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Cell Surface Properties and Biofilm Formation of Pathogenic Bacteria

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Abstract Cell surface properties of slime production, cell hydrophobicity (CH), colony spreading, auto-aggregation, and biofilm formation (BF) of pathogens were investigated. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* generally formed strong biofilms and were positive slime producers. The extracellular polymeric substances isolated from agar-grown biofilms of *Bacillus cereus* and *P. aeruginosa* contained a high proportion of carbohydrates, proteins, and extracellular DNA. High levels of CH were observed for all pathogens when hydrocarbons were combined with ammonium sulfate (AS). Levels of hydrophobicity based on bacterial adhesion to hydrocarbons using nonane with AS were positively correlated with hydrophobic interaction chromatography assay and contact-angle measurement results. BF and CH values of all pathogens, except *E. coli* O157:H7 and *B. cereus,* were highly correlated. Levels of pathogen hydrophobicity differed depending on the measurement method. Factors affecting BF were different depending upon the pathogen.

Keywords: biofilm, cell hydrophobicity, foodborne pathogen

Introduction

Foodborne pathogens are of great concern for food processing industries that aim to provide safe products to consumers. Numerous studies have shown that pathogenic bacteria are able to adhere to and form biofilms on material surfaces found in food processing environments, resulting in an important source of contamination (1,2).

A biofilm can be defined as a sessile community of microbes characterized by cells that are irreversibly associated with a surface and embedded in an extracellular polymeric substance (EPS) matrix. The EPS plays an integral role in enabling microorganisms to initially adhere to a surface and to subsequently form a biofilm. Polysaccharides, proteins, and phospholipids, and teichoic and nucleic acids are the primary components of an EPS. The nucleic acid present in the biofilm EPS matrix has been termed extracellular DNA (eDNA) (3). In previous studies, eDNA has been shown to be essential for biofilm stability during the early stages of biofilm growth as eDNA enhances gene transfer and provides nutrition (4) under oligotrophic conditions (5). Biofilms develop when pathogenic microorganisms attach to food surfaces, equipment, and processing environments and biofilms can be a continuous source of contamination for contacting foods. Increased resistance of biofilm cells to antibacterial agents and sanitizers has also been observed (6). Therefore, biofilms are not easily controlled or eradicated.

The factors of hydrophobicity, surface charge, outer membrane proteins, and material properties play roles in attachment of bacterial cells to abiotic surfaces (7). Surface hydrophobicity is generally associated with bacterial adhesiveness and varies from organism to organism and from strain to strain and is influenced by the growth medium, bacterial age, and bacterial surface structures (1,3). Fimbriae of bacteria contribute to cell surface hydrophobicity and the majority of fimbriae that have been examined contained a high proportion of hydrophobic amino acid residues that play a role in cell surface properties and attachment (8). Previous studies have shown that there is a positive correlation between bacterial cell hydrophobicity and attachment to hydrophobic surfaces (9). Therefore, hydrophobicity and biofilm production are major factors in the adhesive process and in survival of pathogens.

A variety of techniques have been applied for evaluation of cell surface hydrophobicity. Measurement of microbial cell surface hydrophobicity is a subject of debate and commonly used methods of assessment include bacterial adherence to hydrocarbons (10), contact angle measurements (11), and hydrophobic interaction chromatography (12). Considerable variation in hydrophobicity values has been reported depending on the method of determination (13). Large variations have been observed using different measurement methods and a correlation between cell surface hydrophobicity and

bacterial adhesion has been reported (14); however, disagreement exists (15). Studies investigating the relationship between the hydrophobic properties of *E. coli* and attachment ability have shown a positive correlation in some varieties (16) and no correlation in other varieties (17) with no consistent results.

Pathogenic bacteria have been responsible for major outbreaks associated with food products due to a wide distribution and presence in the food industry as biofilms. However, to date, few studies have evaluated and compared cell surface properties and biofilm formation of pathogenic bacteria. Therefore, this study was conducted to investigate biofilm formation on polystyrene and the cell surface properties of cell hydrophobicity and auto aggregation of major pathogenic bacteria. Additionally, correlations between biofilm formation and bacterial cell surface properties were evaluated.

Materials and Methods

Bacterial strains and growth conditions Seven different pathogenic bacterial species were evaluated with 2-3 strains for each bacterium, including *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *Cronobacter sakazakii* (ATCC 12868, ATCC 29544, and ATCC 29004), *Salmonella typhimurium* (ATCC 19585, ATCC 43971, and DT104 phage type), *Pseudomonas aeruginosa* (ATCC 10145 and ATCC 15692), *Listeria monocytogenes* (ATCC 7644, ATCC 19114, and ATCC 19115), *Staphylococcus aureus* (ATCC 49444, ATCC 12692, and ATCC 12600), and *Bacillus cereus* (ATCC 10876 and ATCC 13061) obtained from the Chung-Ang University bacterial culture collection (Anseong, Korea). All cultures were maintained on tryptic soy agar (TSA) (Difco, Detroit, MI, USA) slants at 4°C and subcultured monthly.

Biofilm formation assay Each pathogen strain was cultured individually in tryptic soy broth (TSB) (Difco) for 24 h at 37°C. Wells of sterile polystyrene plates were each filled with 90 μL of TSB and inoculated with 10 μL of a pathogen to form biofilms on the surfaces of the microtiter plates. Negative control wells containing only TSB were included in each assay. Pathogens were incubated at 37°C for 6, 12, and 24 h. After removal of the medium from the microtiter plates wells wells were rinsed three times with distilled water (200 μL/well). After air-drying in a laminar flow biosafety hood, wells were stained with 50 μL of 0.5% crystal violet (CV) for 5 min. Excess stain was removed by washing five times with distilled water (200 μL/well). Dye bound to adherent cells was destained with and without 50 μL of 99% ethanol, and the optical density (OD) of each well was evaluated at 595 nm using a spectrophotometer (Spectronic 20 Genesys; Spectronic Instruments, Rochester, NY, USA).

Analysis of bacterial EPS EPS was extracted using the method of Wingender *et al.* (18). Preparation of bacterial biofilm cells involved culturing cells in TSB at 37°C for 24 h, after which cells were spread-plated onto TSA and incubated at 37°C for 24 h (19). Cells were collected from TSA surfaces using a sterilized cotton swab. The collected slimy biomass was suspended in 0.14 M NaCl at a mass ratio of biofilm wet weight to NaCl solution of 1:15, and bacterial cells were dispersed using vigorous stirring for 30 min. Cell numbers in TSA were then counted. Bacteria were separated from EPS by centrifugation of suspensions at 4,000×*g* for 1 h. Residual bacteria in the supernatants were removed using filtration through cellulose acetate membrane filters (0.2 μm pore diameter; Advantec, Saijo, Japan) to yield cell-free EPS solutions. The amount of protein was assayed using Bradford reagent with bovine serum albumin as a standard (Sigma, St. Louis, MO, USA). The total carbohydrate content was quantified using the phenol-sulfuric acid method with glucose as a standard (20). The quantity of eDNA was estimated based on the absorbance value at 260 nm in the Eq. (1):

$$\text{Quantity of eDNA } (\mu g/\mu L) = A_{260} \times \text{dilution factor} \quad (1)$$

Bacterial adhesion to hydrocarbons (BATH) assay BATH assays were performed as described by Goulter *et al.* (10) with minor modification. Two or three strains each of seven pathogens were harvested at the stationary phase, collected by centrifugation (8,000×*g* for 5 min), washed twice using phosphate buffered solution (PBS, pH 7.2) containing NaCl (0.137 μmol/L), potassium chloride (0.0027 μmol/L), potassium phosphate (0.0018 μmol/L), and sodium phosphate (0.001 μmol/L), and resuspended in PBS. The cell suspensions were adjusted to an OD of 1.0±0.2 at 600 nm. Three milliliter aliquot of the bacterial cell suspensions was overlaid with 1 mL of *n*-hexadecane (Sigma), *n*-xylene (Samchun, Pyeongtaek, Korea), *n*-octane (Junsei, Kanto, Japan), or *n*-nonane (Alfa Aesar, Lancashire, UK) as a hydrocarbon, followed by addition to 1.5 mL of 2 M ammonium sulfate (AS) (Kanto Chemical, Tokyo, Japan). Suspensions were vortexed for 3 min. A tube containing 3 mL of untreated cell suspension was used as a control (Ac). All tubes were allowed to stand at room temperature for 30 min. After the incubation for 30 min, 1 mL of the lower aqueous layer was removed using a pipette and the The OD of each well were measured at 600 nm using a spectrophotometer (Spectronic 20 Genesys). The ratio of the absorbance value of bacterial assay tubes (Ab) to the control suspension (Ac) was calculated as a percentage of cells bound to the hydrocarbon using the following Eq. (2):

$$\text{Hydrophobicity } (\%) = (Ac - Ab)/Ac \times 100 \quad (2)$$

BATH polystyrene microtiter plate assay For BATH polystyrene microtiter plate assays, two or three strains each of seven pathogen were harvested at the stationary phase, collected using centrifugation (8,000×*g* for 5 min), washed twice using PBS, and resuspended in PBS (pH 7.2). The OD value of the suspension was adjusted using PBS to 1.0±0.2 at 595 nm. The OD value of a 50 μL aliquot of the bacterial cell suspension (Ac) was measured using a spectrophotometer (Specronic 20 Genesys). A total volume of 90 μL of the bacterial cell suspension was overlaid with 30 μL of *n*-hexadecane, *n*-xylene, *n*-

octane, or *n*-nonane as a hydrocarbon, and was then added to 45 μL of 2 M AS. Suspensions were vortexed for 5 min (Ab). A plate containing 50 μL of an untreated cell suspension was used as a control (Ac). Plates were allowed to stand at room temperature for 30 min. After the incubation for 30 min, 50 μL of the lower aqueous layer was removed using a pipette, and the $OD_{600\ nm}$ value was measured using a spectrophotometer (Specronic 20 Genesys). The ratio of absorbance values of bacterial assay tubes (Ab) to the control suspension tubes (Ac) was calculated as a percentage of cells bound to the hydrocarbon as Eq. (2).

Hydrophobic interaction chromatography assay (HIC) The HIC method using phenyl-Sepharose (Sigma) was performed as outlined by Rivas *et al.* (12). Cell suspensions were prepared as described above and adjusted to an OD value of 1.0±0.2 at 470 nm (Ai) using 4 M sodium chloride in a 0.01 M sodium phosphate buffer (high salt buffer) at pH 7.0. Plastic Poly-Prep Chromatography Columns (Bio-Rad Laboratories, Hercules, CA, USA) were packed with 1 mL of phenyl-Sepharose CL-4B (Sigma). Columns packed with Sepharose CL-4B were used as controls as cells can become trapped in untreated Sepharose beads. Packed columns were washed with 20 mL of the high salt buffer for removal of excess ethanol used for preservation of gel suspensions. Ten mL of the bacterial cell suspension was applied to the column and allowed to drain into gel beads. The final 5 mL of eluted bacterial suspension was collected, and the $OD_{470\ nm}$ value was measured using a spectrophotometer (Specronic 20 Genesys) (Af). The percentage of cells retained by the phenyl-Sepharose was calculated as; cell hydrophobicity (%)=(Ai–Af)/Ai×100. The percentage of cells retained by the phenyl-Sepharose alone was obtained from the difference between the percentage retained by the phenyl-Sepharose columns and the control column containing Sepharose.

Contact-angle measurement (CAM) The CAM method was used as described by Chia *et al.* (11) using a goniometer (KSV Instruments, Ltd., Helsinki, Finland). A cell suspension was prepared as described above using sterile distilled water (SDW) and adjusted to an OD_{540} value of 1.0±0.2. A bacterial lawn was obtained by filtering a 20 mL cell suspension through an HA filter (0.45 μm pore diameter, 25 mm filter diameter; Millipore, Bedford, MA, USA) using negative pressure filtration. Bacterial cell filters were attached to glass using double sided adhesive tape and dried in a desiccator containing Drierite ($CaSO_4$) (W.A. Hammond Drierite Company Ltd., Xeniam, OH, USA) for 60 min. A drop of SDW was placed onto the filter using a 1 mL syringe (Hamilton, Reno, NV, USA) fitted with a needle and photographed using a digital video camera (30 fps Firewire IEEE 1394) connected to a computer. The contact angle was determined based on measurement of the angle between the filter surface and the tangent to the drop at the solid-solid air point using Image XP contact angle software (PHX-300, ver 5.9. U090902; S.E.O. Co., Suwon, Korea). At least 5 drops of SDW were deposited onto each filter, and 30 images were captured within 5 s for each drop.

Slime producing ability on Congo red agar (CRA) The slime producing ability on CRA was evaluated for all strains. CRA plates were prepared based on addition of 0.8 g of Congo red (Sigma) and 36 g of saccharose to 1 L of TSA. Plates were incubated for 24 h at 37°C, and subsequently overnight at room temperature. Slime-producing strains formed black colonies on CRA, whereas non-producing strains produced red colonies. The original method developed by Freeman *et al.* (21) was implemented for colony color evaluation. An internal reference 6-color scale was used for accurate assessment of all possible chromatic variations exhibited by colonies. Results were interpreted as very black, black, and almost black or reddish-black colonies with a rough, dry, and crystalline consistency on CRA considered as normal slime-producing strains. Very red, red, Bordeaux, and smooth colonies were classified as non-slime-producing strains.

Autoaggregation Autoaggregation assays were performed as described by Collado *et al.* (22) with minor modification. Seven different pathogenic bacteria were harvested at the stationary phase, collected by centrifugation (8,000×*g* for 5 min), washed twice using PBS, and resuspended in PBS (pH 7.2). Suspensions of the pathogens were standardized to OD_{550}=1.0 (2×10^8 CFU/mL) for all experiments. Bacterial suspensions were incubated as 1 mL aliquots at room temperature and the OD value was measured using a spectro-photometer (Multiskan EX; Thermo Electron Corp., Shanghai, China) at regular intervals without disturbing the microbial suspension to determine the kinetics of sedimentation. The autoaggragation coefficient (AC) was calculated at time *t* following the method of Kos *et al.* (23) as: $AC_t=[1-(OD_t/OD_i)]\times100$, where OD_i is the initial optical density of the microbial suspension at 550 nm and OD_t is the optical density at *t* time.

Statistical analysis Data were analyzed using an analysis of variance (ANOVA) with the SAS version 9.1 software package (SAS Institute, Cary, NC, USA) for randomized analysis. Significantly ($p\leq0.05$) different mean values were compared using Duncan's multiple range test. Interdependence between measureable features was evaluated based on Pearson's linear correlation coefficient (r).

Results and Discussion

Pathogen biofilm formation and slime production The ability of pathogens to produce biofilms on polystyrene microtiter plates for 24 h based on CV staining before and after destaining with ethanol is shown in Table 1. *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* were strong biofilm formers, whereas *E. coli* O157:H7 showed a moderate level of biofilm formation. Patterns of pathogen biofilm formation were significantly ($p\leq0.05$) different between biofilm formation measured using CV staining before and after ethanol destaining. *S. aureus* (very black colony), *E. coli* O157:H7 (black), and

Table 1. Optical density at 595 nm of pathogenic biofilms on a 96-well polystyrene microtiter plate at 37°C for 6, 12, and 24 h measured based on staining with and without ethanol destaining

Pathogens	Slime Production[1)]	Before destaining with ethanol			After destaining with ethanol		
		6 h	12 h	24 h	6 h	12 h	24 h
E. coli O157:H7	++	0.342±0.10B[2)]a[3)]	0.286±0.02Bab	0.212±0.03BCb	0.446±0.19CDa	0.352±0.05ABa	0.290±0.05Ba
C. sakazakii	-	0.164±0.04CDa	0.132±0.02Cab	0.116±0.02CDb	0.254±0.04DEa	0.213±0.04CDab	0.164±0.03Cb
S. Typhimurium	---	0.122±0.02Da	0.135±0.02Ca	0.086±0.02Db	0.190±0.04Ea	0.189±0.03Da	0.121±0.02Cb
P. aerugenosa	++	0.439±0.09Aa	0.230±0.10BCb	0.317±0.13Aab	0.679±0.09ABa	0.332±0.12ABb	0.363±0.15ABb
B. cereus	---	0.105±0.01Db	0.199±0.05BCa	0.123±0.02CDb	0.168±0.03Eb	0.303±0.07ABCa	0.161±0.03Cb
L. monocytogenes	---	0.214±0.04Cb	0.169±0.04Cb	0.293±0.07ABa	0.875±0.36Aa	0.278±0.07BCDb	0.409±0.05Ab
S. aureus	+++	0.491±0.10Aa	0.408±0.16Aa	0.371±0.12Aa	0.542±0.11BCa	0.408±0.13Aa	0.399±0.13Aa

[1)]Slime colorimetric scale (24): +++, very black; ++, black; +, almost black; -, bordeaux; --, red; ---, very red.
[2)]Mean values with the same letter within a column are significantly different ($p<0.05$).
[3)]Mean values with the same letter within a row are significantly different ($p<0.05$).

Table 2. Quantitative estimation of the amount of protein, carbohydrate, and eDNA isolated from extracellular polymeric substances (EPS) of pathogenic bacteria

Pathogens	Total cell number ($\log_{10}$ CFU/mL)	EPS[1)]		
		Protein (mg/mL)	Carbohydrate (mg/mL)	eDNA (ng/μL)
E. coli O157:H7	10.75±0.15	0.05±0.02C[2)]	0.28±0.06AB	315.47±92.90B
C. sakazakii	10.33±0.48	0.05±0.02C	0.14±0.02C	395.87±63.08B
S. Typhimurium	11.01±0.16	0.02±0.02C	0.18±0.11C	389.06±50.06B
P. aerugenosa	11.15±0.16	0.50±0.12B	0.36±0.01A	1176.20±280.63A
B. cereus	9.80±0.34	1.17±0.41A	0.37±0.00A	968.59±315.15A
L. monocytogenes	10.27±0.61	0.04±0.01C	0.33±0.02A	292.88±57.41B
S. aureus	10.79±0.40	0.54±0.16B	0.21±0.00BC	301.63±29.94B

[1)]Results are mean values of 3 independent experiments.
[2)]Mean values with the same letter within a column are significantly different ($p<0.05$).

P. aeruginosa (black) were positive slime producers using the CRA method (Table 1). Thus, biofilm formation and slime production in microtiter plates differed depending on the pathogen. A positive correlation was present between biofilm formation and slime production (r=0.5452). Numerous studies have shown that *L. monocytogenes*, *S. aureus*, and *P. aeruginosa* are capable of forming biofilms in a food-processing environment (25,26). *P. aeruginosa* and opportunistic pathogens have a strong tendency to form biofilms and have a known resistance to disinfectant actions (26).

EPS production of pathogens For quantitative comparison of EPS production between different pathogenic bacteria, EPS compositions of pathogens were compared for an investigation into the influence of EPS on biofilm formation (Table 2). EPS isolated from agar-grown biofilms of *B. cereus* contained a high proportion of carbohydrates. *B. cereus* EPS contained significantly ($p\leq0.05$) more protein and eDNA, compared with another pathogens. The carbohydrate content was significantly ($p\leq0.05$) higher in EPS from *P. aeruginosa* and *L. monocytogenes* than from other pathogens. The eDNA content in EPS from *P. aeruginosa* was significantly ($p\leq0.05$) higher than for other pathogens Protein, carbohydrate, and eDNA contents in the EPS from *S. aureus* were significantly ($p\leq0.05$) lower than for other pathogens, although *S. aureus* formed a significantly ($p\leq0.05$) larger biofilm than another pathogens.

High levels of EPS production by *B. cereus* and *P. aeruginosa* have been reported (27). High levels of EPS production by *B. cereus* and *P. aeruginosa*. *B. cereus* on stainless steel, which is commonly used in food processing plants, contributes to biofouling of processed food (27). The presence of adhesions on the cell surface and the adhesive role of EPS components have been thoroughly documented in Gram-positive organisms such as *Bacillus subtilis* (28) and in the Gram-negative organism *P. aeruginosa* (29). Karunakaran and Biggs (30) reported that cell surface properties and EPS proteins drive biofilm formation and maintain *B. cereus*. One of the most common and best-studied biofilm-forming genera is *Pseudomonas*. In the case of mucoid variants of *P. aeruginosa*, EPS contains a high portion of the exopolysaccharide alginate, which is a high-molecular-mass unbranched co-polymer consisting of the (1→4)-linked uronic acid residues of β-D-mannuronate and α-L-glucoronate (31).

Cell hydrophobicity of pathogens measured using the BATH method Surface hydrophobicity is generally associated with bacterial adhesiveness and varies from organism to organism and from strain to strain. Therefore, in this study, cell hydrophobicity values of pathogens measured using the BATH method with different hydrocarbons with and without AS were expressed as BATH% based on chemicals in the pathogens (Table 3). High levels of cell hydrophobicity were observed for all tested pathogens when hydrocarbons were combined with AS.

Table 3. Cell hydrophobicity of pathogenic bacteria measured based on bacterial adhesion to hydrocarbon (BATH) assays using xylene, octane, hexane, and nonane as hydrocarbons with and without ammonium sulfate (AS)

Pathogens	Chemical							
	Xylene	Octane	Hexadecan	Nonane	Xylene+AS	Octane+AS	Hexadecan+AS	Nonane+AS
E. coli O157:H7	11.29±5.50CD[1)]d[2)]	0.00Cd	4.69±1.24Cd	0.00Cd	60.45±7.62ABa	47.40±11.24ABCb	4.70±4.61Bd	35.50±9.62ABc
C. sakazakii	0.00Da	0.00Ca	0.00Ca	0.00Ca	5.22±4.98Ca	41.46±49.85ABCa	26.01±32.37ABa	2.17±2.98Ca
S. Typhimurium	8.97±6.43CDbc	0.00Cc	1.14±1.84Cc	0.00Cc	47.30±12.61Ba	38.93±2.82BCa	6.31±7.63Bc	19.14±7.18BCb
P. aeruginosa	14.59±13.71BCDab	0.00Cb	17.26±10.86Bab	34.61±11.60Aa	20.25±12.66Cab	16.64±11.32Cab	41.05±31.65Aa	42.04±6.64Aa
B. cereus	22.95±15.43ABCcd	0.00Cd	30.03±8.87Abc	16.58±0.90Bcd	50.03±30.54Ba	48.18±12.53ABCab	60.65±2.73Aa	56..90±7.71Aa
L. monocytogenes	34.72±4.85ABbc	24.49±4.73Bcd	16.44±3.92Bd	15.62±2.12Bd	61.05±7.80ABa	61.18±1.91ABa	42.85±9.12Ab	51.25±21.50Aab
S. aureus	35.85±18.05Ab	58.09±22.13Ab	2.03±2.47Cc	2.78±3.72Cc	80.90±14.40Aa	81.79±17.49Aa	60.65±2.73Ab	54.84±13.70Aab

[1)]Mean values with the same letter within a column are significantly different ($p<0.05$).
[2)]Mean values with the same letter within a row are significantly different ($p<0.05$).

The BATH assay with AS showed that weakly hydrophobic bacteria exhibited higher levels of hydrophobicity in the presence of AS. Levels of *S. aureus* cell hydrophobicity measured using BATH with xylene and AS were significantly ($p \leq 0.05$) higher than for other pathogens, whereas levels of *C. sakazakii* cell hydrophobicity were significantly ($p \leq 0.05$) lower than for other pathogens. A significant ($p \leq 0.05$) difference was observed between levels of hydrophobicity for both Gram-positive and Gram-negative pathogens measured using BATH with nonane and AS, compared with use of chemicals. Thus, levels of pathogen hydrophobicity differed depending on the measurement method, and the BATH assay using nonane with AS is recommended for consistent results.

The BATH assay showed variations in results that were dependent on hydrocarbons used. Dillon *et al.* (32) reported that xylene produced the best response. A difference in the degree of adherence to different hydrocarbons has been noted for some organisms (33). This difference may be due to the amount of surface area created during mixing of 2 liquid phases, mixing conditions, or the size and number of hydrocarbon droplets obtained in the aqueous phase (34). In this study, the BATH method using nonane with AS produced consistent results. However, to date, no study has compared BATH using nonane with and without AS supplementation for measurement of pathogen cell hydrophobicity. Rosenberg (35) reported that simple ionic compounds, such as AS, promote microbial adhesion to a hydrophobic surface. Adherence of *E. coli* was enhanced in the presence of increasing AS concentrations. AS similarly enhanced adherence to other tested hydrocarbons, and to the solid hydrophobic surface of polystyrene.

Pathogen cell hydrophobicity The BATH method using a tube can be simplified with microtiter plates. Therefore, polystyrene microtiter plates using different hydrocarbons supplemented with AS were tested. The HIC and CAM methods were also evaluated (Fig. 1).

BATH using nonane with AS on a polystyrene microtiter plate showed a clear difference in the levels of hydrophobicity between Gram-positive and negative pathogens, compared with other chemicals. When a polystyrene microtiter plate based on the BATH method using different hydrocarbons supplemented with AS was correlated with the results of other methods, BATH using nonane with AS was positively correlated with HIC (r=0.7328) and CAM (r=0.5790) results. However, no such relationship was observed for polystyrene microtiter plates tested using xylene, octane, or hexane with AS. A high correlation was observed between BATH using nonane with AS and polystyrene microtiter plate test results using nonane with AS (r=0.7989). Therefore, the polystyrene microtiter plate test for BATH using nonane with AS can be used as a simplified method in place of the tube method. Polystyrene microtiter plate test results using nonane with AS were positively correlated with biofilm formation (r=0.7856) when the correlation between cell hydrophobicity measured using different methods and biofilm formation was evaluated. Correlation values between biofilm formation and the CAM and HIC methods were lower (r=0.4467 and r=0.0137, respectively). Therefore, correlations

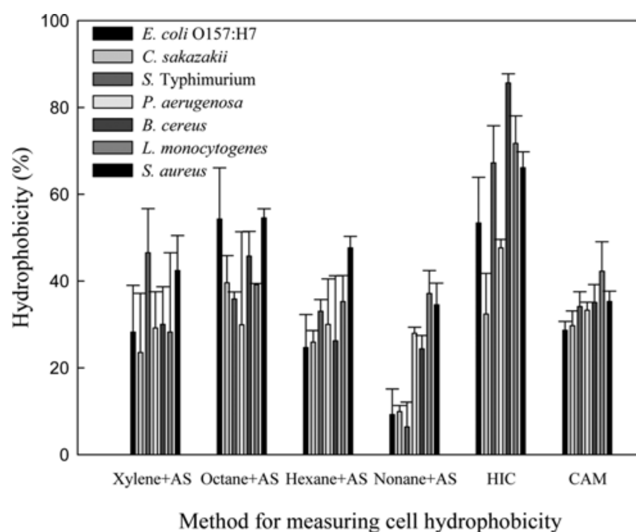

Fig. 1. Cell hydrophobicity of pathogenic bacteria measured using xylene, octane, hexane, and nonane with ammonium sulfate (AS) in a 96-well plate test, hydrophobic interaction chromatography assay (HIC), and contact angle measurement (CAM) (xylene+AS; octane+AS; hexane+AS; nonane+AS). Error bars indicate 95% confidence intervals (n=3).

of hydrophobicity with biofilm formation levels can be different depending on the method used for measuring hydrophobicity.

Hamadi and Latrache (36) reported no correlation between hydrophobicity values of *P. aeruginosa, B. subtilis, S. aureus,* and *E. coli* determined based on the water contact angle and BATH methods using hexadecane. However, Ahimou *et al.* (37) reported correlations between the 3 methods of BATH using toluene, HIC, and CAM for evaluation of the hydrophobicity of *B. subtilis*. Results of the BATH and HIC methods were better correlated with each other than with water contact angle results. The r value between the BATH and HIC methods was 0.80, whereas r values between the water contact angle method were 0.50 for HIC and 0.43 for BATH. A high degree of correlation (r=0.7989) was, thus, observed between BATH using nonane with AS and the polystyrene microtiter plate test method using nonane with AS.

Cell hydrophobicity of pathogens depending upon incubation time Evaluation of cell hydrophobicity for different pathogens depending on growth stage involved measurement of pathogenic cell hydrophobicity values using the polystyrene microtiter plate test method with nonane and AS for 24 h (Table 4). *P. aeruginosa* and *S. aureus* in the exponential phase were significantly ($p \leq 0.05$) more hydrophobic than other pathogens. In particular, *P. aeruginosa* showed a greater hydrophobicity (33.87%) after 6 h than after 12 h (18.44%) and 24 h (28.00%), compared with other pathogens. The hydrophobicity of *B. cereus* was significantly ($p \leq 0.05$) higher in the stationary phase (12 h) than the hydrophobicity of other pathogens. In a study by Wang and Han (38), the hydrophobicity of *Bacillus* sp. was significantly ($p<0.05$) higher in the exponential phase (18 h) than after 96 h. Jana *et al.* (31)

Table 4. Cell hydrophobicity of pathogenic bacteria depending on the incubation time for 6, 12, and 24 h

Pathogens	Incubation time (h)		
	6	12	24
E. coli O157:H7	0.000±0.00C[1)]c[2)]	9.260±8.07BCa	9.240±5.90Da
C. sakazakii	0.000±0.00Cb	6.200±10.74Cb	9.900±1.43Da
S. Typhimurium	0.000±0.00Cb	4.230±4.00Ca	6.400±5.70Da
P. aerugenosa	33.870±0.00Aa	18.440±24.06ABCa	28.000±1.36BCa
B. cereus	0.000±5.92Cc	35.430±12.73Aa	24.380±3.05Cab
L. monocytogenes	25.910±3.93Ba	29.290±9.12ABa	37.120±5.29Aa
S. aureus	33.630±2.32Aa	30.030±5.36ABa	34.510±5.04ABa

1)Mean values with the same letter within a column are significantly different (p<0.05).
2)Mean values with the same letter within a row are significantly different (p<0.05).

Table 5. Autoaggregation coefficients of pathogenic bacteria during 24 h

Pathogens	1 h	4 h	6 h	12 h	18 h	24 h
E. coli O157:H7	0.34±0.59C[1)]b[2)]	1.09±1.34Cb	2.02±0.6CDb	2.12±0.74Cb	2.25±0.55Db	5.44±3.00Ea
C. sakazakii	0.99±0.50Cc	2.64±1.62BCc	4.23±2.03BCDc	4.61±2.36BCc	10.42±2.61BCb	14.49±2.98CDa
S. Typhimurium	0.06±0.11Cb	1.19±2.06Cb	1.50±1.85Db	2.15±1.6Cb	3.44±1.38CDab	5.74±2.99Ea
P. aeruginosa	3.95±0.92Bc	4.98±0.93Bbc	4.92±1.38BCDbc	5.67±1.78BCbc	8.27±2.05BCDab	9.55±3.64DEa
B. cereus	7.91±1.62Ad	14.63±3.41Acd	20.73±3.32Abc	29.49±6.78Ab	42.11±5.44Aa	50.76±6.91Aa
L. monocytogenes	1.28±0.47Cd	4.20±2.28BCcd	7.02±3.78Bcd	9.89±3.27Bbc	16.06±7.74Bb	24.95±4.94Ba
S. aureus	0.83±0.47Cd	3.50±0.59BCcd	6.13±0.78BCcd	7.79±1.66BCc	14.03±4.76Bb	21.68±5.22BCa

1)Mean values with the same letter within a column are significantly different (p<0.05).
2)Mean values with the same letter within a row are significantly different (p<0.05).

reported that the cell surface hydrophobicity of *P. aeruginosa* in the early to mid-log exponential phase was higher than in the stationary phase. Other studies have also reported different adhesive natures of cells in different growth phases (39). Bacteria are inherently dynamic organisms and protein coverage and lipopolysaccharides (LPS) on cell surfaces will be different depending upon the growth phase (39).

Correlation between biofilm formation and cell hydrophobicity Correlations between biofilm formation measured using CV staining with ethanol decolinization, and cell hydrophobicity, were measured using the polystyrene microtiter plate test method with nonane and AS based on incubation time. Biofilm formation for pathogens incubated for 6, 12, and 24 h was positively correlated with the hydrophobicity level of the pathogens (r=0.8090, r=0.5500, and r=0.7856, respectively) (data not shown). *B. cereus* showed low levels of biofilm formation but high levels of hydrophobicity, whereas *E. coli* O157:H7 showed high levels of biofilm formation but low levels of hydrophobicity. Therefore, these pathogens behaved counter to the observed correlation. However, biofilm formation and cell hydrophobicity (r=0.9934) were highly correlated for all other pathogens. Auger *et al.* (40) demonstrated that the hydrophobicity of *Bacillus cereus* was not significantly positively correlated with biofilm formation on PVC microtiter plates (r= −0.23). *E. coli, P. aeruginosa, Streptococcus pyrogenes,* and *S. aureus* were included in the study of Rad *et al.* (41) with hydrophobicity values of 49.68±26.02 determined using the BATH method with xylene (41) with a positive correlation between hydrophobicity and attachment ability.

Pathogen autoaggregation ability Several studies have reported that autoaggregation was related to adhesion of bacteria to different surfaces (41). Therefore, the pathogen autoaggregation ability was measured in this study. The autoaggregation ability increased over time (Table 5). In particular, the autoaggregation ability of *B. cereus* was significantly (p≤0.05) higher than the ability of other pathogens after 24 h. Pathogen autoaggregation did not correlate with biofilm formation after 1, 4, 6, 12, 18, and 24 h. Autoaggregation plays important roles in development of several different multispecies of biofilms into integrated biological structures (42). Properties that enhance bacterial attachment include increased whole cell hydrophobicity and the ability to autoaggregate (43). A larger percentage of biofilm strains are able to autoaggregate than planktonic counterparts (43). However, no correlation between autoaggregation and biofilm formation was observed in this study.

In conclusion, biofilm formation was influenced by cell surface properties and EPS production. EPS played an integral role in enabling pathogens to form biofilms. Among cell surface properties, cell hydrophobicity was probably affected by pathogen biofilm formation. Levels of hydrophobicity of pathogens differed depending on measurement methods, but the BATH method using nonane with AS is recommended for consistent results. Biofilm formation varied depending on the type of pathogen and showed different levels of cell hydrophobicity and different EPS compositions of polysaccharides, proteins, and DNA. Biofilm formation, cell hydrophobicity, and EPS production were highly correlated in some pathogens, but not in others. Therefore, factors affecting biofilm formation differed depending

on the bacterial strain. Changes in environmental conditions can affect biofilm formation due to changes in cell hydrophobicity and EPS production. However, this study was conducted using a mixture of only 2-3 strains of pathogenic bacteria. Therefore, biofilm formation factors of pathogenic bacteria cannot be generalized as factors affecting biofilm formation can be different depending on the bacterial strain. Therefore, further studies are needed to understand pathogenic biofilm formation on surfaces and correlations with cell surface properties of different bacterial strains.

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