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Growth Rate Control of Adherent Bacterial Populations

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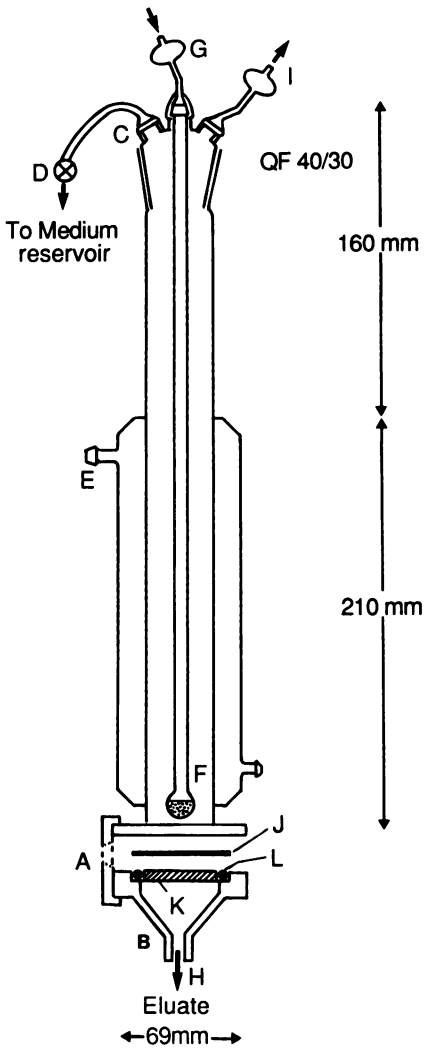
We report a novel in vitro method which, through application of appropriate nutrient limitations, enables growth rate control of adherent bacterial populations. Exponentially growing cells are collected by pressure filtration onto cellulose acetate membranes. Following inversion into the bases of modified fermentors, membranes and bacteria are perfused with fresh medium. Newly formed and loosely attached cells are eluted with spent medium. Steady-state conditions (dependent upon the medium flow rate) at which the adherent bacterial biomass is constant and proportional to the limiting nutrient concentrations are rapidly achieved, and within limits, the growth rate is proportional to the medium flow rate. Scanning electron microscopic studies showed that such populations consist of individual cells embedded within an extracellular polymer matrix.

Bacterial biofilms may be considered as highly structured, functional consortia of cells attached to substrata and within extensive polysaccharide matrices (glycocalyxes) (5). In recent years, such associations have been recognized as being critical for successful colonization of numerous natural habitats by bacteria. These include freshwater systems (17), contaminated pipe work (6, 19), and infections of mucosal surfaces (5, 7, 14) and indwelling medical devices (4, 5, 11, 13). In many of these instances, the problematic nature of the presence of microorganisms is compounded by the recalcitrance of such populations to treatment with chemical antimicrobial agents (9, 18) and antibiotics (5, 9). Since studies of the properties of biofilms are difficult both to conduct and to interpret in situ, many workers have attempted to model such populations in the laboratory. The most widely used of these models is the Robbins device (15). Actively growing cultures in appropriate media are passed through a cylinder until a biofilm, which may periodically be sampled through removal of retractable pistons forming part of the cylinder wall, develops on its inner surface. Typical studies (5, 8, 10, 16) using this model compare the properties of adherent cells with those of the equivalent planktonic population of cells passing through the device. Although this technique has provided much valuable information concerning bacterial biofilms, the interpretation of such data has recently been brought into question (2) since the adherent cells in such models grow extremely slowly, yet they are compared to planktonic controls which are in a state of rapid growth. Since systems such as the Robbins device lack effective growth rate control, when used in this way they do not differentiate between properties attributable to the growth rate and those associated with adherence. There is, however, strong evidence to suggest that many of the properties currently attributed to the growth of cells as biofilms are actually related to slow growth (3, 5, 9).

Helmstetter and Cummings (12) described a method for the selection of synchronous populations of bacteria in which the cells are attached to a filter membrane and eluted with fresh medium. Most of the cells remain attached to the filter matrix, whereas progeny are lost to the eluate. Although the method has found widespread use in synchronization studies, the facts that the parent population represents a biofilm and that the continuous flow of fresh medium offers the potential, as in a chemostat, to control the growth rate have been overlooked. By suitable adaptation, we evaluated such potential in the technique of Helmstetter and Cummings to control the growth rate in a biofilm.

Escherichia coli ATCC 8739 was grown for 16 h at 35°C in



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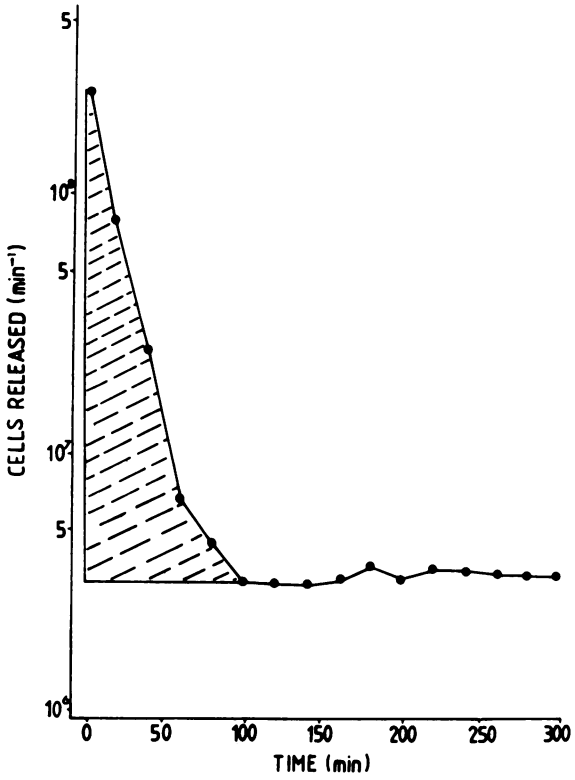

FIG. 2. Elution of bacteria from a 0.22-μm-pore-size Millipore filter at a constant flow rate of 1 ml/min. Irrespective of the flow rate, all loosely bound cells were removed within the first 100 ml of eluate, as represented by the shaded area.

shaken (150 oscillations per min) Erlenmeyer flasks (250 ml) containing 100 ml of a chemically defined simple-salts liquid medium (1) in which glycerol was the growth-limiting nutrient. After transfer to fresh medium, 60 ml of an exponential culture (ca. 10^8 cells per ml) was passed through a 47-mm-diameter cellulose acetate membrane filter (Oxoid; 0.22-μm pore size) with a Millipore pressure filtration unit (35 kN/m^2). The filter had been prewashed by passing sterile medium (50 ml) through it. The cell-impregnated filter membrane was removed, inverted, and carefully placed into the base of a modified continuous-fermentation apparatus (Fig. 1) maintained at 35°C by a water jacket. Fresh medium was passed into the fermentation chamber via the peristaltic pump at a rate of 1 ml/min. A hydrostatic head develops above the membrane filter which at steady state perfuses the filter at the rate of medium addition to the vessel. The eluate passing through the filter was collected, and viable counts were made by serial dilution in sterile physiological saline and plating in triplicate onto predried nutrient agar (Oxoid CM3) plates. These were incubated at 35°C for 16 h before counting. The viable counts obtained in the eluate are presented in Fig. 2. This shows that the numbers of eluted cells decreased rapidly to attain a steady state after approximately 100 min; this level was maintained for up to 14 days. The initial decrease, represented by the shaded area in Fig. 2, corresponds to the removal of loosely attached cells from the filter bed. According to the technique of Helmstetter and Cummings (12), the cells eluted during the steady-state period should correspond to newly formed daughter cells. This was evaluated by taking the eluate produced over a 5-min period (5 ml) and transferring it directly to fresh

FIG. 1. Schematic representation of the biofilm device. This consists of a glass fermentor and a clamped (A) Teflon base (B). Fresh medium is delivered (C) via a peristaltic pump (D) and maintained at 35°C by a water jacket (E) with appropriate control systems. Aeration was achieved by using a grade O sinter (F) connected (G) to an air filter and pump. The medium and air outlets are at H and I, respectively. The fermentor-base assembly shows the positioning of the 0.22-μm-pore-size filter membrane (J) impregnated with cells on the hydrophobic surface adjacent to the stainless steel sintered support (K) and O-ring seal (L). The direction of medium flow is shown; this removes cells from the underside of the membrane into the collected eluate.

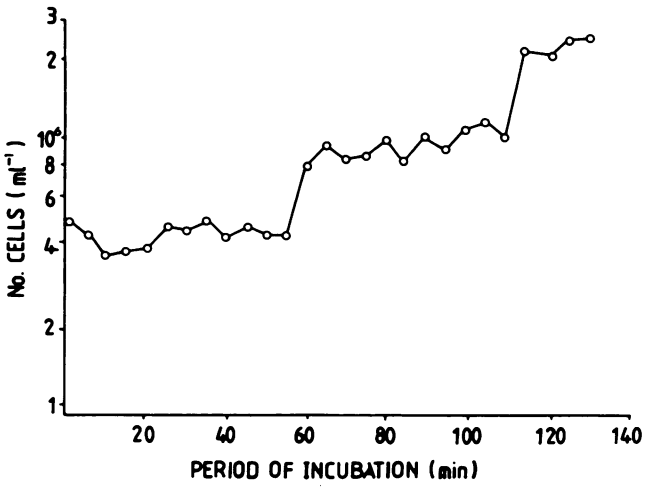

FIG. 3. Synchronized growth of a cell sample collected from the membrane eluate 90 min after a steady state was reached. The sharp increases in viable cell number at 60 and 120 min correspond to the generation time for *E. coli* in the chemically defined medium used.

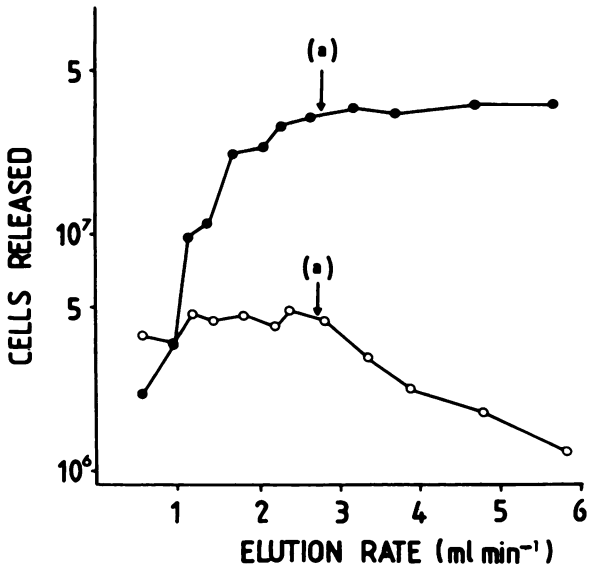

FIG. 4. Relationship between the rate of elution of the filter membrane and either the viable count per milliliter of filtrate (○) or the rate of release of organisms from the system per minute (●). From the graph it is possible to calculate the critical medium flow rate (a) which at steady state is equivalent to μ_{max}. Growth rate control is exerted in the period up to a. By definition, the biofilm population is analogous to a steady-state chemostat culture at values below the critical flow rate (2.67 ml/min). The rate of cell division in the biofilm at slow elution rates is regulated by the rate of fresh medium flow.

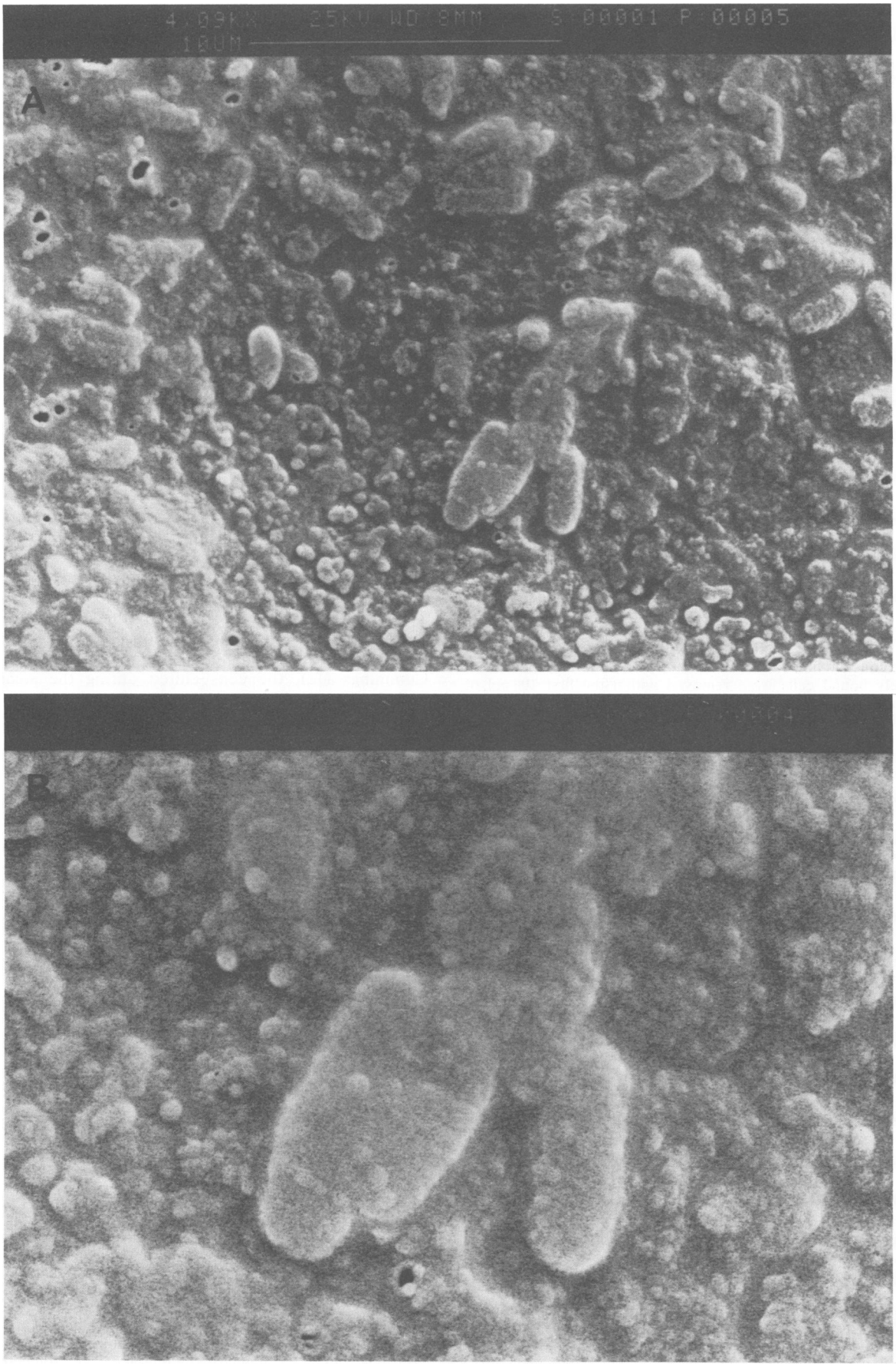
4.09KX 25KV WD 8MM S 00001 P 00005
10UM
A
B

FIG. 5. Scanning electron micrographs of a sample *E. coli* biofilm grown at a specific growth rate of 0.4 per h on cellulose acetate membrane filters. Samples were rapidly frozen in supercooled liquid nitrogen, transferred under vacuum to a low-temperature stage, heated to −80°C to remove surface water, gold coated, and examined by scanning electron microscopy with an S200 microscope (Cambridge Instruments). Panel B in an enlargement of a portion of panel A.

medium (45 ml) contained in an Erlenmeyer flask (250 ml) shaken at 150 oscillations per min at 35°C. Viable counts made over a 140-min period indicated that these cells grew synchronously with a synchronization index of >0.9 (Fig. 3).

Repeat experiments in which the membrane filter was sacrificed at various times up to 14 days after achievement of a steady state and the adherent population was estimated by viable counts showed that the size of the adherent population increased only slightly (less than twofold) over this period. The height of the hydrostatic head above the filter, however, increased up to ca. 20 cm. In subsequent experiments, the rate of flow of fresh medium through the filter bed was altered after achievement of the initial steady state, and viable counts in the eluate were estimated up to 40 h later. The results are presented in Fig. 4 both as viable counts per milliliter of eluate and as number of viable cells eluted per minute against the medium flow rate. These results, which have been repeated on numerous occasions and in two separate laboratories, show that the cell density in the eluate remains constant up to a critical medium flow rate (2.67 ml/min) and thereafter decreases. Correspondingly, the rate of evolution of cells from the filter bed was directly related to the medium flow rate up to the same critical value, when it was maximized. These results bear a strong similarity to those of a chemostat culture in which the critical flow rate might correspond to achievement of μ_{max}. Indeed, when the maximal rate of cell elution from the filter bed (4.5×10^7 cells per min) was related to the size of the adherent population (1.2×10^8 cells), the growth rate was seen to equal the μ_{max} for that medium determined in batch culture (1 per h). The entire adherent population must therefore grow actively and be under a form of growth rate control, analogous to that of a chemostat, brought about by the rate of perfusion of the filter. Sample biofilms prepared by low-temperature stage freeze techniques for scanning electron microscopy showed dispersed cells attached to the filter matrix yet embedded within an extracellular polymer matrix (Fig. 5). Thus, although the initial method of attachment by pressure filtration is artificial, the biofilms so produced at steady state resemble those isolated in vivo.

The method described here offers the ability to control the rate of growth of adherent populations of microorganisms and might prove a useful model of in vivo biofilms. It is important to note that in these systems the biofilm populations are perfused with nutrients from their underside. While this is atypical of in situ biofilms on inanimate surfaces, it is representative of bacterial surface infections of soft tissues. Perfusion of the biofilms with antibiotics would also mimic antibiotic therapy in such instances. It is relevant to note, however, that the residual adherent population in this growth system is enriched in older dividing cells relative to that of batch cultures. This might, however, also be true in vivo, when less-adherent, newly formed cells are able to relocate and eventually colonize new surfaces, thereby contributing to the spread of infection. With control populations grown in a chemostat using media and nutrient deprivations identical to those of the adherent populations, this method offers the possibility of separating the physiological effects of growth rate and adhesion. We used the technique successfully to examine the effects of growth rate upon the cell surface hydrophobicity and biocide and antibiotic susceptibility of bacterial biofilms. While techniques such as the Robbins device more closely model natural biofilms, they do not offer such possibilities.

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LITERATURE CITED

1. **Al-Hiti, M. M. A., and P. Gilbert.** 1983. A note on inoculum reproducibility: solid versus liquid culture. J. Appl. Bacteriol. **55**:173–176.
2. **Brown, M. R. W., D. G. Allison, and P. Gilbert.** 1988. Resistance of bacterial biofilms to antibiotics: a growth rate related effect. J. Antimicrob. Chemother. **22**:777–780.
3. **Brown, M. R. W., and P. Williams.** 1985. The influence of environment on envelope properties affecting survival of bacteria in infections. Annu. Rev. Microbiol. **39**:527–556.
4. **Costerton, J. W.** 1984. The etiology and persistence of cryptic bacterial infections: a hypothesis. Rev. Infect. Dis. **6**(Suppl. 3):S608–S616.
5. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
6. **Costerton, J. W., and E. S. Lashen.** 1984. Influence of biofilm efficacy of biocides on corrosion-causing bacteria. Mater. Prot. **23**:34–37.
7. **Ellwood, D. C., and D. W. Tempest.** 1972. Effects of the environment on bacterial wall content and composition. Adv. Microb. Physiol. **7**:83–117.
8. **Evans, R. C., and C. J. Holmes.** 1987. Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. Antimicrob. Agents Chemother. **31**:889–894.
9. **Gilbert, P., M. R. W. Brown, and J. W. Costerton.** 1987. Inocula for antimicrobial sensitivity testing: a critical review. J. Antimicrob. Chemother. **20**:147–154.
10. **Gristina, A. G., C. D. Hobgood, L. X. Webb, and Q. N. Myrvik.** 1987. Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials. **8**:423–426.
11. **Gristina, A. G., M. Oga, L. X. Webb, and C. D. Hobgood.** 1985. Adherent bacterial colonization in the pathogenesis of osteomyelitis. Science **228**:990–993.
12. **Helmstetter, C. E., and D. J. Cummings.** 1963. An improved method for the selection of bacterial cells at division. Biochim. Biophys. Acta **82**:608–610.
13. **Jacques, M., T. J. Marrie, and J. W. Costerton.** 1986. *In vitro* quantitative adherence of microorganisms to intrauterine contraceptive devices. Curr. Microbiol. **13**:133–137.
14. **Marrie, T. J., and J. W. Costerton.** 1985. Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. J. Clin. Microbiol. **22**:924–933.
15. **McCoy, W. F., J. D. Bryers, J. Robbins, and J. W. Costerton.** 1981. Observations in fouling biofilm formation. Can. J. Microbiol. **27**:910–917.
16. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of cells of *Pseudomonas aeruginosa* growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27**:619–624.
17. **Pringle, J. H., and M. M. Fletcher.** 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Appl. Environ. Microbiol. **45**:811–817.
18. **Ruseska, I., J. Robbins, J. W. Costerton, and E. S. Lashen.** 1982. Biocide testing against corrosion-causing oilfield bacteria helps control plugging. Oil Gas J. **1982**:253–264.
19. **Shaw, J. C., B. Bramhill, N. C. Wardlaw, and J. W. Costerton.** 1985. Bacterial fouling in a model core system. Appl. Environ. Microbiol. **49**:693–701.