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Plugging of a Model Rock System by Using Starved Bacteria

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The effects of starvation on bacterial penetration through artificial rock cores were examined. *Klebsiella pneumoniae* was starved in a simple salts solution for a duration of up to 4 weeks. These cell suspensions were injected into sintered glass bead cores, and the resulting reductions in core permeabilities were recorded. Vegetative cell cultures of *K. pneumoniae* grown in a sodium citrate medium were injected into other, similar cores, and the reductions in core permeabilities were recorded. The starved cell suspensions did not completely block the core pores, whereas the vegetative cultures reduced core permeability to less than 1%. Scanning electron microscopy of core sections infiltrated with either vegetative or starved cells showed that the former produced shallow "skin" plugs and copious amounts of glycocalyx at the inlet face, whereas the latter produced very little glycocalyx and the cells were distributed evenly throughout the length of the core. The use of a DNA assay to produce a cell distribution profile showed that, compared with the vegetative cells, starved bacteria were able to penetrate deeper into the cores. This was due to the smaller size of the cells and the reduction in biofilm production. This ability of starved bacteria to penetrate further into cores than the normal-size vegetative cells can be usefully applied to selective plugging for enhanced oil recovery. To further test the suitability of starved cells for use in selective plugging, the activities of starved cells present within cores were monitored before and after nutrient stimulation. Our data indicate that with nutrient stimulation, the starved cells lose their metabolic dormancy and produce reductions in core permeability due to cell growth and polymer production.

Conventional oil production methods characteristically recover 8 to 30% of the total oil present in a petroleum reservoir (21). Methods of increasing recovery rates by using bacterial injections into reservoirs have been examined (14, 20). One such technique, called selective plugging, is directed toward improving the sweep efficiency of a waterflood. Typically, when water injection is used to recover additional oil, water "fingers" develop because of the presence of high-permeability zones in the reservoir (17). These high-permeability zones act as water thief zones and prevent the injected water from contacting the oil-bearing, low-permeability regions of the reservoir. The sealing of these high-permeability zones with a bacterial biofilm would permit an ensuing waterflood to contact bypassed low-permeability regions. This increased volumetric sweep would improve the oil recovery efficiency.

Laboratory studies have demonstrated that living bacteria form confluent biofilms that seal a simulated reservoir matrix (31). Selective plugging of high-permeability zones and subsequent flow diversion to low-permeability zones have been successfully demonstrated in parallel core laboratory models by using different permeability sand packs (16) and Berea sandstone cores (29).

In the reservoir formation, water injections sweep the low-permeability zones to the depth of the biofilm plugs in the high-permeability zones. However, beyond the plugs, water is lost by crossflow to the high-permeability zones. Therefore, the effectiveness of this technique in improving oil recovery efficiency is dependent on the depth of the bacterial plugs in the rock matrix.

Ultramicrobacteria (UMB), defined as having a diameter of less than 0.3 μm (33), may penetrate deeper into rock strata by virtue of their size. They develop as a consequence of a starvation-survival response when environmental conditions become unfavorable for growth. This response produces a dormant organism that permits the preservation of the bacterial genome until suitable growth conditions are established (23). UMB have been observed in both terrestrial and aquatic environments (2, 24, 26) and have been produced under laboratory conditions (3, 25). Here we report on the use of bacteria which approach the size of UMB for improving the depth of bacterial penetration by comparing their plugging abilities with those of normal-size cells in simulated rock matrices.

MATERIALS AND METHODS

Culture conditions. The bacterium used for all experimental work was isolated from produced water (water coproduced with the oil) obtained from a battery (Shell, Didsbury, Alberta, Canada) and identified as *Klebsiella pneumoniae*. Streptomycin resistance was induced as a genetic marker, and two separate reservoirs of microorganisms were periodically prepared. The first, referred to as the vegetative culture, contained *K. pneumoniae* grown at 22°C at 200 rpm (on a Fisher Thermix model 220T stirrer) in sodium citrate medium. The medium contained the following, in grams liter^{-1} of glass-distilled water: $Na_3C_6H_5O_7 \cdot 2H_2O$, 7.36; $(NH_4)_2SO_4$, 3.30; KH_2PO_4, 7.30; K_2HPO_4, 9.22; $MgSO_4$, 0.12; $FeCl_3$, 0.0041; streptomycin, 0.1 (pH 7.0; Sigma Chemical Co., St. Louis, Mo.) (Ron Read, Ph.D. thesis, University of Calgary, Calgary, Alberta, Canada, 1983). The second reservoir contained starved cells of *K. pneumoniae*. The bacteria were grown to the stationary phase in sodium citrate medium; harvested by centrifugation (10,000 × *g*, 15 min, 4°C); and washed twice in phosphate-buffered saline (PBS), which contained the following, in grams liter^{-1} of distilled water: NaCl, 8.5; KH_2PO_4, 0.61; K_2HPO_4, 0.96 (pH 7.0). The bacteria were suspended at a known concentration in PBS. The starved cell suspension was stirred at 22°C at 200 rpm (on a Fisher Thermix model 220T stirrer) for 2 to 4 weeks. Starved cell suspensions were filtered through 11.0-

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cm-diameter Whatman no. 1 filter paper (W & R Balston Ltd.) prior to their use in core injections. All media were filter sterilized into acid-washed, autoclaved glassware.

Core plugging. Starved suspensions or vegetative cultures held in 4-liter glass reservoirs were injected into sintered glass bead cores (length, 5 cm; diameter, 1.0 cm) by the procedure described by Shaw et al. (31). Effluent volumes and flow rates were measured at various time intervals, and the percent plugging was calculated as follows: (final flow rate [K] × 100)/initial flow rate (K_i). Viable cell concentrations of effluent and reservoir fluids were determined by viable plate counting by using plates which contained the following, in grams liter^{-1} of glass-distilled water: brain heart infusion broth (Difco Laboratories, Detroit, Mich.), 18.5 g; Bacto-Agar (Difco), 20.0 g. Total cell numbers in sample fluids were counted by acridine orange epifluorescence microscopy, as described by Zimmerman and Myer-Reil (36) and as modified by Geesey et al. (11).

Scanning electron microscopy. To examine biofilm development at the different core depths, the cores were injected with vegetative or starved cells, fractured into six sections along their lengths, and prepared for scanning electron microscopy as described elsewhere (31).

Determination of cell distribution in plugged cores by using a fluorometric DNA assay. To determine the number of cells at the various core depths, plugged cores were cut into 10 0.5-cm-long sections. The 10 sections of each core were further subdivided into four equal portions. The amount of DNA in each quarter section was measured by using an adaptation of the methods described by Labarca and Paigen (18) and Paul and Myers (27). The DNA concentrations were then translated into cell number values by using conversion data prepared with samples of starved and vegetative cell cultures.

The equipment and solutions used to extract the DNA were kept at 2 to 4°C. Each quarter section was placed in a stainless steel core crusher together with 1.0 ml of filter-sterilized standard saline citrate, which consisted of the following, in grams liter^{-1} of distilled water: NaCl, 9.0; $Na_3C_6H_8O_7 \cdot 2H_2O$, 4.41 (pH 7.0). The core crusher was a modified mortar and pestle. It consisted of three pieces. The interior of the mortar piece had bevelled edges which sloped to a circular base that was 1.0 cm in diameter. A cover was threaded onto this mortar. The crushing of the core was performed with a threaded pestle which entered the mortar through the center of the cover. The crushed core slurry was placed in a 50-ml glass beaker with 4 ml of standard saline citrate, which was used to rinse the mortar and pestle. The mixture was sonicated with a cell disruptor (Virsonic 16-850; The Virtis Co., Inc., Gardiner, N.Y.) equipped with an intermediate tip for 3 min with 60-s bursts. Core material was removed by centrifuging the mixture twice at 500 × g for 10 min each time at 4°C. A solution of 20 μl of 1.6×10^{-4} M Hoechst 33258 (Hoechst-Roussell Pharmaceuticals Inc., Somerville, N.J.) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added to 2-ml portions of core supernatant or *Escherichia coli* DNA standards (Sigma). The fluorescence was measured by using a spectrophotofluorometer (Aminco-Bowman; American Instrument Co. Ltd.). The DNA to cell number conversion data were obtained by placing 1.0 ml of either a starved or a vegetative cell culture with a sterile core section into the core crusher. The DNA extraction procedure was performed as described above. The measured DNA concentration was then converted to cell numbers by using cell concentrations of the starved and vegetative cell cultures, as determined by epifluorescence microscopy.

Respiration of glutamic acid prior to nutrient stimulation. The metabolic activity of the *K. pneumoniae* cells that were present in cores and that were not exposed to a nutrient injection was determined by measuring the respiration of radiolabeled glutamic acid over a 1-h period. The procedure used was an adaptation of the method described by Hobbie and Crawford (15). Plugged cores were cut into 10 0.5-cm-long sections. Each section was further subdivided into four equal portions. Quarter sections of each core depth were placed into sterile, 50-ml stopped bottles which contained 10 ml of PBS and 11.2 μg of L-[U-^{14}C]glutamic acid (specific activity, 280 mCi mmol^{-1}; New England Nuclear Corp., Boston, Mass.) liter^{-1}. After the bottles were incubated at 23°C for 1 h, respiration was terminated with 0.5 ml of 10 M H_2SO_4. Respired $^{14}CO_2$ was flushed from the bottles with a closed-loop circulation system (35) and collected in 10 ml of ethanolamine-methanol (1:3; vol/vol; Fisher Scientific Co., Pittsburgh, Pa.). The flushing efficiency, determined with $NaH^{14}CO_3$, was 95%. Portions (10 ml) of 0.4% (wt/vol) Omnifluor (New England Nuclear Corp.) in toluene were added to each sample, and the radioactive counts were measured with a scintillation counter (LS8000; Beckman Instruments, Inc., Fullerton, Calif.). All counts were corrected for quenching by the channels ratio method and for nonspecific binding by using acid-killed controls.

Assimilation of acetic acid during nutrient stimulation. The activity of *K. pneumoniae* over 24 h of nutrient stimulation was determined by measuring the assimilation of L-[U-^{14}C]acetic acid. Cores were plugged to the appropriate flow rate decrease that allowed the passage of 2 liters of the acetic acid solution in 24 h. The bacterial reservoir was then replaced with a sterile flask which contained 3 liters of PBS and 3.25 μg of L-[U-^{14}C]acetic acid (specific activity, 56 mCi mmol^{-1}; New England Nuclear Corp.) liter^{-1}. A 0.2-μm-pore-size filter (Acro 50A; Gelman Sciences, Inc., Ann Arbor, Mich.) was placed in line between the reservoir and the core to prevent any backflow contamination. The core effluent was transported in latex tubing (catalog no. 14-178-5B; Fisher Scientific Co., Pittsburgh, Pa.) which terminated with a 1.5-inch (3.8-cm) 21-gauge needle (Becton Dickinson and Co., Paramus, N.J.). The needle was inserted through a rubber serum stopper (catalog no. 14-126DD; Bittner Corp.) which sealed an effluent collector bottle. As a safety precaution, air was slowly bled off the acetic acid and effluent reservoirs and passed through 10-ml volumes of an ethanol-amine-methanol mixture (1:3; vol/vol) to trap any $^{14}CO_2$ before it was vented off into a laboratory fume cabinet.

The assimilation of acetic acid into bacterial lipids and a measure of the total assimilation were used to quantify the bacterial activity at different core depths. Lipids were extracted by using an adaptation of the single-phase, chloroform-methanol method of White et al. (34). Cores were cut into 10 0.5-cm-long sections. Each section was placed in 5 ml of methanol and 2.5 ml of chloroform overnight. Water (2.5 ml) and chloroform (2.5 ml) were added to each section 18 h later. The mixture was shaken and left for 2 h. The chloroform phase of each section was collected and placed in a scintillation vial.

The chloroform was removed from the lipid by evaporation at 35°C in an analytical evaporator (model No. 111; Organomation Associates Inc.) with nitrogen gas. Lipids were stored under nitrogen at −2°C and counted in the presence of 10 ml of Omnifluor in toluene (0.4%; wt/vol). Values were corrected for quenching and collection efficiency, which was determined to be 80% by using [^{14}C]oleic acid.

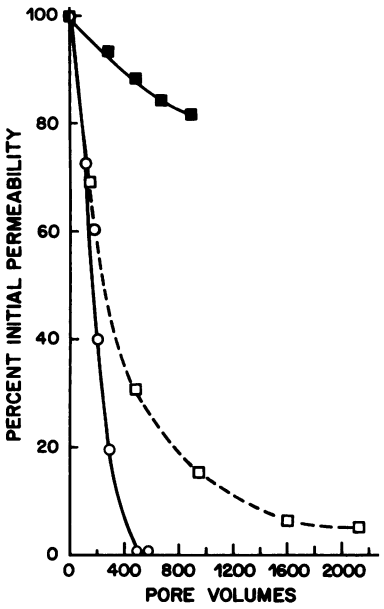


FIG. 1. The different permeability reduction profiles of fused glass bead cores injected with vegetative or starved *K. pneumoniae* cells. Symbols: ○, vegetative culture; □, cell suspension starved in PBS for 2 weeks; ■, cell suspension starved in PBS for 4 weeks.

A measure of the total assimilation was obtained by combining values of lipid incorporation with the counts present in the water phase and those associated with the core sections after lipid extraction. Counts which remained present in the water phase after lipid extraction were determined following the addition of 10 ml of ScintiVerse II. By using [^{14}C]glutamic acid, polar counts were detected at an efficiency of 80% and were corrected for quenching. To measure the labeled material that was still associated with the core after lipid extraction, core sections were crushed in 5.0 ml of sterile distilled water and treated with 10 M H_2SO_4 at a final concentration of 5% (vol/vol). The sections were left overnight, and then the radioactivity was measured in the presence of 10 ml of ScintiVerse II. The labeled material associated with the crushed core sections was extracted and counted at a 72% efficiency rate, as determined with [^{14}C]glutamic acid. Values were also corrected for quenching.

RESULTS

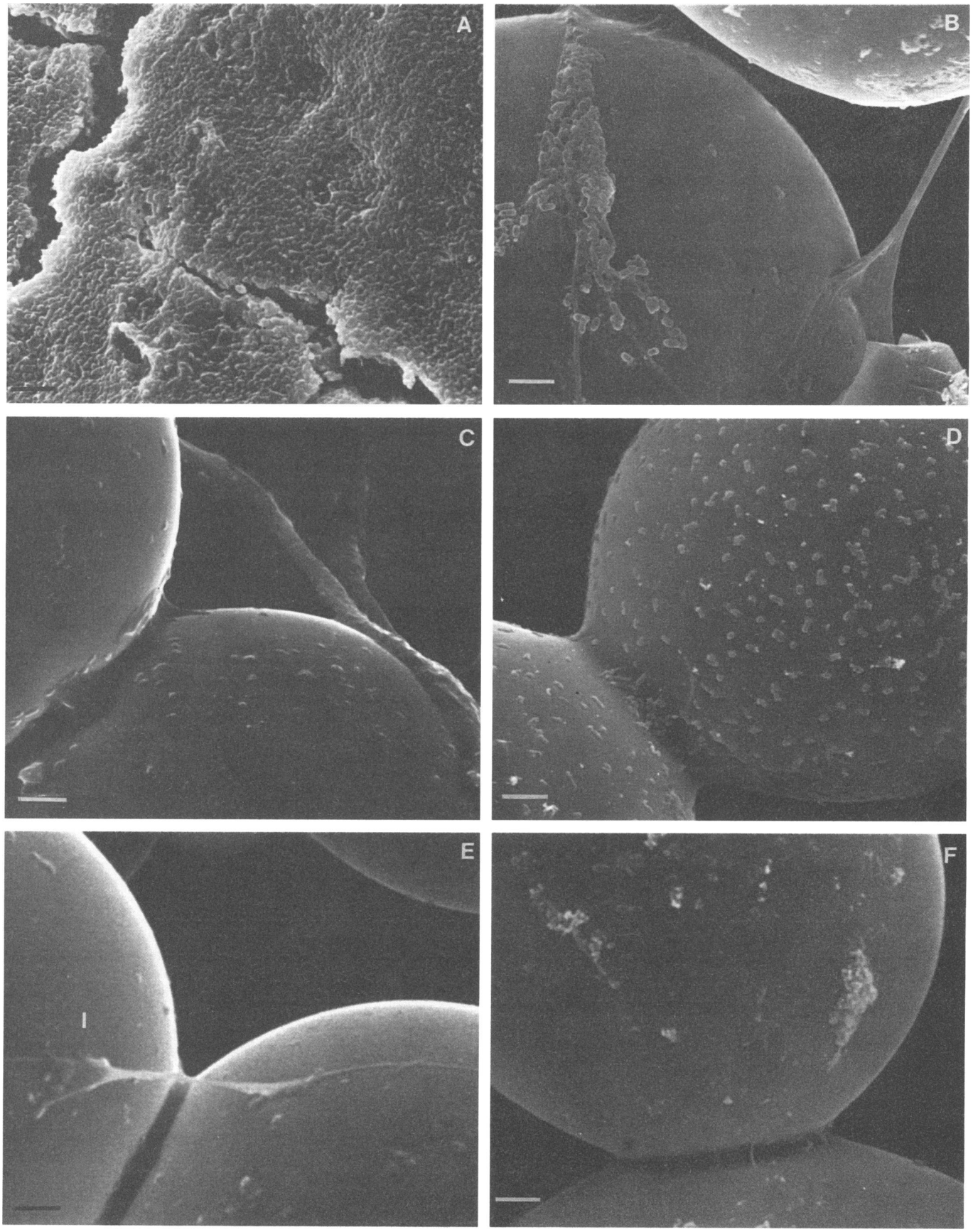
Core plugging. The ability of the starved cell suspensions to reduce core permeability was demonstrated to be dependent on the length of the starvation regimen and the volume of cells injected through the core (Fig. 1). The injection of 500 pore volumes (PV) of *K. pneumoniae* (10^8 ml^{-1}) starved for 2 or 4 weeks reduced core permeabilities by 71 and 11%, respectively. However, under the same injection conditions, that is, 500 PV containing 10^8 cells ml^{-1}, the vegetative culture reduced the core permeability by over 99%. The injection of cells until no further permeability losses were recorded indicated that cultures starved for 2 weeks were not capable of reducing core permeability beyond 95%. The maximum reduction value of cultures starved for 4 weeks was not determined because of the massive volumes of culture which would have been required.

Scanning electron microscopy of the plugged cores. Scanning electron microscopy revealed significant differences in biofilm production between the cores plugged with approximately 500 PV of either vegetative or starved cell cultures. Examination of cores plugged with the vegetative cell culture (10^8 ml^{-1}) showed that the inlet pores were sealed with a thick biofilm of bacteria and glycocalyx (Fig. 2A). The glass bead surfaces of sections from deeper in the core were colonized with *K. pneumoniae*, and biofilm development was noted (Fig. 2B). The bacterial morphology was either bacillus or coccibacillus, with cell lengths ranging from 1.0 to 2.7 μm. In contrast, cores injected with *K. pneumoniae* starved for 2 weeks demonstrated very incomplete sealing of the inlet pore spaces (Fig. 2C). In starved cell cultures, biofilm bridging of the pore spaces and glycocalyx production appeared to be much lower throughout the core length (Fig. 2D) compared with those in the vegetative cell culture. Finally, scanning electron microscopic examination of cores injected with *K. pneumoniae* starved for 4 weeks showed that there was very little biofilm sealing of the inlet end (Fig. 2E). An examination of the core surface at the deeper core depths revealed that the core surface was often coated with large numbers of cells (Fig. 2F). The morphology of these bacteria ranged from cells in which the protoplasm appeared to fill the entire cell wall structure to ghost cells, which appeared to consist only of cell wall material. The protoplasmic component of a number of these cells possessed diameters of less than 0.3 μm.

Cell distribution in plugged cores. Cell numbers within different core sections assessed in cores plugged with either vegetative cell cultures or starved cell suspensions were assessed by determination of DNA content. The distribution of starved and vegetative cells in the cores differed substantially (Fig. 3). In cores plugged with approximately 2,100 PV of bacteria starved for 2 weeks, the cell numbers were more uniform throughout the length of the core, with only a slight decrease with increased core depth. In contrast, cell numbers decreased with an increase in core depth in cores plugged with approximately 500 PV of the vegetative cell culture, and the majority of bacteria were close to the core inlet. Moreover, cell numbers were higher at each section of the core injected with starved cells. More starved cells were detected at the core inlet (3×10^{10} cells g^{-1}) than at the corresponding core section injected with vegetative cultures (approximately 4×10^9 cells g^{-1}). At lower core sections, the differences in the cell numbers were even more pronounced, with approximately 1×10^{10} cells g^{-1} in cores injected with starved cells and 2×10^8 cells g^{-1} in cores injected with vegetative cell cultures.

Respiration of glutamic acid prior to nutrient stimulation. A comparison of glutamic acid respiration in cores plugged by cell suspensions starved for 2 weeks or vegetative cell cultures of *K. pneumoniae* revealed a similar pattern of metabolic activity in both cultures. The activity was maximum at the core inlet, with respiratory activities of over 75,000 dpm g^{-1} in both cores (Fig. 4). This decreased with increased core depth to less than 30,000 dpm g^{-1} at depths of 3 cm and greater. However, subtle differences were also apparent in the two profiles. Compared with experimental runs with starved cells, the vegetative cell runs exhibited a level of activity which was consistently higher (approximately 16%) near the inlet end and consistently lower (approximately 67%) near the outlet end.

When the data of respiratory activity (Fig. 4) and DNA-derived cell density (Fig. 3) were combined to yield a profile of respiratory activity per cell, very different activity patterns were noted for the two populations (Fig. 5). It was apparent that in relation to starved cells, the vegetative cells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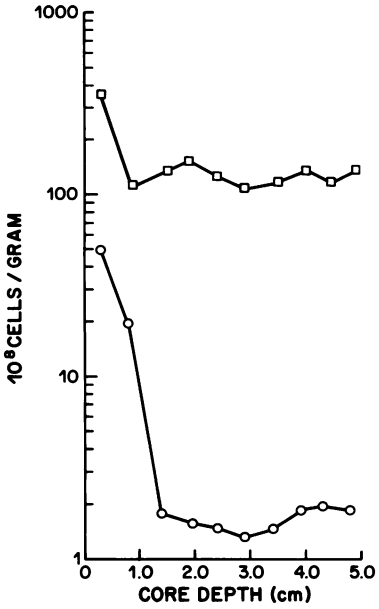

FIG. 3. Differences in the DNA-derived cell distribution in cores injected with either a vegetative cell culture of *K. pneumoniae* (○) or a cell suspension that was starved in PBS for 2 weeks (□).

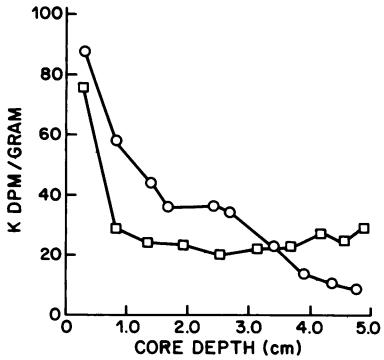

FIG. 4. Glutamic acid respiratory activity in cores injected with either a vegetative culture of *K. pneumoniae* (○) or a cell suspension that was starved in PBS for 2 weeks (□).

demonstrated higher respiratory activity per cell at every core depth. The maximum activity of the vegetative cells per cell (26,289 dpm/10^8 cells) was approximately 100 times higher than the maximum value exhibited by cells starved for 2 weeks (260 dpm/10^8 cells). Respiratory values were also observed to be more constant at the different depths in cores plugged with starved cells compared with those in cores plugged with vegetative cells.

Assimilation of acetic acid during nutrient stimulation. The assimilation of acetic acid represented a measure of bacterial activity after exposure to nutrients. The acetic acid nutrient injection was observed to induce bacterial growth and permeability reductions in cores containing either starved or vegetative cells (Fig. 6). Total assimilation and lipid assimilation values were observed to follow the same trend with respect to core depth (Fig. 7). Assimilation was highest near the inlet, and then it dropped sharply with increasing depth. The actual values of total assimilation were similar for both the vegetative and starved cell populations. Total assimilation values of approximately 35,000 and 1,000 dpm/g were recorded near the inlet and at the deepest core depths, respectively. However, equivalent lipid assimilation levels were not observed for the two cell populations. The incorporation of acetic acid into lipids occurred at a much higher rate near the inlet of cores colonized by starved cells compared with that near the inlet of cores colonized by vegetative cells.

To obtain a measure of total assimilation per cell, it was necessary to determine the cell density at the various core depths. This was done by the fluorescent DNA assay described above. To ensure that the injection of acetic acid would not change the DNA to cell number conversion value, 20-ml volumes of cells starved for either 24 h or 2 weeks were incubated in the presence of 6.5 μg of acetic acid for 24 h. No changes were detected in the DNA/cell ratios of these two populations compared with those of the vegetative cells and cell suspensions starved for 2 weeks. It is apparent that the DNA-derived cell density data after acetic acid nutrient stimulation (Fig. 8) were very similar to those observed after cell injection alone (Fig. 3). Starved cells were observed to maintain higher numbers than the vegetative cells throughout the core length. Likewise, starved cells maintained their numbers more uniformly throughout the entire core length.

The combination of the total assimilation data obtained during the acetic acid nutrient stimulation (Fig. 7) with the DNA-derived cell density data (Fig. 8) revealed that a similar total assimilation per cell trend occurred in cores injected with either vegetative or starved cell suspensions and stimulated by the injection of acetic acid (Fig. 9). We observed that assimilatory activity was highest near the inlet end and decreased thereafter. However, vegetative cells were always more active than starved cells. The maximum assimilation by vegetative cells per cell (1,084 dpm/10^8 cells) was almost 5 times higher than that exhibited by previously starved cells (226 dpm/10^8 cells).

DISCUSSION

K. pneumoniae isolated from oil well-produced water has previously been shown (H. M. Lappin-Scott, F. Cusack, A. MacLeod, and J. W. Costerton, J. Appl. Bacteriol., submitted for publication) to be capable of producing UMB. It was our premise that the use of this size reduction phenomenon should enhance the penetration and decrease the plugging tendencies of bacteria injected into an artificial rock matrix. This supposition is based on the findings of Gruesbeck and Collins (12) and Davis and Updegraff (8). Results of those

FIG. 2. Scanning electron micrographs of cores injected with either vegetative cultures or cell suspensions of *K. pneumoniae* starved in PBS. (A) The inlet pores were sealed with a thick layer of vegetative bacteria encased in exopolysaccharide. (B) Colonization by vegetative cells at a depth of 2.0 cm was less severe than that at the inlet face; however, biofilm bridging of the pore spaces was still apparent. Most cells demonstrated a short rod morphology. (C) The injection of cell suspensions starved for 2 weeks still produced biofilm bridging of the inlet pores, but pore blockage was incomplete. (D) *K. pneumoniae* starved for 2 weeks produced no significant plugging of the pore spaces at a depth of 3.0 cm; however, large numbers of cells, often cocci, were present on the core surface. (E) Biofilm development at the inlet was very low after the injection of *K. pneumoniae* starved for 4 weeks. (F) A large number of cells was present on the core surface at a depth of 3.0 cm after the injection of *K. pneumoniae* that were starved for 4 weeks. Bars, 5.0 μm.

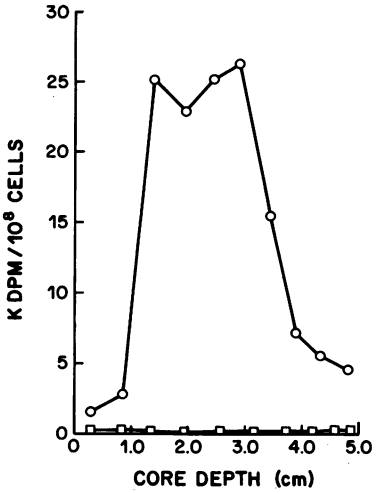

FIG. 5. Differences in the glutamic acid respiratory activity per cell in cores injected with either a vegetative culture of *K. pneumoniae* (○) or a cell suspension that was starved in PBS for 2 weeks (□).

studies indicated that there is a direct correlation between the size of bacteria and the minimum pore size through which the cells pass without causing a significant reduction in permeability.

The injection of starved cells reduced core permeability less than did the injection of vegetative cells; this effect was diminished by larger periods of starvation. The permeability reduction value produced by the starved cells resembled that produced by inert carbonate particles (7). This similarity, together with the observation of glycocalyx loss during starvation, suggests that unlike the vegetative cell cultures, starved cells are not capable of forming a confluent plugging biofilm. Rather, one can envision that the starved cells behaved as simple particles. The cells became packed in the pores of the core, but because of the spaces in the packing, a residual amount of fluid continued to pass through the core. It appears that as the average cell size decreases with the length of the starvation period, the cells are less capable of causing permeability reductions in the artificial cores.

A predominant observation was that the glycocalyx was negligible after 4 weeks of starvation (Fig. 2). The loss of glycocalyx as a result of starvation has also been reported for a marine vibrio (25). Several investigators (10, 13, 31)

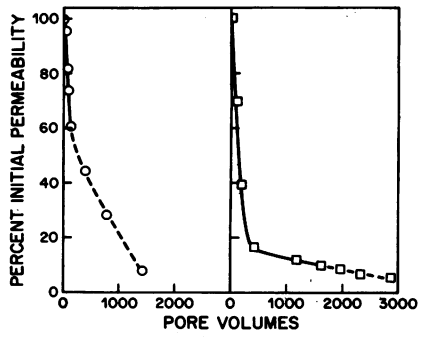

FIG. 6. The permeability reduction profiles of cores injected with bacterial cells (solid line) followed by nutrient stimulation with a [^{14}C]acetic acid solution (dotted line). Symbols: ○, vegetative culture; □, cell suspension starved in PBS for 2 weeks.

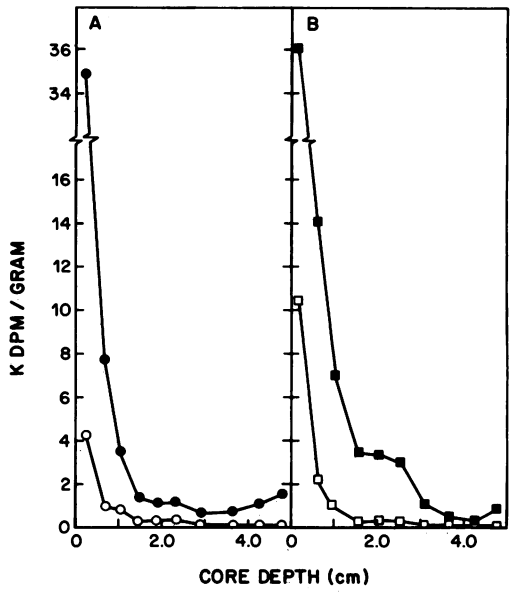

FIG. 7. Assimilation of [^{14}C]acetic acid over a 24-h period in cores colonized by *K. pneumoniae*. (A) Cores colonized by vegetative cells. Symbols: ○, lipid assimilation; ●, total assimilation. (B) Cores colonized by cells starved for 2 weeks. Symbols: □, lipid assimilation; ■, total assimilation.

have reported a strong correlation between glycocalyx production and the plugging of porous matrices. Since it is well documented that the glycocalyx is involved in the irreversible adhesion of bacteria to surfaces (1, 5, 22, 32), it is understandable that a reduction of this extracellular polymer would decrease bacterial adhesion and the subsequent core plugging.

Very few studies have dealt with the distribution of bacteria in three-dimensional matrices. In previous reports (10, 13) it has been indicated that the greatest numbers are present near the inlet. The cell distribution of vegetative cells in the present study (Fig. 3) complements the previous findings (10, 13). In contrast, cells starved for 2 weeks prior to core injection were observed to be distributed uniformly

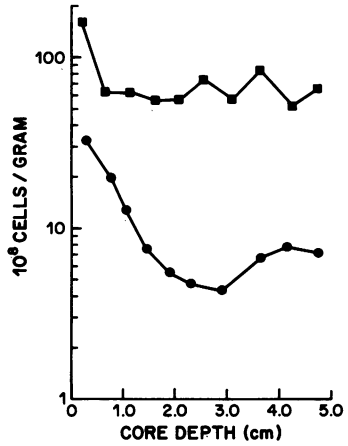

FIG. 8. DNA-derived cell distribution in cores stimulated with a [^{14}C]acetic acid nutrient injection after the previous injection of a vegetative culture of *K. pneumoniae* (●) or a cell suspension that was starved in PBS for 2 weeks (■).

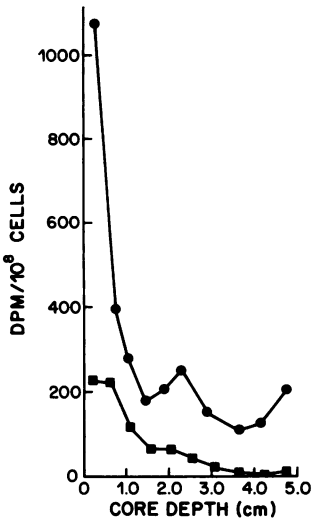

FIG. 9. [^{14}C]acetic acid assimilation per cell values in cores colonized with either vegetative *K. pneumoniae* cells (●) or cells that were starved in PBS for 2 weeks (■).

throughout the core length (Fig. 3). Thus, the analysis of cell numbers confirms the previous conclusions that the starvation-induced reduction in cell size and loss of the thick glycocalyx layer permit the deeper penetration of a greater number of bacterial cells.

The development of a bacterial selective plugging technique requires an understanding of the metabolic response of the injected bacteria once they are present within the formation and are stimulated by an injection of nutrients. The measurement of the metabolic activity in bacterial biofilms on two-dimensional surfaces has been the subject of many reviews (6, 9, 19). In contrast, the metabolic activity of bacterial biofilms in porous matrices has not been investigated as thoroughly. Studies dealing with the latter surface environment have tended to be directed toward wastewater treatment systems (4, 28, 30). A number of the assumptions used in those studies are not applicable to reservoir formations.

In an endeavor to elucidate the metabolic response of bacteria within the porous core structure to nutrient stimulation, two measures of metabolic activity were used in this study. The first technique, glutamic acid respiration, was used to provide a measure of metabolic activity before nutrient stimulation. The second technique, acetic acid assimilation over a 24-h period, provided a measure of metabolic activity which occurred as a result of a nutrient stimulation.

The glutamic acid respiration technique demonstrated that the respiration per cell of starved bacteria was fairly constant throughout the core length (Fig. 5). This indicated that transport through the porous matrix does not result in a segregation of starved bacteria into levels with different activity potentials. Furthermore, the respiration per cell produced by starved cells was always lower than that produced by vegetative cells. This suggests that a level of metabolic dormancy is present in the starved cell population. The endogenous respiration rates of starved marine vibrio populations indicated that there was a similar level of metabolic dormancy (25). The respiration per cell values in cores colonized by vegetative cells yielded a metabolic profile entirely different from those in cores colonized by starved cells. Respiration per vegetative cell was highest in the middle section of the core and decreased as the inlet or outlet was approached. This was a surprising finding, since one would expect that a position near the core inlet would be ecologically advantageous. It is possible that the average respiration per cell near the inlet was lowered by the selective trapping of dead cells in the biofilm (Fig. 2A). It is also possible that the method was limited by substrate exclusion. The thick aggregation of bacteria and glycocalyx at the inlet area may have physically prevented the substrate from reaching all the cells, or it may have reduced dissolved oxygen levels; consequently, a lower measure of cell respiration would have been obtained. This premise is supported by results by Ladd et al. (19), who demonstrated that glutamic acid uptake is about 4 times higher when a biofilm is dispersed compared with its in situ activity.

To gain an understanding of the metabolic response to nutrient stimulation for 24 h, the acetic acid assimilation technique was used. The results presented in Fig. 7 demonstrate that lipid assimilation cannot be used as a measure of bacterial activity if meaningful comparisons are to be made between populations with different nutritional statuses. This conclusion was based on the observation that while total assimilation by the two populations of vegetative and starved cells was very similar, lipid assimilation occurred at a much higher level in starved cells. For this reason, the total assimilation data were used with the DNA-derived cell density data to produce a measure of assimilation per cell (Fig. 9). In agreement with the glutamic acid respiration results (Fig. 5), the total assimilation of acetic acid per cell data (Fig. 9) indicated that the starved cells are metabolically less active than the vegetative cells at all core depths. However, after the acetic acid nutrient stimulation, the maximum assimilatory activity per vegetative cell was less than 5 times higher than that exhibited per starved cell. This difference is considerably less than the 100-fold difference in the maximum glutamic acid respiratory activity per cell that was detected between vegetative and starved cells prior to nutrient stimulation. This observation suggests that the starved cells lose their metabolic dormancy on nutrient stimulation. The ability of bacteria starved for 2 weeks to produce permeability reductions after nutrient stimulation (Fig. 6) provides further evidence of a nutrient-induced loss of metabolic dormancy by the starved bacteria.

The acetic acid assimilation results also provide several conclusions that are applicable to the development of a selective plugging technique. First, the cell distribution in cores injected with starved cells followed by nutrient injection demonstrates that the injected cells are not simply washed away. Rather, the cells remain uniformly distributed in the core (Fig. 8). Second, the finding that assimilatory activity per cell was highest near the core inlet (Fig. 9) suggests that the injection of a readily metabolized substrate such as acetic acid stimulates bacterial growth and plugging near the injection zone instead of in the deeper regions. It therefore seems reasonable to suggest that the development of a deep plug in a bacterial selective plugging technique requires the use of an injected nutrient which is slightly recalcitrant.

To date, normal-size bacteria have been used to investigate selective plugging (16, 29). We conclude that the general starvation of the bacterial cultures prior to core injection can improve penetration. Further investigations dealing with the controlled nutrient stimulation of UMB injected into petroleum reservoirs may provide a new bacterial selective plugging technique.

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LITERATURE CITED

1. **Allison, D. G., and I. W. Sutherland.** 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. J. Gen. Microbiol. **133**(P5):1319–1327.
2. **Bae, H. C., E. H. Cota-Robles, and L. E. Casida, Jr.** 1972. Microflora of soil as viewed by transmission electron microscopy. Appl. Microbiol. **23**:637–648.
3. **Baker, R. M., F. L. Singleton, and M. A. Hood.** 1983. Effects of nutrient deprivation on *Vibro cholerae*. Appl. Environ. Microbiol. **46**:930–940.
4. **Chang, H. T., and B. E. Rittman.** 1987. Verification of the model of biofilm on activated carbon. Environ. Sci. Technol. **21**:280–288.
5. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35**:299–324.
6. **Costerton, J. W., J. C. Nickel, and T. I. Ladd.** 1986. Suitable methods for the comparative study of free-living and surface associated bacterial populations, p. 49–84. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature, vol. 2. Plenum Publishing Corp., New York.
7. **Cusack, F., D. R. Brown, J. W. Costerton, and D. M. Clementz.** 1987. Field and laboratory studies of microbial/fines plugging of water injection wells: mechanism, diagnosis and removal. J. Petrol. Sci. Eng. **1**:39–50.
8. **Davis, J. B., and D. M. Updegraff.** 1954. Microbiology in the petroleum industry. Bacteriol. Rev. **18**:215–238.
9. **Fletcher, M.** 1985. Effect of solid surfaces on the activity of attached bacteria, p. 339–362. *In* D. C. Savage and M. Fletcher (ed.), Bacterial adhesion, mechanisms and physiological significance. Plenum Publishing Corp., New York.
10. **Geesey, G. G., M. W. Mittelman, and V. T. Lieu.** 1987. Evaluation of slime-producing bacteria in oil field core flood experiments. Appl. Environ. Microbiol. **37**:67–72.
11. **Geesey, G. G., R. Mutch, J. W. Costerton, and R. B. Green.** 1978. Sessile bacteria: an important component of the microbial population in small mountain streams. Limnol. Oceanogr. **23**: 1214–1223.
12. **Gruesbeck, C., and R. E. Collins.** 1982. Entrainment and deposition of fine particles in porous media. Soc. Petrol. Eng. J. **22**:847–856.
13. **Gupta, R. P., and D. Swartzendruber.** 1962. Flow-associated reduction in the hydraulic conductivity of quartz sand. Soil Sci. Soc. Am. Proc. **26**:6–10.
14. **Hitzman, D. O.** 1983. Petroleum microbiology and the history of its role in enhanced oil recovery, p. 162–218. *In* E. C. Donaldson and J. B. Clark (ed.), Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. U.S. Department of Energy, Bartlesville, Okla.
15. **Hobbie, J. E., and C. C. Crawford.** 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. **14**:528–532.
16. **Jack, T. R., and E. Diblasio.** 1985. Selective plugging for heavy oil recovery, p. 205–212. *In* J. E. Zajic and E. C. Donaldson (ed.), Microbes and oil recovery. Bioresources Publications, El Paso, Tex.
17. **Jack, T. R., B. G. Thompson, and E. Diblasio.** 1983. The potential for use of microbes in the production of heavy oil, p. 88–93. *In* E. C. Donaldson and J. B. Clark (ed.), Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. U.S. Department of Energy, Bartlesville, Okla.
18. **Labarca, C., and K. Paigen.** 1980. A simple and sensitive DNA assay procedure. Anal. Biochem. **102**:344–352.
19. **Ladd, T. I., J. W. Costerton, and G. G. Geesey.** 1979. Determination of the heterotrophic activity of epilithic microbial populations, p. 180–195. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology. ASTM STP 695. American Society for Testing and Materials, Philadelphia.
20. **Lazar, I., and P. Constantinescu.** 1985. Field trials results of microbial enhanced oil recovery, p. 122–143. *In* J. E. Zajic and E. C. Donaldson (ed.), Microbes and oil recovery. Bioresources Publications, El Paso, Tex.
21. **Leonard, J.** 1986. Annual production report. Increased rate of EOR brightens outlook. Oil Gas J. **April**:71–101.
22. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge.
23. **Morita, R. Y.** 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. **6**:171–198.
24. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
25. **Novitsky, J. A., and R. Y. Morita.** 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Mar. Biol. **48**:289–295.
26. **Oppenheiner, C. H.** 1952. The membrane filter on marine microbiology. J. Bacteriol. **64**:783–786.
27. **Paul, J. H., and B. Myers.** 1982. Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. Appl. Environ. Microbiol. **43**:1393–1399.
28. **Pirt, S. J.** 1973. A quantitative theory of the action of microbes attached to a packed column: relevant to trickling filter effluent purification and to microbial action in soil. J. Appl. Chem. Biotechnol. **23**:289–400.
29. **Raiders, R. A., M. J. McInerney, D. E. Revus, H. M. Torbati, R. M. Knapp, and G. E. Jenneman.** 1986. Selectivity and depth of microbial plugging in Berea sandstone cores. J. Ind. Microbiol. **1**:195–203.
30. **Saunders, P. T., and M. J. Bazin.** 1973. Attachment of microorganisms in a packed column: metabolite diffusion through the microbial film as a limiting factor. J. Appl. Chem. Biotechnol. **23**:847–853.
31. **Shaw, J. C., B. Bramhill, N. C. Wardlaw, and J. W. Costerton.** 1985. Bacterial fouling in a model core system. Appl. Environ. Microbiol. **50**:693–701.
32. **Sutherland, I. W.** 1980. Polysaccharides in the adhesion of marine and freshwater bacteria, p. 329–338. *In* R. C. W. Berkeley, J. W. Lynch, J. Milling, P. R. Butler, and B. Vincent (ed.), Microbial adhesion to surfaces. Ellis Horwood Ltd., Chichester, England.
33. **Torrella, F., and R. Y. Morita.** 1981. Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl. Environ. Microbiol. **41**:518–527.
34. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40**:51–62.
35. **Wyndham, R. C., and J. W. Costerton.** 1981. Heterotrophic potentials and hydrocarbon biodegradation potentials of sediment microorganisms within the Athabasca Oil Sands deposit. Appl. Environ. Microbiol. **41**:783–790.
36. **Zimmermann, R., and L. Myer-Reil.** 1974. A new method for fluorescence staining of bacterial populations on membrane filters. Kiel. Meeresforsch. **30**:24–27.