



Note

Selective adhesion of extremophiles for scanning electron microscopy

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Abstract

Polyethyleneimine (PEI)-coated slides were used for attachment of unknown extremophiles and their cultures grown at high temperature and low pH. Selective adhesion of cells in natural samples and cultural isolates was compared to polylysine (PL) and several other coatings. PEI is superior to PL for adhesion of extremophiles.

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Extremophiles from continental solfataric environments (Brock, 1978; Stetter, 1995; Pace, 1997) do not readily bind to an adhesive layer of poly-L-lysine (PL) (Mazia et al., 1975). An earlier study (Razatos et al., 1998) used polyethyleneimine (PEI) adhesion to form a confluent lawn of *Escherichia coli* for a study of bacterial adhesion by atomic force microscopy (AFM). Similarly, PEI provides selective adhesion of extremophiles for scanning electron microscopy (SEM) of natural samples and cultures isolated from acid hot springs; it is superior to PL as an adhesive for extremophiles.

A 0.1-ml inoculum ($1-2 \times 10^7$ cells ml^{-1}) was added to a 20 ml screw cap vial (28×58 mm) with 10

ml of Brock's medium (Brock et al., 1972; Seegerer and Stetter, 1992). Cultures were grown to $2-3 \times 10^7$ cells ml^{-1} at 70 °C, pH 3, in a water bath.

Slides were cleaned by wiping with a Kimwipe. PEI, obtained as a 50% (w/v) aqueous solution (Sigma), was dissolved in 0.5% (v/v) Photo-Flo 200 (PF; Eastman Kodak) in distilled water (1 mg PEI ml^{-1}). A 2- μl drop, applied 1–2 mm from the end of a slide, was spread like a blood smear. Controls, PL hydrobromide, MW 100 000, polyethylene glycol MW 6000 (PEG), and dextran MW 40 000 (DEX) were used at 0.1% with 0.5% PF. Plain slides and 0.5% PF-coated slides were also used as controls.

Shortened slides scored and separated lengthwise were coated with adhesive and placed in a 15-ml screw cap vial (21×70 mm) filled with culture. Samples were attached horizontally at a concentration of 1.8×10^7 cells ml^{-1} for 30 min at 23 °C. Attached samples were washed $3 \times$ in 10 mM *N'*-2''-hydroxy-

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Table 1
Binding of microorganisms to PEI-coated slides

Microorganism	Response	
	PEI	PL
<i>Extremophiles (thermoacidophiles)</i>		
<i>Sulfolobus</i> -like organism in mineral particles (85.5 °C; pH 2.1) ^a	+	–
<i>Sulfolobus</i> -like organism (68 °C; pH 1.8) ^b	+	+/-
Rod-shaped microbes (78 °C; pH 2)	+	+/-
Rod-shaped and <i>Sulfolobus</i> -like (79.5 °C; pH 2) ^c	+	–
Sulfur crystals (79.5 °C; pH 2) ^c	–	–
Rod-shaped mixed microbial cultures (65 °C; pH 3)	+	+/-
Rod-shaped culture (70 °C; pH 3)	+	+/-
<i>Proteobacteria</i>		
<i>Escherichia coli</i>	+	+
Unidentified proteobacteria	+	+
<i>Other bacteria</i>		
Cyanobacteria ^d	+	+
<i>Eucarya</i>		
<i>Cyanidium caldarium</i> (45 °C; pH 2) ^c	+/-	+/-
Diatoms, dinoflagellates ^d	+	+
<i>Chlamydomonas reinhardtii</i> wild type	+	+
<i>Chlamydomonas reinhardtii</i> cw 15 wall-less mutant	+	+
<i>Saccharomyces cerevisiae</i>	+	+
Unidentified yeast (23 °C; pH 3)	+	+/-

(+) indicates binding, (–) indicates no binding, (+/-) indicates infrequent binding.

^a Great Sulfur Spring, Crater Hills, YNP.

^b Moose Pool, Mud Volcano, YNP.

^c Amphitheater Springs, YNP.

^d Red tide, San Diego and Salton Sea, CA.

thylpiperazine-*N'*-2''-ethanesulfonic acid, pH 7.0, buffer (HEPES), fixed at 23 °C for 30 min each in 3% glutaraldehyde and 1% OsO₄ in HEPES. Washed samples were dehydrated in ETOH. Fixed samples were dried by the critical point method (Tousimis Samdri 790) from ETOH, coated with Au–Pd in an Anatech Hummer VI sputtering system, and photographed in a Hitachi Model 2700 scanning electron microscope.

A coated slide was placed vertically in a 15-ml vial (21 × 70 mm) with a cell suspension or horizontally on a countertop, and a 200-μl drop of cells was placed on the slide. After cell suspensions (2×10^7 cells ml⁻¹) adhered for 10 min at 23 °C, slides were washed 3 × with medium. A solution of 4',6-diamidino-2-phenylindole, HCl (DAPI) ($5 \mu\text{g ml}^{-1}$) was prepared in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 6.8, 1 mM ethyleneglycol-bis-(β-amino-ethyl

ether) *N,N'*-tetraacetic acid, 0.5 mM MgCl₂·6H₂O. After attachment, cells were rinsed 3 × with MES buffer and stained with 10 μL DAPI on a slide for 5 min in the dark. Stained cells were counted on a Leitz Dialux 20 phase-contrast microscope with a 40 × objective, epi-illumination, a 100 W Hg-UV bulb, a Cohu 1332–1000 camera, and 13 in Sony monitor. Four slides ($n = 30$) were sampled for each mean value. Numbers reported are for 5.6×10^{-2} mm².

PL is widely used for adhering cells to slides (Mazia et al., 1975). As shown in Table 1, extremophiles bound infrequently to PL-coated slides compared to proteobacteria and eucarya. In contrast, when PEI was used, all of the thermoacidophiles tested bound to slides. Extremophile samples from mixing pools, *Sulfolobus*-like organisms near the upper temperature limit (85.5 °C) and in milder conditions (68 °C), showed infrequent binding to PL and significant binding to PEI, leaving little doubt about its effectiveness. Samples from a flowing spring, wide rod shaped microbes (78 °C), and a mixture of thin rod-shaped and *Sulfolobus*-like organisms (79.5 °C) showed a similar response, whereas sulfur (79.5 °C) showed no binding. A mixed enrichment of rod-shaped cells grown in culture at 65 °C, pH 3, and an uncharacterized rod-shaped pure culture isolated at 70 °C, pH 3, showed infrequent PL binding and specific adhesion to PEI. Binding of the pure culture to controls was less than 4% that of PEI, including PL that was 3.4%. Occasional *E. coli* and *Sulfolobus*-like cells (< 1 cell/ 5.6×10^{-2} mm²), and no other extremophile cells, bound to the controls, PEG, DEX, PF, or plain glass slides. The same result was observed with unidentified proteobacteria, cyanobacteria, and red tide organisms. Occasional cyanobacteria and diatoms bound to controls were gliding cells that produced their own adhesive. Controls for the single-cell biflagellate green alga, *Chlamydomonas*

Table 2
Effect of orientation on cell binding to PEI-coated half-slides

Half-slide orientation	Vertical		Horizontal	
	No. bound cells	Length (μm)	No. bound Cells	Length (μm)
<i>Escherichia coli</i> ^a	53	5	56	4
Extremophile ^b	25	11	58	10

^a Cell concentration, 2×10^7 cells ml⁻¹.

^b Cell concentration, $2-3 \times 10^7$ cells ml⁻¹.

reinhardtii, all bound less than 1% PEI binding. Similarly, binding of the wall-less mutant cw 15 to controls was less than 1% that of PEI, except for DEX, which was 4.7%. With the exception of plain glass that was 5.5% of PEI binding, the controls for *Saccharomyces*

cerevisiae and the unidentified yeast were all less than 1% PEI binding. The final exception was *Cyanidium caldarium*, a single cell flagella-less red alga that consistently showed infrequent binding to PEI, PL, and the controls.

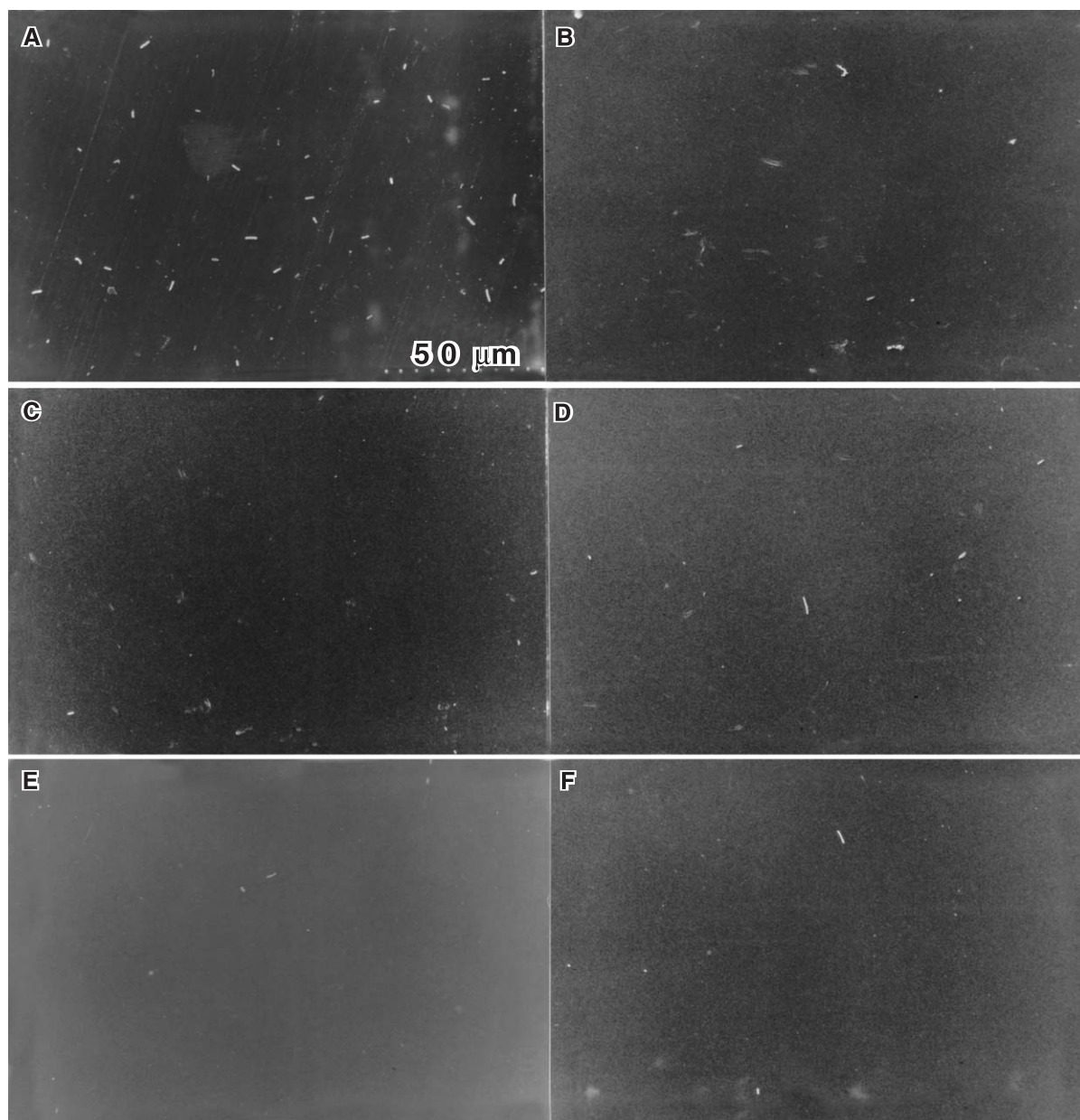


Fig. 1. Selective attachment of extremophile cells. PEI shows attachment of cells; mainly clear areas appear with the other slide coatings. (A) PEI, (B) PL, (C) PEG, (D) DEX, (E) PF, and (F) plain glass. Marker bar in (A) applies to all figures.

The next question considered was whether slide orientation (vertical vs. horizontal) influenced cell binding. The results (Table 2) show that *E. coli* bound with greater effectiveness than the extremophile in vertical, but not horizontal sample applications. Filament length of bound extremophiles did not change significantly as a result of orientation. Orientation had no effect on binding of *E. coli* cells, i.e. about the same amount of binding was seen in vertical and horizontal slide orientation. In contrast, extremophile vertical binding was about half that of horizontal binding. Significantly, increasing the binding time of extremophiles in vertical orientation from 10 to 20 min approximately doubled the number of attached extremophile cells (25 vs. 52 cells/ 5.6×10^{-2} mm²). No binding to the control slides was observed with the extremophile; occasional bound cells of *E. coli* (< 1 cell/ 5.6×10^{-2} mm²) were observed with control slides, PEG, DEX, PF, or plain glass.

Selective adhesion of extremophiles to PEI is shown in Fig. 1. Cell filaments bound to PEI were readily seen while examining samples. In contrast, clear areas with few cells were seen for the binding result with PL and other coatings, even after extensive searching. Representative fields of view shown at the same magnification under identical binding conditions illustrate greater adhesion with PEI (Fig. 1A) compared to PL (Fig. 1B) and other coatings, PEG (Fig. 1C), DEX (Fig. 1D), PF (Fig. 1E), and plain glass (Fig. 1F).

PEI is shown to be a selective agent for attaching extremophile cells, whereas PL or other coatings repeatedly yielded only low numbers of attached cells. These results extend the findings of Razatos et al. (1998) who used PEI to form a confluent monolayer of *E. coli* cells in an investigation of bacterial adhesion using AFM. PEI has also served as a binding agent for immunofluorescent microscopy (Sanders and Salisbury, 1995). A plausible explanation for PEI to act as a selective binding agent for extremophile cells is that it has a higher positive charge density than PL. At low pH, every third atom is a protonatable amino nitrogen that imparts a reactive site on or above the glass slide surface. It is recognized that PEI has a high positive

charge at low pH (Claesson et al., 1997). That this is a possible means of binding is consistent with the lower ability of negatively charged PEG- and DEX-coated slides to bind cells. This suggests that PEI may bind extremophiles through electrostatic forces.

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References

- Brock, T.D., 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag, New York. 465 pp.
- Brock, T.D., Brock, K.M., Belly, R.T., Weiss, R.L., 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Mikrobiol. 84, 54–68.
- Claesson, P.M., Paulson, O.E.H., Blomberg, E., Burns, N.L., 1997. Surface properties of poly (ethylene imine)-coated mica surfaces-salt and pH effects. Colloids Surf., A. 123–124, 341–353.
- Mazia, D., Schatten, G., Sale, W., 1975. Adhesion of cells to surfaces coated with polylysine. J. Cell Biol. 66, 198–200.
- Pace, N.R., 1997. A molecular view of microbial diversity and the biosphere. Science 276, 734–740.
- Razatos, A., Ong, Y.-L., Sharma, M.M., Georgiou, G., 1998. Molecular determinants of bacterial adhesion monitored by atomic force microscopy. Proc. Natl. Acad. Sci. U. S. A. 95, 11059–11064.
- Sanders, M.A., Salisbury, J.L., 1995. Immunofluorescence microscopy of cilia and flagella. Methods Cell Biol. 47, 163–169.
- Seeger, A.H., Stetter, K.O., 1992. The order Sulfolobales. In: Ballows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes, 2nd ed. Springer-Verlag, New York, pp. 684–701.
- Stetter, K.O., 1995. Microbial life in hyperthermal environments. Am. Soc. Microbiol. News 61, 285–290.