INFECTION AND IMMUNITY, Apr. 1982, p. 227-234
0019-9567/82/040227-08$02.00/0

Vol. 36, No. 1

Isoelectric Points and Surface Hydrophobicity of Gram-Positive Cocci as Determined by Cross-Partition and Hydrophobic Affinity Partition in Aqueous Two-Phase Systems

HÅKAN MIÖRNER,[1]* PER-ÅKE ALBERTSSON,[2] AND GÖRAN KRONVALL[1]

Departments of Medical Microbiology[1] and Biochemistry,[2] University of Lund, S-223 62 Lund, Sweden

Received 6 August 1981/Accepted 16 November 1981

Thirty-nine streptococcal strains belonging to groups A, C, and G and 12 staphylococcal strains were investigated with respect to surface charge and hydrophobicity. Isoelectric points of the bacteria were determined by cross-partition experiments in dextran-polyethylene glycol two-phase systems containing charged polymers. The results obtained indicate that group A, C, and G streptococci have isoelectric points of pH 3.75 ± 0.15 standard deviation. Staphylococci show an isoelectric point of around pH 2 and thereby differ markedly from the streptococci. Pretreatment of bacteria with human serum resulted in a significant change in the isoelectric points of streptococci. In a second series of experiments, an aqueous dextran-polyethylene glycol two-phase system containing polyethylene glycol palmitate or stearate was used to study the hydrophobic surface properties of the bacterial cells. The partition of the staphylococci was not influenced by the addition of up to 1% (wt/wt) polyethylene glycol palmitate or stearate, whereas the streptococci showed a large variation in affinity for polyethylene glycol-bound hydrophobic groups. The bacterial strains included in the study were also tested for uptake of human serum proteins. A positive correlation was found between the hydrophobic affinity of group A streptococci and the density of receptors for aggregated beta-2-microglobulin.

The interaction of bacteria with mammalian cells is of great importance in pathogenicity (31). Recently, surface properties of bacteria, such as charge and hydrophobicity, have received increased attention. Studies on *Salmonella typhimurium* have shown that the phagocytosis-sensitive R-mutants are liable to hydrophobic and ionic interactions, whereas the smooth phagocytosis-resistant strains have a hydrophilic and uncharged surface (22). Investigations with hydrophobic interaction chromatography suggest that the M-protein of *Streptococcus pyogenes* and protein A of *Staphylococcus aureus* contribute to the hydrophobic character of the bacterial cell surface (37, 38, 41). Specific binding of human proteins to receptors on gram-positive bacteria have been shown to change their physico-chemical surface properties (24).

Partition between two immiscible liquid phases composed of different aqueous polymer solutions has been used to investigate surface properties of *Salmonella typhimurium*, *Escherichia coli*, *Neisseria gonorrhoeae*, and various streptococcal and staphylococcal strains (5, 8, 19, 22, 24, 27, 28, 33–36). Such phase systems containing dextran and polyethylene glycol (PEG) in water are highly useful for the separation and study of biological substances (2–4, 42). Particles, such as bacteria, distribute between the two phases and the interphase according to their surface properties, such as electrical charge and hydrophilic-hydrophobic character (4). To increase the selectivity of a polymer two-phase system, charged and hydrophobic groups have been covalently bound to the phase polymers (1, 10, 43, 44). With aqueous two-phase systems containing charged PEG, it is possible to perform a so-called cross-partition whereby the isoelectric points of the bacteria can be estimated (1, 2, 6, 9, 14). The hydrophobic surface properties of the bacteria were studied by hydrophobic affinity partition in two-phase systems containing PEG palmitate (P-PEG) or PEG stearate (St-PEG) (10, 20–22, 43, 44).

In the present investigation the isoelectric points of various group A, C, and G streptococci and staphylococci were determined by cross-partition. Pretreatment of bacteria with serum had a marked effect on the isoelectric point of the exposed bacterial surface and might have a bearing on the outcome of host-parasite interactions in vivo. The observed differences in hydro-

phobicity among the streptococci were correlated to the presence of specific receptors for human proteins.

MATERIALS AND METHODS

Bacterial strains. A total of 43 laboratory-maintained strains, 8 freshly isolated strains, and 8 heat-inactivated strains were included in the study. Laboratory-maintained strains were kindly supplied by D. Mirelman, Rehovot, Israel (one strain), by I. Rotta, Prague, Czechoslovakia (one strain), and by L. W. Wannamaker, Minneapolis, Minn. (six strains) or were obtained consecutively from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. The bacterial species and numbers of laboratory-maintained strains were as follows: *Staphylococcus aureus*, 6; *Staphylococcus epidermidis*, 2; *Staphylococcus saprophyticus*, 2; group A streptococci, 13; group C streptococci, 11; and group G streptococci, 10. All strains were kept at 4°C on blood agar plates before use. Todd-Hewitt broth cultures (Difco Laboratories, Detroit, Mich.) were incubated overnight at 37°C. Bacteria were harvested by centrifugation at 3,000 rpm for 15 min and washed twice in phosphate-buffered saline (PBS; 0.03 M sodium phosphate, 0.12 M NaCl, pH 7.2). The optical density at 620 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml. Heat-inactivated bacteria were prepared by heat treatment at 80°C for 5 min.

Radiolabeling of bacteria. Bacteria were labeled with ^{51}Cr (The Radiochemical Centre, Amersham, England). A 50-μCi portion of ^{51}Cr was added to 10^9 live bacteria in PBS and incubated at 37°C for 3 h. Heat-inactivated bacteria were labeled with 100 μCi of ^{51}Cr per 10^9 bacteria and incubated overnight. The bacteria were then deposited by centrifugation, washed four times in PBS, and suspended in sterile water to contain 5×10^8 bacteria per ml. Radioactivity in the last supernatant never exceeded 2% of that in the pellet. Radioactivity per bacterial cell was in the range of 10^{-3} cpm.

Human protein preparations. Human serum albumin, human polyclonal immunoglobulin (immunoglobulin G [IgG]), and human fibrinogen were purchased from Kabi AB (Stockholm, Sweden). IgA was prepared from a serum containing a high concentration of an IgA myeloma protein as described previously (25). Preparations of aggregated human beta-2-microglobulin were kindly supplied by L. Björck, Lund, Sweden. Human serum albumin, IgG, IgA, and aggregated beta-2-microglobulin were labeled with ^{125}I (The Radiochemical Centre) by using the chloramine-T method (23). Fibrinogen was radiolabeled by using the electrolytic method of Rosa et al. (29), as described by Harboe and Fölling (11).

Human protein-binding assay. Overnight cultures of bacterial strains in Todd-Hewitt broth were harvested and washed twice in PBS. The bacterial concentration was adjusted to 10^9 organisms per ml. The bacteria were suspended in PBS with 0.05% Tween 20. Binding studies were carried out in plastic tubes (70 by 12 mm; AB CERBO, Trollhättan, Sweden) by adding 2×10^8 bacteria (200-μl suspension containing 10^9 organisms per ml) to radiolabeled protein. The amounts used were 0.08 to 0.17 μg except for beta-2-microglobulin (2.12 μg). After 1 h at room temperature, 2 ml of PBS containing Tween 20 was added to each tube, and the bacteria were centrifuged. The supernatants were removed, and the radioactivity of the bacterial pellets was measured in a gamma counter (LKB Rack Gamma, model 1270; Biotec, Stockholm, Sweden). The uptake of human serum proteins in percentage of total radioactivity added was calculated (Table 1). An uptake of <6% was considered negative. All uptake assays were performed in duplicate.

Polymer two-phase systems. PEG 4000 was obtained from Union Carbide, New York, N.Y., and Dextran T500 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Positively charged trimethylamino PEG (TMA-PEG), negatively charged PEG sulfonate (S-PEG), and P-PEG synthesized from PEG 4000 (15) were kindly supplied by G. Johansson, University of Lund, Lund, Sweden. St-PEG 4000 was purchased from Serva, Heidelberg, West Germany. The phase systems were prepared from stock solutions of dextran (20%), PEG 4000 (40%), TMA-PEG (10%), S-PEG (10%), P-PEG (2.5%), and St-PEG (2.5%). Salt and buffer stock solutions were made in concentrations 10 to 20 times higher than that of the final concentration in the phase system. All phase systems were prepared and all experiments were carried out at room temperature.

Cross-partition experiments. Single-tube partitions were performed in plastic tubes (70 by 12 mm; AB Cerbo) with 1.00-g phase systems. Two series of phase systems were prepared, one with positively charged TMA-PEG and one with negatively charged S-PEG. The compositions of the phase systems are given in Table 2 (phase systems A and B). Batch systems were prepared from stock solutions of dextran, PEG 4000, and TMA-PEG 4000 or S-PEG 4000. A 0.85-g sample was taken from the batch systems immediately after mixing, using a Cornwall pipette (Becton, Dickinson & Co., Rutherford, N.J.). Adding 50 μl of buffer solution and 100 μl of bacteria (5×10^7 bacteria) in sterile water gave the desired final concentration of all components. Sodium citrate-citric acid and citrate HCl buffers were used to obtain pH values between 3.0 and 7.2 and between 1.3 and 4.8, respectively. The phase systems were mixed thoroughly by inverting the tubes about 50 times. After 40 min of settling time, 100 μl of the top or bottom phase was withdrawn from each tube with a calibrated pipette (Oxford Laboratories, Foster City, Calif.). The radioactivity in each sample was measured in a gamma scintillation counter (LKB-Wallace 1280 Ultro Gamma; Biotec). The percentage of bacterial cells in the top phase (in some experiments in top and bottom phases) was calculated. The pH was measured on the residual phase system.

Hydrophobic affinity partition experiments. Single-tube partitions were performed with 1.00-g phase systems of the compositions given in Table 2 (phase systems C and D). A batch system was prepared from stock solutions of dextran, PEG 4000, sodium phosphate buffer, and sodium chloride. A 0.80-g sample was taken from the batch system immediately after mixing. Adding 100 μl of esterified PEG (P-PEG or St-PEG) or sterile water and 100 μl of bacterial suspension gave the final concentration of all components. The phases were mixed thoroughly and allowed to separate for 40 min. From the bottom phase or the top and bottom phases 100 μl was withdrawn, and deter-

TABLE 1. Binding of radiolabeled human proteins to bacterial strains[a]

Bacterial strains	Uptake (% total radioactivity added to cells)				
	IgG	IgA	Beta-2-microglobulin	Albumin	Fibrinogen
Group A streptococcal strains					
A1	24	7	19	6	65
A207	39	15	42	2	18
AM1	53	20	53	2	52
AM18	18	22	93	3	62
AM23	47	19	77	2	33
AM24	19	5	19	3	65
AM28	12	7	17	6	68
AR1	86	6	17	5	68
AW1	14	43	28	3	43
AW4	14	21	86	3	58
AW25	39	7	18	6	68
AW33	15	34	45	3	45
AW43	16	23	29	3	47
HA1	16	5	32	3	55
HA2	28	11	59	3	35
Group C and G streptococcal strains					
C16	82	7	3	30	51
C48	83	8	22	33	55
G34	86	11	4	44	51
G41	92	10	6	33	49
Staphylococcal strains					
Cowan 1	78	2	2	2	5
L603	5	3	4	3	4

[a] Includes all investigated group A streptococci and representative strains of group C and G streptococci as well as straphylococci.

mination of the bacteria in the phases was made by measuring the radioactivity in a gamma scintillation counter. In the phase systems used for hydrophobic affinity partition experiments, the bacteria partitioned between bottom phase and interphase. For comparison of the relative hydrophobicities among the various bacterial strains, a hydrophobicity index (C) was calculated by using the following formula (see Fig. 5): C = [counts per minute in bottom phase in system with 0.10% (wt/wt) P-PEG/counts per minute in bottom phase in system without P-PEG].

RESULTS

Isoelectric point of streptococci. The isoelectric point of 28 streptococcal strains was determined by cross-partition and included 16 laboratory-maintained strains, 6 freshly isolated strains, and 6 heat-inactivated strains. The laboratory-maintained strains showed cross-points between pH 3.50 and 4.10, with a mean of 3.75 (Table 3). Figure 1 shows representative results from a cross-partition of four strains of streptococci. The freshly isolated streptococcal strains showed cross-points between pH 3.60 and 3.95 (mean, 3.74) and thereby did not differ from the laboratory-maintained strains. The cross-points of the heat-inactivated strains were in all instances lower than the corresponding live bacteria tested on the same occasion. Two bacterial strains (G148 and ARI) tested on five different occasions showed a standard deviation in cross-

TABLE 2. Composition of phase systems for single-tube partition

Phase system	% (wt/wt)						mM		
	Dextran T500	PEG 4000	TMA-PEG	S-PEG 4000	P-PEG 4000	St-PEG 4000	Buffer (see text)	Sodium phosphate buffer (pH 6.9)	NaCl
A	6.8	4.8	2.0				2.5		
B	6.8	4.8		2.0			2.5		
C	6.1	6.1			0–0.5			5.0	40.0
D	6.1	6.1				0–0.5		5.0	40.0

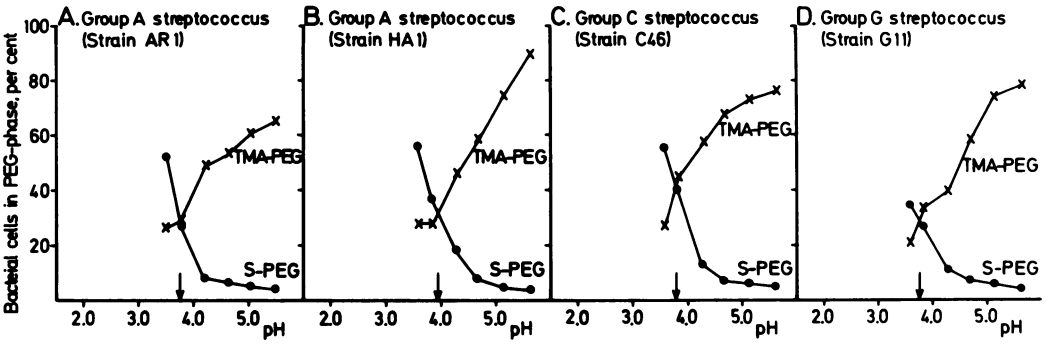

FIG. 1. Cross-partition of two group A streptococcal strains, AR1 and HA1 (A and B), one group C streptococcal strain, C46 (C), and one group G streptococcal strain, G11 (D). Symbols: ×, partition with TMA-PEG (positively charged); ●, phase systems with S-PEG (negatively charged). Arrows indicate cross-point values.

point values of not more than 0.11 and 0.025 pH units, respectively.

Isoelectric point of staphylococci. The isoelectric point of nine staphylococcal strains was determined by cross-partition and included five laboratory-maintained strains, two freshly isolated strains, and two heat-inactivated strains. Table three summarizes the mean cross-point value for the staphylococcal strains. For the laboratory-maintained strains and the freshly isolated strains, a cross-point below pH 2.2 was obtained (mean, pH 1.95). Figure 2 shows representative results from cross-partition of two staphylococcal strains. The cross-point values of the heat-inactivated strains were in both instances significantly higher than those for the corresponding live bacteria tested on the same occasion (mean, pH 2.65).

Effect of human serum on bacterial cross-partition. Five bacterial strains capable of binding different human serum proteins were preincubated with human serum before analysis. A 100-μl portion of normal human serum, diluted 1:10 in PBS, was added to 8×10^8 bacterial organisms and incubated at room temperature for 30 min. The bacteria were then washed once in PBS and suspended in sterile water. The isoelectric point of the bacteria pretreated with serum was determined by cross-partition and compared with the cross-point values obtained with the same bacteria pretreated with PBS alone as a control. The cross-partition of all four streptococcal strains studied was strongly influenced by pretreatment with human serum, resulting in less acidic cross-points (Table 4; Fig. 3). In contrast, pretreatment of *S. aureus* with human serum did not lead to any detectable change of isoelectric point by this method (Table 4).

Hydrophobic affinity partition. Relative surface hydrophobicity of 12 staphylococcal strains, 15 group A streptococci, 12 group C streptococci, and 12 group G streptococci was determined by hydrophobic affinity partition, using P-PEG (phase system C) and St-PEG (phase system D; Table 2). All bacteria studied were affected to the same extent by P-PEG and St-PEG.

The streptococci varied greatly in affinity for the PEG-bound groups. Most of the group A streptococci studied (11 of 15) gave results indicating a high affinity for P-PEG (Fig. 4A and 5). The remaining group A streptococci showed no

TABLE 3. Cross-point values of streptococcal and staphylococcal strains[a]

Bacteria	No. of strains[b]	Cross-point (mean pH ± SD)
Group A streptococci	8	3.67 ± 0.15
Group C streptococci	7	3.80 ± 0.12
Group G streptococci	7	3.80 ± 0.16
Staphylococci	7	1.95 ± 0.16

[a] The cross-point pH is taken as an estimate of the isoelectric point of the exposed bacterial surfaces.

[b] Includes both laboratory-maintained and freshly isolated strains.

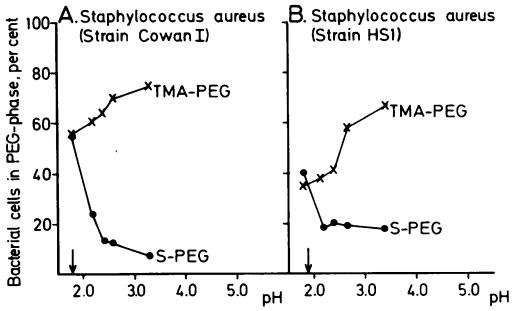

FIG. 2. Cross-partition of two *Staphylococcus aureus* strains. Symbols as in Fig. 1.

TABLE 4. Effect of human serum on cross-point values of bacteria

Bacterial strain	Cross-point (pH)	
	Cells pretreated with human serum	Cells untreated
Group A streptococci, ARI	4.80	3.75
Group A streptococci, AMI	4.20	3.50
Group C streptococci, CI6	4.35	3.95
Group G streptococci, G148	4.80	3.90
Staphylococcus aureus Cowan I	<1.80[a]	1.80

[a] No cross-point was obtained within the pH range used in this experiment.

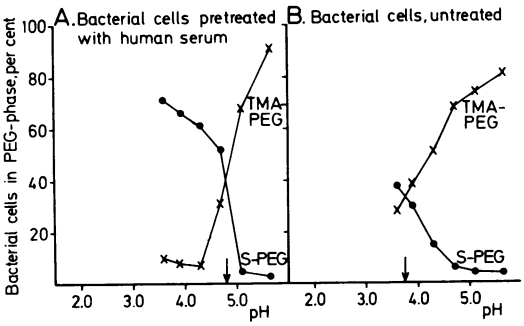

FIG. 3. Effect of normal human serum on cross-partition of one group A streptococcal strain (AR1). The bacteria were pretreated with human serum (A) or with PBS as a control (B) before cross-partition experiments. Symbols as in Fig. 1.

affinity for P-PEG in concentrations up to 0.25% (wt/wt). Among the group C and G streptococci there was a more even distribution among the strains with high, medium, and low affinity for P-PEG (Fig. 4B and 4C and 5). The partition of the staphylococci was not influenced by the addition of up to 1% (wt/wt) P-PEG or St-PEG (Fig. 5). Figure 4D shows the partition of three staphylococcal strains as a function of the concentration of P-PEG.

The freshly isolated streptococcal strains all had intermediate to high affinity for P-PEG, whereas the freshly isolated staphylococcal strains showed no affinity for P-PEG. Heat-inactivated streptococcal strains showed, when tested on the same occasion as the live bacteria, a lower hydrophobic affinity than the live bacteria in all instances. Hydrophobic affinity partition of heat-inactivated staphylococci was not altered compared with live bacteria.

DISCUSSION

In the present investigations the isoelectric points and surface hydrophobicity of gram-positive cocci have been determined by cross-partition and hydrophobic affinity partition in aqueous two-phase systems. Phase partition provides a useful tool for the study of surface charge and hydrophobicity in particles such as bacteria (3, 5, 8, 19–22, 24, 27, 33–36). Isoelectric points of the bacteria were determined by cross-partition experiments with dextran-PEG two-phase systems containing charged polymers (6, 9; Table 2). The results obtained indicate an isoelectric point for streptococci of about pH 3.75 (Fig. 1; Table 3). The staphylococci showed an isoelectric point of around pH 2, thereby differing markedly from the streptococci (Fig. 2; Table 3). Results from cross-partition of the ribitol teichoic acidless mutant 52 A5 (kindly supplied by D. Mirelman, Rehovot, Israel) indicated an isoelectric point of <pH 2. This observation suggests that teichoic acid makes only a minor contribution to the highly acidic cross-point of staphylococci.

The surface hydrophobicity of gram-positive cocci was assessed by determining the affinity

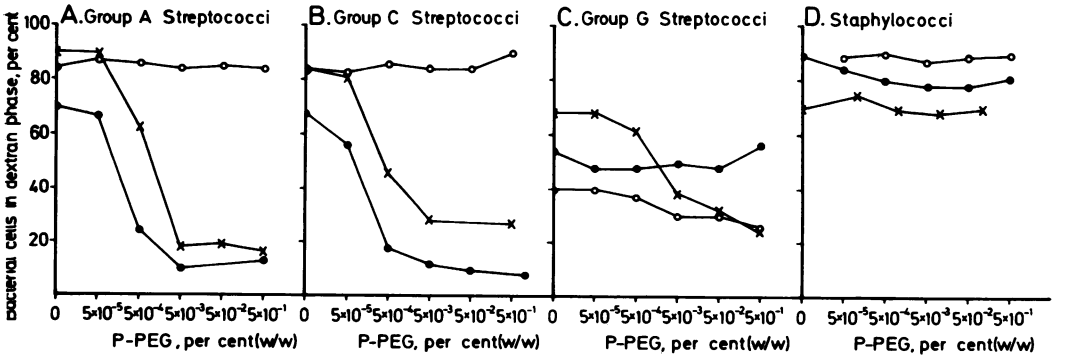

FIG. 4. Hydrophobic affinity partition in a dextran-PEG phase system at different P-PEG concentrations. Symbols: (A) ×, group A streptococcal strains AM23; ●, AM1; ○, AM28. (B) ×, Group C streptococcal strains C48; ●, C42; ○, C16. (C) ×, Group G streptococcal strains G41; ●, G34; ○, G51. (D) ×, Staphylococcal strains DO4975; ●, S48; ○, L602.

for PEG-bound hydrophobic groups. Two groups with different hydrophobic reactivities were identified among the group A streptococci. Most group A streptococci tested showed a high hydrophobic affinity, whereas partition of the remaining group A streptococci was not influenced by P-PEG (Fig. 5). Among the group C and G streptococci, there were several strains with intermediate reactivities giving a wider distribution of hydrophobic affinity (Fig. 5). The partition of the staphylococcal strains was not influenced by the addition of up to 1% (wt/wt) P-PEG or St-PEG (Fig. 4D and 5). *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* are known to have a poor hydrophobic interaction liability (8). In our investigation this was also shown to be true for all *S. aureus* strains studied. It might be a general phenomenon that when a bacterial surface is highly negatively charged, it also shows a low hydrophobic affinity even if hydrophobic sites are present. The large number of negative charges might prevent the interaction of PEG-bound palmitoyl groups with hydrophobic structures on the bacterial surface.

An increasing number of specific interactions between surface structures of streptococci and human proteins have recently been discovered (17; G. Kronvall, L. Björk, H. Miörner, E. Myhre, and K. Widebäck, *in* S. E. Holm and P. Christensen [ed.], *Basic Concepts of Streptococci and Streptococcal Diseases*, in press). The bacterial strains included in this study were tested for uptake of human serum proteins (Table 1). A positive correlation was found between the hydrophobic affinity of group A streptococci and the density of specific receptors for aggregated beta-2-microglobulin (Table 1; Fig. 5). All group A streptococcal strains with low hydrophobic affinity showed a low uptake of radiolabeled aggregated beta-2-microglobulin (mean uptake, 17.5%), whereas the strains with high hydrophobic affinity showed a medium or high uptake of aggregated beta-2-microglobulin (mean uptake, 56%). It has been suggested that M-protein carries a receptor for aggregated beta-2-microglobulin (7). The results obtained indicate a relationship between M-protein and the hydrophobic properties of group A streptococcal cells which has previously been suggested by Tylewska et al. (37). The surface hydrophobicity of streptococci did not correlate with the presence of surface receptors for other human proteins tested (e.g., albumin, IgG, IgA, and fibrinogen).

We have previously described marked changes of surface characteristics of gram-positive cocci after specific binding of human proteins (24). Pretreatment of bacteria with human serum leads to an increase of the isoelectric

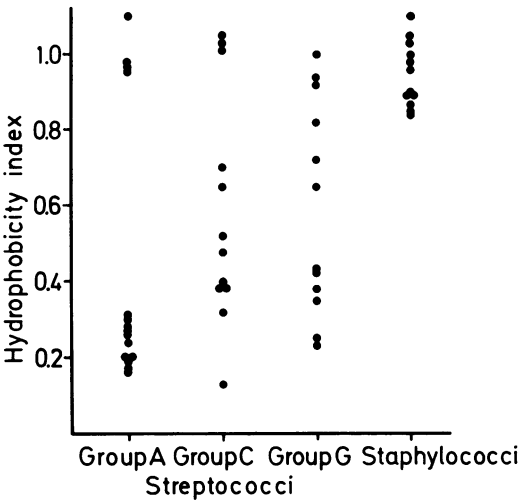

FIG. 5. Hydrophobicity index (*C*) of 50 human strains of group A, C, and G streptococci and staphylococci as calculated from the formula: C = (counts per minute in bottom phase in system with 0.10% P-PEG/counts per minute in bottom phase in system without P-PEG). The group A streptococci with low hydrophobic affinity include strains A1, AM28, AR1, and AW25.

points of the streptococci investigated, whereas no such increase could be observed with the *S. aureus* strain (Fig. 3; Table 4). We have previously suggested that the binding of protein to bacteria leads to a masking of the bacterial cell surface properties (24). Experiments have been initiated to further characterize the change in surface charge of gram-positive cocci after specific binding of various human serum proteins.

An aqueous dextran-PEG two-phase system containing P-PEG or St-PEG was used for hydrophobic affinity partition (phase systems C and D, Table 2). These phase systems have an interfacial potential close to zero; therefore, the bacteria distribute according to the ratio between hydrophilic and hydrophobic sites exposed on their surface. Most of the bacteria partitioned into the bottom phase in the absence of esterified PEG. The main change observed when the concentration of P-PEG or St-PEG was increased was the transfer of bacterial cells from the bottom phase into the interphase. The bacteria studied were affected to a similar extent by P-PEG and St-PEG.

No single method can adequately describe surface hydrophobicity, and there is not always a correlation between hydrophobic affinity partition and other methods of assessing surface hydrophobicity. A possible explanation is that hydrophobic affinity partition measures an overall quantitative expression of the hydrophobic and hydrophilic sites exposed on the bacterial

surface whereas with other methods (hydrophobic interaction chromatography, binding of hydrocarbons and fatty acids to cells; 16, 30, 31, 37–39, 41) a few strongly hydrophobic sites on the bacterial surface will allow the bacteria to adsorb to the hydrophobic ligand.

Cross-partition has previously been used for the determination of the isoelectric points of proteins, mitochondrial membranes, spinach thylakoid membranes, and rat liver peroxisomes (4, 6, 9, 14). Two curves were obtained when the percentage of bacteria in the top phase was plotted against the pH (Fig. 1). The obtained cross-point of the curves was taken as the approximate isoelectric point of the bacteria. In the present investigation the bacteria were grown in a defined medium overnight. Variations of the electrophoretic mobility of streptococci harvested during different growth phases or prepared in different growth media have been reported (12, 13). The possible effect of different growth media and growth conditions on the surface properties of the bacteria have not yet been studied in polymer two-phase systems.

Several reports indicate that the surface charge of either the mammalian cells or the bacteria plays an important role in their mutual interaction (26, 33, 36, 40). Gram-positive cocci are negatively charged at neutral pH due to an excess of carboxyl and phosphate groups. In addition, some bacteria contain highly negatively charged teichoic and teichuronic acids. Most mammalian cells also carry a net negative charge which could give rise to a net repulsive force between bacteria and mammalian cells. Previous attempts have been made to measure the isoelectric point of bacteria. Electrokinetic studies of different strains of *Streptococcus pyogenes* indicate an isoelectric point of between pH 4.3 and 4.7 (12). The isoelectric point of *Streptococcus sanguis* has been determined by isoelectric focusing and was found to be pH 5.3 (18). Preliminary results from isoelectric focusing of M-protein-positive and M-protein-negative strains of *Streptococcus pyogenes* suggest isoelectric points of pH 5.8 and 6.0, respectively (37). M-proteins of streptococci play a major role in the pathogenicity of these bacteria. It appears that the presence of M-protein does not lead to any major change in surface charge, whereas the indicated hydrophobic properties of M-protein might be of importance in the interaction between vertebrate cells and *Streptococcus pyogenes*.

ACKNOWLEDGMENTS

The excellent technical assistance of Elisabeth Nilsson, Ing-Britt Gustavsson, and Åke Christensson and the secretarial assistance of Ingela Berggren are gratefully acknowledged.

This project was supported by the Swedish Medical Research Council, grant 05210, and by the A. Österlunds Foundation.

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