JOURNAL OF BACTERIOLOGY, Sept. 1986, p. 1016–1019
0021-9193/86/091016-04$02.00/0

Vol. 167, No. 3

Determination of Hydrophobicity on Bacterial Surfaces by Nonionic Surfactants

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Received 3 January 1986/Accepted 29 May 1986

The hydrophobicity of the bacterial cell surface was determined by using nonionic surfactants. The method is based on the adsorption of nonionic surfactants at the hydrophobic sites of the cell surface. Among many nonionic surfactants, $C_{18}H_{37}O(CH_2CH_2O)_{13}H$ was preferred. The surfactant was added in excess to a bacterial suspension, and the suspension was mixed by sonication or mechanical stirring. The amount of surfactant remaining in the supernatant after centrifugation was determined spectrophotometrically by measuring the absorbance of tetrabromophenolphthalein ethylester. Effective dispersion of bacterial cells such as *Staphylococcus aureus* and *Mycobacterium smegmatis* was achieved by sonication in the presence of the nonionic surfactant. Adsorption measurements coincided with Langmuir's equation, indicative of monolayer adsorption. The method is useful for the determination of the hydrophobicity of various bacterial cell surfaces.

The bacterial cell surface contains hydrophilic as well as hydrophobic sites. The former is formed mainly by charged groups such as the carboxyl, phosphate, amino, or guanidyl group and the noncharged hydroxyl group. The latter consists of lipids and lipopolysaccharides. Previously (15), we determined the surface charge by colloid titration. More recently, the hydrophobicity of bacterial cell surfaces was reported (8). Methods used were based on bacterial adherence to hydrocarbons (17), interaction with hydrophobic chromatography (2, 19), aqueous two-phase partition (1, 10–13), measurement of the contact angle (14, 21, 22), salt aggregation (3, 7), and other factors (5, 16). Binding of a radioactive probe (4) is quantitative. However, it is uncertin whether the bonding between the active probe and the cell surface reflects correctly the hydrophobicity of the cell. Other effects may be involved.

A nonionic surfactant contains a hydrophobic alkyl group and a hydrophilic polyethyleneoxide chain. The alkyl group can combine with hydrophobic sites such as lipids and lipopolysaccharides on the bacterial cell surface, and the polyethyleneoxide chain will be in water. If a cationic or anionic surfactant is used, the alkyl group can combine with the hydrophobic sites on the bacterial cell surface and at the same time the cationic or anionic charge can also combine with the oppositely charged sites on the bacterial cell surface. Therefore, the hydrophobicity cannot be determined exactly by the cationic or anionic surfactant.

In this study, we determined quantitatively the hydrophobicity on the surfaces of various kinds of gram-positive and gram-negative bacteria by using a nonionic surfactant. An excess amount of the nonionic surfactant was added to the bacterial suspension and agitated. After centrifugation, the excess of the surfactant in the supernatant reacted with potassium ion to form a cationic complex which was extracted into dichlorobenzene as an ion pair with tetrabromophenolphthalein ethylester potassium salt. The A_{620} of the tetrabromophenolphthalein ethylester ion in the solvent was measured to determine the surfactant (20), and the adsorbed surfactant on the bacterial cell surface was calculated.

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MATERIALS AND METHODS

Chemicals. Tetrabromophenolphthalein ethylester potassium salt obtained from Wako-junyaku-kogyo Co., Ltd., Osaka, Japan, was dissolved in ethanol to make a 1.5×10^{-3} M solution. As a standard nonionic surfactant, $C_{18}H_{37}O(CH_2CH_2O)_{13}H$ (polyethylene glycol mono-*n*-oleylether) (Emalgen 420; Kao Co., Ltd.) was dissolved in distilled water to give a stock solution of 200 mg/ml and was diluted to the desired concentration. All reagents used in this work were of analytical reagent grade.

Bacteria. The following strains were used: *Staphylococcus aureus* 209P, *Staphylococcus aureus* FRI243, *Staphylococcus saprophyticus* H131, *Staphylococcus saprophyticus* H138, *Mycobacterium smegmatis* ATCC 14468, *Micrococcus luteus* ATCC 4698, *Escherichia coli* K-12, and *Salmonella typhi* ATCC 33458. Cells of the stock strains cultivated in a nutrient broth at 37°C with gentle shaking were harvested at the late logarithmic phase by centrifugation and washed twice with physiological saline solution.

Experiments. (i) Pretreatment of bacterial cells. Gram-positive bacteria were suspended in physiological saline solution and transferred to a 100-ml conical flask. The suspension was sonicated in a bath-type sonicator (model no. 41-4000, 60 Hz, 40 W; Branson Sonic Power Co., Danbury, Conn.) at 4°C for 3 min and centrifuged at $170 \times g$ for 5 min to remove the nondispersed cells as a pellet. The dispersed cells were collected by centrifugation at $1,500 \times g$ for 10 min, and the supernatant was discarded. The cells (about 1,500 mg [wet weight]) were suspended in 100 ml of 0.02 M phosphate buffer (pH 7.8). Duplicate samples from the suspension were used for the dry weight determination (dried at ~105 to 110°C for 24 h). The residual suspension was used for the adsorption experiment with the nonionic surfactant.

(ii) Adsorption of the nonionic surfactant on the bacterial cells. Samples (5 ml) of a freshly prepared cell suspension (15 mg [dry weight] of bacteria) were transferred into five test tubes (1.8 cm [inside diameter] by 18 cm). A 3-ml portion of 1.35 M glucose solution and 2 ml of 0.02 M phosphate buffer (pH 7.8) were added to each to give a total volume of 10 ml. Next, 5-ml samples of the surfactant solutions in concentrations of 50, 70, 100, 140, and 200 μg/ml were added to each

TABLE 1. Equilibrium concentration and adsorbed amount of the nonionic surfactant

Organism	Equilibrium concn (*C*) (μg/15 ml)	Adsorbed surfactant (*X*) (μg/15 mg [dry wt])
M. smegmatis ATCC 14468	164	186
	261	239
	417	283
	704	296
S. aureus 209P	155	95
	235	115
	370	130
	558	142
	852	148
S. aureus FRI243	191	59
	278	72
	415	85
	609	91
	904	96
Micrococcus luteus ATCC 4698	170	80
	255	95
	397	103
	594	106
	892	108
S. saprophyticus H131	170	80
	262	88
	407	93
	904	96
S. saprophyticus H138	174	76
	268	82
	412	88
	609	91
	906	94
E. coli K-12	213	37
	303	47
	444	56
	635	65
	931	69
Salmonella typhi ATCC 33458	207	43
	301	49
	445	55
	642	58
	940	60

to give a final volume of 15 ml. (Final nonionic surfactant concentrations were 250, 350, 500, 700, and 1,000 μg/15 ml.) These individual samples were mixed with a vortex mixer (TM-105; Thermonics Co., Ltd., Tokyo, Japan) before sonication. The test tubes were placed in a 300-ml beaker containing water and sonicated at 25°C for 2 min; this was to prevent the bacteria from breaking during sonication. The cells were not destroyed, as determined by observation with a microscope. By this procedure, the cells were dispersed sufficiently, and the surfactant was adsorbed on the individual bacterial cell surface.

For gram-negative bacteria, the adsorption of the surfactant was performed without pretreatment by mixing with a vortex mixer for 1 min, because the bacteria were suspended completely; the sonication process was omitted.

A portion of the suspension was used for counting the bacteria with Petroff-Hauser and Helber counting chambers. After adsorption, each suspension was centrifuged at 22,000 × *g* for 15 min to recover the supernatant.

(iii) Spectrophotometric determination of the nonionic surfactant in the supernatant. For the 50-μg/ml surfactant (250 μg/15 ml), 2 ml of the supernatant was used for the determination of the amount of surfactant. For the surfactants at 350, 500, 700, and 1,000 μg/15 ml, the supernatants were diluted with 0.02 M phosphate buffer (pH 7.8) to make almost the same concentration as the 50-μg/ml surfactant. A 2-ml portion of the nondiluted or diluted surfactant was pipetted into a 25-ml stoppered test tube. Then, 1 ml of 1 M phosphate buffer (pH 7.8), 2 ml of 2 M KCl, 3.3 ml of distilled water, and 0.7 ml of tetrabromophenolphthalein ethylester potassium salt were added (resulting in a final volume of 9 ml). A 5-ml portion of *o*-dichlorobenzene was added, and the chromophore was extracted into the organic phase with a vortex mixer. After phase separation, the A_{620} of the organic phase was measured with a spectrophotometer (UV-200S; Shimadzu, Kyoto, Japan) in a glass cell of path length 10 mm, and the adsorbed surfactant on the bacterial cell was calculated. The results are shown in Fig. 1 and Table 1.

RESULTS

Selection of nonionic surfactants. The various nonionic surfactants examined were as follows: $C_{12}H_{25}O(CH_2CH_2O)_6H$, $C_{12}H_{25}O(CH_2CH_2O)_{13.7}H$, $C_{18}H_{37}O(CH_2CH_2O)_{13}H$, $C_{18}H_{37}O(CH_2CH_2O)_{20}H$, n-$C_9H_{19}C_6H_4O(CH_2CH_2O)_8H$, n-$C_9H_{19}C_6H_4O(CH_2CH_2O)_{17}H$, $CH_3C(CH_3)_2CH_2C(CH_3)_2C_6H_4O(CH_2CH_2O)_{10}$, and $CH_3C(CH_3)_2CH_2C(CH_3)_2C_6H_4O(CH_2CH_2O)_{40}H$. For *S. aureus* 209P (from 0 to 20 mg [dry weight] of bacteria), every surfactant showed the ideal linearity between the amount of adsorbed surfactant and the amount of bacteria. Therefore, this method can be applied to clumping cells such as *S. aureus* 209P. A comparison of the calibration curves of the eight surfactants indicated that when n of $RO(CH_2CH_2O)_nH$ was ~6 to 20 and chain length was ~C_{12} to C_{18}, the surfactants for the adsorption were most suitable. Among them, $C_{18}H_{37}O(CH_2CH_2O)_{13}H$ (Emalgen 420) had the highest sensitivity and therefore was used in this experiment.

Effect of sonication time. Sonication of bacteria with the nonionic surfactant separated *Staphylococcus*, *Micrococcus*, and *Mycobacterium* strains into single cells. The quantity of adsorbed surfactant was constant at 2 min or more. Accordingly, sonication was performed for 2 min.

Effect of surfactant concentration. Various concentrations of the surfactant (250, 350, 500, 700, 1,000, 2,500, and 5,000 μg/ml) were examined for adsorption on the bacterial cells. (The bacterial suspensions used were 0, 2, 3, 5, and 7 ml/15 ml.) As the surfactant concentration increased, the quantity of the adsorbed surfactant on the cells tended to increase. It was found that the total amount of surfactant should be larger than the adsorbed amount on the bacterial cells, but not too much larger. Therefore, the following concentrations were used: 250, 350, 500, 700, and 1,000 μg/15 ml. The amount of adsorbed nonionic surfactant was expressed as the adsorbed amount of the surfactant per 15 mg (dry weight) of bacteria (Table 1 and Fig. 1).

DISCUSSION

Langmuir's equation. The quantity of the adsorbed surfactant on the cell surface tended to increase as the equilibrium concentration of the surfactant increased and to

approach a maximum gradually (Fig. 1). This was modeled by the Langmuir adsorption isotherm (6) as follows:

$$\theta = \frac{X}{X_{max}} = \frac{a \cdot C}{1 + a \cdot C} \quad (1)$$

or

$$\frac{1}{X} = \frac{1}{a \cdot C} \cdot \frac{1}{X_{max}} + \frac{1}{X_{max}} \quad (2)$$

where X is the quantity of the adsorbed surfactant, X_{max} is the maximum quantity of the adsorbed surfactant at infinite concentration of the added surfactant and should be insensitive to temperature, a is the ratio of the rate of sticking per collision to the rate of evaporation and should be constant at constant temperature, and C is the equilibrium concentration of the surfactant in the supernatant. In equation (2), X_{max} and a are the characteristic values of the bacterium under the experimental conditions. The results of the experiment summarized in Table 1 are shown in Fig. 2, in which X^{-1} is expressed as the ordinate and C^{-1} is expressed as the abscissa. The straight lines in Fig. 2 indicate that the adsorption is described by Langmuir's equation at the concentrations investigated. This fact is consistent with the proposition that the nonionic surfactant is adsorbed on the hydrophobic sites, such as lipids in the membrane and lipopolysaccharides on the cell surface, as a monolayer of the surfactant molecule. From the slope and the intercept of the lines in Fig. 2, X_{max} and a can be calculated. These values are summarized for the various organisms in Table 2.

Determination of hydrophobic sites on the bacterial cell surfaces. *S. aureus* 209P showed a higher hydrophobicity than *S. saprophyticus*, which indicates that the hydrophobicity of the cells is measured strictly in the same genus. Hydrophobicity as measured by adherence to hydrocarbons (17) and by hydrophobic affinity partition (12) supports this indication. The surface of *M. smegmatis* is covered with hydrophobic constituents such as mycolic acid (18), and the X_{max} for this species is higher than those for the other bacteria. Our method can also be applied to the gram-negative bacteria (9). The quantity of the surfactant adsorbed on each bacterium can be calculated from the relationship between the number of cells and the dry weight of the bacteria. The results are shown in Table 3. The surface hydrophobicity is measured by our method with a good reproducibility. The charged groups, such as guanidinium or

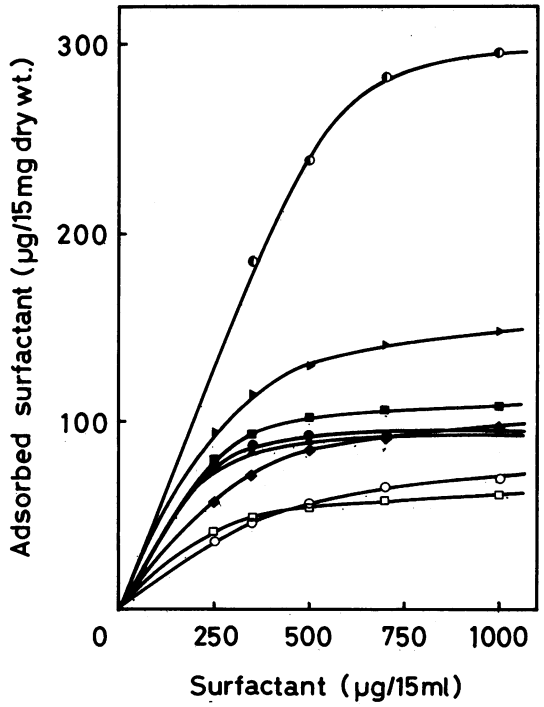

FIG. 1. Adsorbed amount of nonionic surfactant on the various bacterial cells. Concentrations of 250, 350, 500, 700, and 1,000 μg/15 ml of the surfactant were examined. Symbols: ◐, *M. smegmatis* ATCC 14468; ▶, *S. aureus* 209P; ◆, *S. aureus* FRI243; ■, *Micrococcus luteus* ATCC 4698; ●, *S. saprophyticus* H131; ▲, *S. saprophyticus* H138; ○, *E. coli* K-12; □, *Salmonella typhi* ATCC 33458.

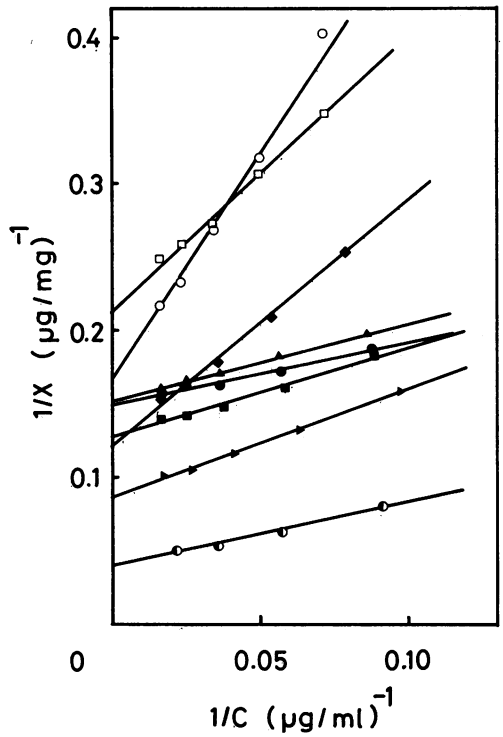

FIG. 2. The relationship between $1/C$ and $1/X$. Symbols: ◐, *M. smegmatis* ATCC 14468; ▶, *S. aureus* 209P; ◆, *S. aureus* FRI243; ■, *Micrococcus luteus* ATCC 4698; ●, *S. saprophyticus* H131; ▲, *S. saprophyticus* H138; ○, *E. coli* K-12; □, *Salmonella typhi* ATCC 33458.

TABLE 2. X_{max} and a of the nonionic surfactant adsorbed on the various bacterial cells

Organism	X_{max} [μg/mg (dry wt)] (10^{-1})	Mean ± SD [(μg/ml)$^{-1}$] (10^{-3})
M. smegmatis ATCC 14468	256	88 ± 12[a]
S. aureus 209P	116	116 ± 9[b]
S. aureus FRI243	81	75 ± 8[b]
Micrococcus luteus ATCC 4698	78	233 ± 36[b]
S. saprophyticus H131	67	389 ± 51[a]
S. saprophyticus H138	66	287 ± 15[b]
E. coli K-12	60	55 ± 4[b]
Salmonella typhi ATCC 33458	47	109 ± 11[b]

[a] $n = 4$.
[b] $n = 5$.

TABLE 3. Amount of nonionic surfactant adsorbed on one bacterial cell

Organism	Adsorbed surfactant	
	$\mu g/10^{11}$ Cells	Molecules/cell (10^4)
M. smegmatis ATCC 14468	773	552
S. aureus 209P	155	110
S. aureus FRI243	90	64
Micrococcus luteus ATCC 4698	80	57
S. saprophyticus H131	89	64
S. saprophyticus H138	70	50
E. coli K-12	173	124
Salmonella typhi ATCC 33458	79	56

phosphate ion, cannot influence adsorption, because the surfactant has no charge, and only the hydrophobic group can combine firmly with the hydrophobic sites of the bacteria; the polyethyleneoxide chain serves only to make the molecule soluble in water. Thus, our method for the determination of hydrophobic sites of bacterial surfaces is more useful than other methods.

The nonionic surfactant method for determining hydrophobicity is simple and quantitative and gives good reproducibility. Tests can be performed with living bacteria. The surfactant is adsorbed on the bacterium surface as a monolayer. A specific maximum concentration, X_{max}, and an a value can be obtained for each bacterial species.

ACKNOWLEDGMENTS

We thank Kyoji Tôei and Norio Nishimura, Faculty of Science, Okayama University, for many useful suggestions and valuable discussions, and Kazuro Tsuji, Kao Co. Ltd., for sending the data on the physical and chemical properties of Emalgen 420.

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