was now a high degree of binding to the hydrocarbon (Table 3). Adhesion was high over a wide range of initial cell densities. Controls performed in the presence of carbodiimide and ethanolamine

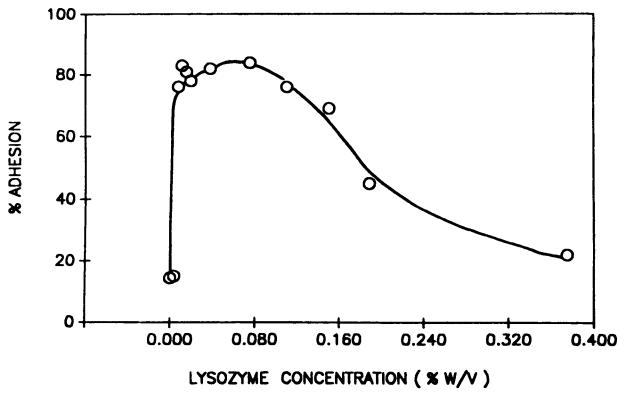

FIG. 4. Effect of lysozyme on adhesion of *E. coli* to *n*-octane. Adhesion of *E. coli* (at an initial OD of 22) to *n*-octane was carried out in the presence of different lysozyme concentrations as explained in Materials and Methods. Adhesion is expressed as the percent decrease in turbidity of the aqueous phase compared with the turbidity prior to mixing.

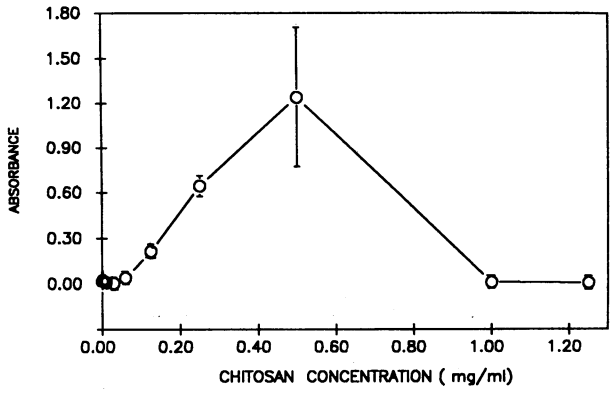

FIG. 5. Chitosan modifies adhesion of *E. coli* to polystyrene. Adhesion of *E. coli* to polystyrene microdilution wells was carried out as described in Materials and Methods. Results are plotted as the average absorbance (± standard deviation) as a function of chitosan concentration.

resulted in cells with intermediate adhesion properties (data not shown). Ethanolamine would give rise to the introduction of a neutral hydroxyl group instead of the positive ammonium residue.

DISCUSSION

The present study demonstrates that several polycationic agents (chitosan, poly-L-lysine, and lysozyme) confer hydrophobic properties on various microbial cells. Strains with no observable hydrophobic surface properties adhered avidly to hydrocarbon droplets in the presence of low polycation concentrations. At optimal concentrations, over 90% of the cells were removed from the bulk aqueous phase to the oil-water interface, as opposed to 0% in the absence of added polycation. Polycations, including those employed in the present study, have been shown to adsorb to solid surfaces and thus modulate the adhesion of microbial and mammalian cells. To our knowledge, this is the first report demonstrating their ability to mediate microbial adhesion at the oil-water interface.

The results suggest that polycations mediate microbial adhesion to hydrocarbon by binding to the cell surface via electrostatic interactions. This process is inhibited by salts and in some cases by protons. The adsorbed polymer may increase cell surface hydrophobicity by reducing the polar effects of negatively charged surface components. Concomitantly, outward orientation of hydrophobic residues present on the polymers may also increase cell-surface hydrophobicity. In the case of poly-L-lysine, the alkyl region of the side chain may contribute to hydrophobicity. Lysozyme is an amphipathic protein and has been shown to bind to hexadecane (15). In the case of chitosan, *N*-acetyl groups uncleaved by acidic hydrolysis may contribute to its hydrophobic properties.

As we have demonstrated here, substitution of ammonium groups for carboxylate residues also promotes hydrophobicity in *E. coli*. Klotz and co-workers have reported increased hydrophobicity of *C. albicans* following blocking of carboxylate groups (9). Ferris and Beveridge have demonstrated that exposure of cation-depleted *E. coli* cells to magnesium ions resulted in increased hydrophobicity (4). E. Rosenberg and co-workers have previously shown that the cationic antibiotic gramicidin S confers adhesion to hexadecane on *B. brevis* cells (11). Goldberg et al. (4a) recently reported that cationic surfactants promote microbial hydrophobicity, as measured by adhesion to hexadecane and polystyrene. Similarly, foam flotation of microorganisms in the presence of cationic surfactants may be related to increased cell surface hydrophobicity (7, 8).

Together with the results of the present study, these data suggest a general microbial phenomenon by which a variety of nonadherent bacteria and yeasts can be rendered hydrophobic by a wide range of cationic agents. Moreover, the results presented here raise the possibility that a similar phenomenon occurs in vivo. The finding that lysozyme, a naturally occurring cationic polypeptide, mediates adhesion to hydrocarbons suggests that microorganisms may increase their cell surface hydrophobicity by adsorbing organic cations from the environment. Experiments to test this premise are under way.

In most instances studied here, higher concentrations of polycations brought about a decrease in adhesion to hydrocarbons and polystyrene. Similar observations have been noted for the effect of cationic surfactants on microbial adhesion to hexadecane (4a), foam flotation of *E. coli* (5), and flocculation of yeast cells (7). This phenomenon may be due to inhibition by unadsorbed polycations of cell-cell interaction. Cell-cell bridging may be of importance in stabilizing adhesion to hydrophobic substrata (M. Rosenberg, I. A. Buivids, and R. P. Ellen, manuscript submitted for publication). Indeed, polycation concentrations which enhanced adhesion in the present study frequently caused some microbial aggregation when added prior to the assay itself, in a manner similar to that observed for cationic surfactants (4a). Furthermore, the increase in percent adhesion of the chemically treated cells with increasing initial cell density (Table 3) suggests cooperative interactions among the adhering bacteria.

Microbial adhesion to hydrocarbons has frequently been used as a model for studying microbial cell surface hydrophobicity. A wide variety of bacteria and fungal cells have been shown to adhere avidly to hydrocarbons (14), yet many microorganisms of considerable scientific interest, such as *E. coli* and vegetative *Bacillus* cells, show little or no hydrophobic surface properties when tested by hydrocarbon adhesion and other hydrophobicity tests (13). The data suggest that hydrophobic properties can be conferred on such microorganisms by appropriate concentrations of cationic moieties, including nontoxic polymers such as chitosan. The potential effect of this acquired hydrophobicity on microbial adhesion and related phenomena warrants further study.

TABLE 3. Adhesion of *E. coli* CSH57 to hexadecane following substitution of ammonium groups onto surface carboxylate residues[a]

Initial OD	% Adhesion
17.5	97
10	97
3.7	92
1.5	83
0.73	51

[a] *E. coli* was treated with dicyclohexyl carbodiimide and ethylenediamine as described in Materials and Methods. Following treatment, cells were washed three times and suspended in 34 mM NaCl to various initial turbidities, after which adhesion to hexadecane was determined. No adhesion to hexadecane was observed with untreated cells.

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