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Nutrient Resuscitation and Growth of Starved Cells in Sandstone Cores: a Novel Approach to Enhanced Oil Recovery

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***Klebsiella pneumoniae*, which was reduced in size (0.25 by 0.5 μm) by carbon deprivation, was injected into a series of sandstone cores and subjected to separate treatments. Scanning electron microscopy of 400-mD cores showed these small starved cells in nearly every core section. The cells were a mixture of small rods and cocci with little or no biofilm production. Continuous or dose stimulation with sodium citrate allowed the cells to grow throughout the sandstone and completely plug the length of the core. The resuscitated cells were larger than the starved cells (up to 1.7 μm) and were encased in glycocalyx. Scanning electron microscopic results of resuscitation in situ with half-strength brain heart infusion broth showed that a shallow "skin" plug of cells formed at the core inlet and that fewer cells were located in the lower sections. Starved cells also penetrated 200-mD cores and were successfully resuscitated in situ with sodium citrate, so that the entire core was plugged. Nutrient resuscitation of injected starved cells to produce full-size cells which grow and block the rock pores may be successfully applied to selective plugging and may effectively increase oil recovery.**

Selective plugging in enhanced oil recovery involves the physical blocking of the high-permeability rock strata that has already been drained of oil. This ensures that subsequent water flooding to push out more oil is diverted to the oil-bearing, low-permeability zones. The major criterion for successful plugging is the depth of penetration into the high-permeability zones. Small particles travel further into the reservoir and achieve deeper plugging. Available materials and methods for selective plugging include the injection of finely dispersed solids or fibers (diameter, less than 1 μm) that are carried in oil or water (4) or in situ chemical reactions that form insoluble precipitates (8). However, these methods often lack the ability to penetrate deep into the rock strata. Microorganisms may offer a feasible alternative to current methods because they can penetrate deeper into the reservoirs and can selectively plug the high-permeability zones (6, 7).

In work undertaken at the University of Oklahoma, the ability of microorganisms to grow in situ was investigated within the rock strata (9, 13, 14). Jenneman et al. (9) have reported that the addition of nutrients to pure cultures injected into sandstone allows the bacteria to grow and reduces the core permeability. Raiders et al. (13, 14) used parallel systems incorporating two cores with different permeabilities to demonstrate that the bacteria selectively grow and plug the higher-permeability core first. Our interest is in increasing the depth of bacterial penetration and plugging by injecting bacteria that are reduced in size by carbon deprivation.

Our research has established that a *Klebsiella pneumoniae* isolate from oil well waters is able to be reduced in size (down to 0.5 by 0.25 μm) when it is starved for 24 days in laboratory batch cultures (H. M. Lappin-Scott, F. Cusack, A. MacLeod, and J. W. Costerton, submitted for publication) and then resuscitated and returned to full size on the addition of nutrients. As a consequence of the size reduction and reduced glycocalyx production, the starved cells are able to penetrate deeper into fused glass bead cores (permeability, 6 to 7 D) than are the full-size bacteria, thus giving deeper penetration (11).

In this study we injected starved bacteria into sandstone cores and then resuscitated them using nutrient stimulation. In this manner, the bacteria were resuscitated and returned to full size, forming a deep plug throughout entire cores as a result of microbial growth and glycocalyx production. The cores had a permeability of 200 or 400 mD, which is more representative of oil well formation strata than the previously used glass bead cores (11, 15). However, the sandstone cores were not entirely homogeneous in structure, and so results with the sandstone cores lacked the reproducibility of those with the glass bead cores.

MATERIALS AND METHODS

Preparation of microorganisms. Natural populations of bacteria were obtained in the water recovered with the oil (produced water) in the Shell production battery in Harmattan, Alberta, Canada. These indigenous organisms were filtered through a 0.8-μm-pore-size cellulose filter (Millipore Corp., Bedford, Mass.) to yield a natural population of small bacterial cells.

K. pneumoniae was isolated from this produced water as the dominant slime-producing, facultative anaerobe (11). Cells were grown in sodium citrate growth medium (SCM) to the stationary phase, harvested, suspended in phosphate-buffered saline, and starved for periods of up to 3 months, as described previously (Lappin-Scott et al., submitted).

Injection of cells into sandstone cores. Fourteen cylindrical sandstone cores (length, 5.0 cm; diameter, 1.0 cm) with known permeabilities were cut from bricks from the Cleveland Quarries, Cleveland, Ohio. Each core was sterilized for 4 h with ethylene oxide gas and soaked in filtered phosphate-buffered saline in a vacuum chamber for 40 min. The cores were then wrapped in Teflon tape (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), covered in silicon grease, and inserted into rubber core holders (15).

The starved cells or filtered produced water, which were held in separate 4-liter reservoirs, were injected through the cores under nitrogen pressure of 25 kPa with the apparatus described by Shaw et al. (15). Changes in core permeabilities

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TABLE 1. Summary of core treatments

Core no.	Initial permeability (mD)	Treatment	Initial viable cell count (ml^{-1})	PV[a]	Reduction in permeability (% of original)	Nutrient treatment	Permeability reduction after nutrient treatment (% of original)
1	200	Produced water	4.8×10^6	582	40		
2	200	Produced water	2.1×10^7	460	25	27 PV of SCM, continuous	<1
3	400	Produced water	2.1×10^7	4,000	13		
4	400	Produced water	2.1×10^7	1,291	17	2,627 PV of SCM, continuous	0.1
5	400	Starved cells	1.1×10^6	630	4		
6	400	Starved cells	8.4×10^4	286	18	364 PV of SCM, continuous	0.1
7	400	Starved cells	3.7×10^5	455	13	47 PV of SCM, locked in	3.9
8	400	Starved cells	7.1×10^5	318	15	95 PV of 0.5 BHI, continuous	0.5
9	400	Starved cells	3.7×10^5	380	16	4 PV of 0.5 BHI, locked in	2.2
10	200	Starved cells	1.0×10^5	1,800	16		
11	200	Starved cells	1.0×10^5	640	24	170 PV of SCM, continuous	4.0
12	400	Starved cells	2.0×10^6	340	40	415 PV of SCM, continuous	0.2
13	400	Starved cells	1.2×10^6	930	60	1,260 PV of SCM, continuous	0.2
14	400	Starved cells	2.4×10^6	150	81	1,160 PV of SCM, continuous	1.0

[a] PV, Pore volume.

were determined by measuring effluent volumes and dividing the flow rate through the core (K) by the initial flow rate (K_i).

The 14 cores were subjected to separate treatments, as summarized in Table 1. Cores 1 and 3 were injected with filtered produced water until no further reductions in permeability were recorded. Cores 2 and 4 were injected with filtered produced water until the permeabilities were reduced to approximately 20%, and then they were injected with SCM until no further reductions were noted. Cores 5 to 14 were injected with starved cells of *K. pneumoniae*. Cores 5 and 10 were controls and were treated only with starved cells until the maximum reductions in core permeability were achieved. Cores 6 to 9 and 11 were plugged with starved cells until the permeability was approximately 20%. Each core was then injected with either SCM or one-half-strength brain heart infusion (0.5 BHI) medium. The nutrients were injected either as a continuous flow until the cores were plugged or as multiple pore volumes to displace the original fluid and to leave retained bacteria in contact with full-strength nutrient solutions. This format is referred to as locked-in nutrient stimulation.

The permeabilities of cores 12, 13, and 14 were reduced to approximately 40, 60, and 80%, respectively, with starved cells. Each core was then injected with a continuous flow of SCM until the final core permeability was less than 2%.

Analysis of core sections. After each core treatment was completed, the cores were removed from the holders and cut along their length by shallow scoring with a diamond blade saw, followed by scoring with a razor blade. Each section was further divided into subsections. Core pieces were prepared for scanning electron microscopy (SEM) by dehydration and coating with gold-palladium (15). Viable cell counts of core pieces with known weights were undertaken by sonicating the crushed core pieces in a sonic bath in 5 ml of PBS for three 30-s bursts and then spread plating the solution onto 0.5 BHI agar. The plates were incubated for 24 to 48 h at 23 ± 1°C before the CFU were counted.

RESULTS

The indigenous bacterial population present in filtered produced water was capable of rapidly blocking the 200- and 400-mD sandstone cores. The permeability of core 1 (200 mD) was reduced to 40% with the injection of 500 pore volumes (PV) of filtered produced water. A further 100 PV was added but failed to cause any further plugging (Fig. 1). Core 2 (200 mD) was plugged to 25% with produced water, and then 27 PV of SCM was injected through the core. The nutrient was quickly utilized by the microorganisms, and growth blocked the core and reduced permeability to less than 1% (Fig. 1). Cores 3 and 4 (400 mD) required the injection of much larger volumes before substantial permeability reductions were observed. Core 3 received 4,000 PV of produced water, but the permeability failed to fall below 13%, whereas the injection of SCM to the partially plugged core 4 stimulated in situ growth and reduced the permeability to 0.1%.

SEM results demonstrated that both 200-mD cores (cores 1 and 2) were plugged with a heavy colonization by a mixed population of bacteria (up to 2 μm long) especially around the core inlets (Fig. 2A and F). Fewer cells were noted lower down in the cores (Fig. 2B to E and G to J); this was confirmed by the viable cell counts of core 2 (Table 2). Although filtered produced water was also injected into both 400-mD cores (cores 3 and 4), the distribution of microorganisms at each core section differed substantially from those of the same core sections in cores 1 and 2 (Fig. 3). These cores had confluent areas of bacteria at the top sections, and more cells were seen at every core section than were seen in the 200-mD cores. Viable cell counts demonstrated (Table 2) that more cells were located at every section in the higher-permeability core (core 4) than in the lower-permeability (core 2).

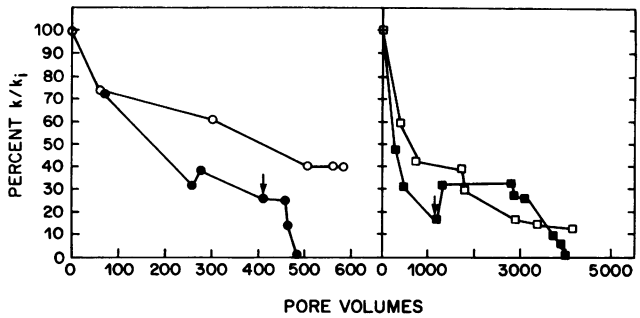

FIG. 1. Reductions in permeabilities of cores 1 to 4 injected with filtered produced water. Symbols: ○, core 1; ●, core 2; □, core 3; ■, core 4. The commencement of SCM injection through cores 2 and 4 is marked by the arrows.

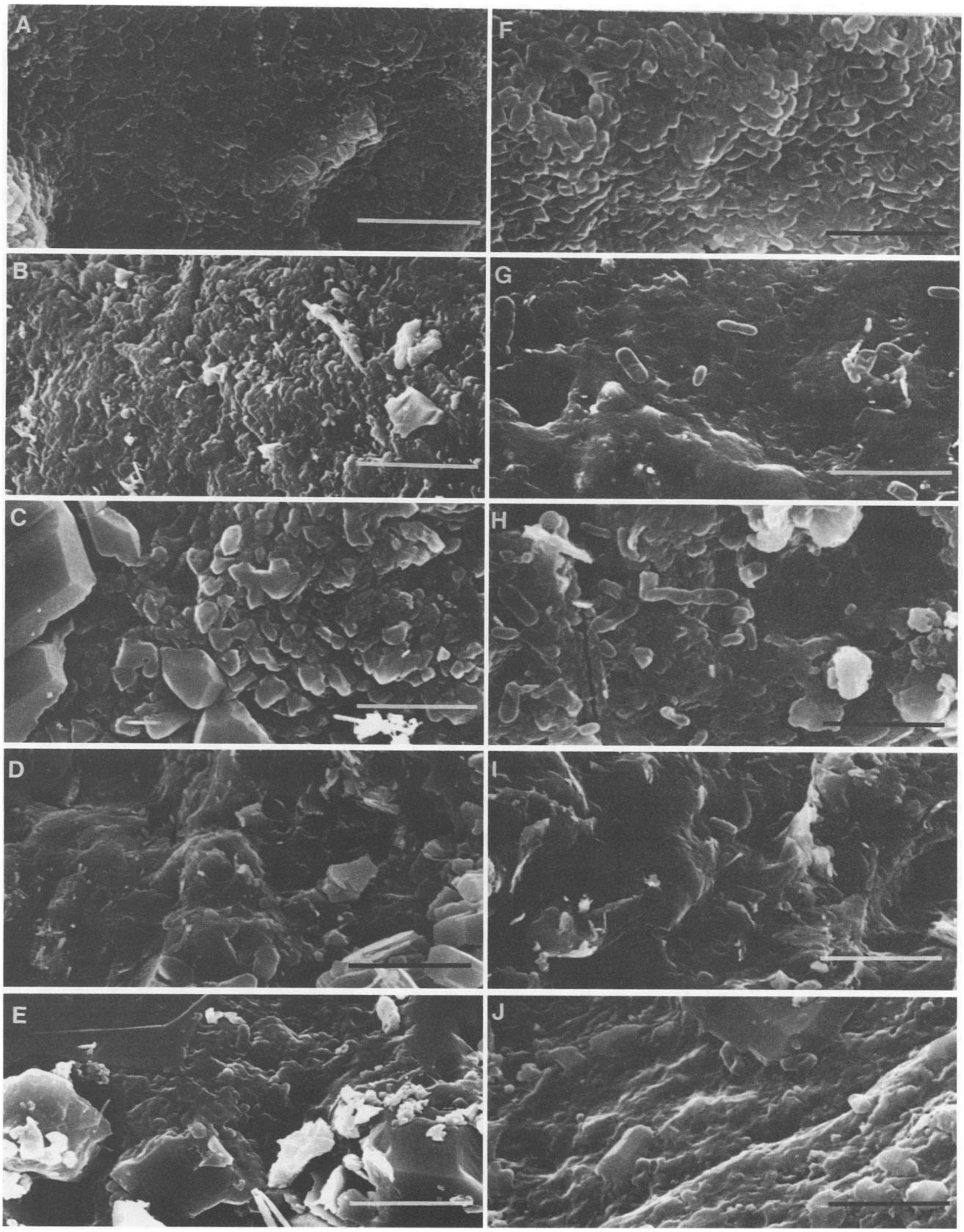

FIG. 2. Micrographs from SEM of sections of cores 1 (A to E) and 2 (F to J) after cores were plugged with filtered produced water. Samples from core 1 were examined at 1.0 cm (A), 2.0 cm (B), 3.0 cm (C), 4.0 cm (D), and 5.0 cm (E). Samples from core 2 were examined at 1.0 cm (F), 2.0 cm (G), 3.0 cm (H), 4.0 cm (I), and 5.0 cm (J). Bars, 5.0 μm.

TABLE 2. Viable counts of *K. pneumoniae* at various core depths

Core depth (cm)	Viable cell counts (cells/ml, 10^8) in[a]:						
	Core 2	Core 4	Core 9	Core 11	Core 12	Core 13	Core 14
1.0	8.1	14.0	8.4	15.3	22.7	28.6	42.7
2.0	2.5	8.2	2.1	8.9	22.0	23.8	23.1
3.0	2.6	8.2	7.6×10^6	7.4	21.7	24.7	21.7
4.0	1.4	7.1	3.1×10^6	4.0	21.6	27.5	17.9
5.0	1.0	6.2	3.6×10^5	6.0	16.4	22.4	19.4

[a] Sandstone core pieces (1.0 g) were crushed and sonicated in 5.0 ml of PBS, and the viable cell counts are reported per milliliter of the resultant suspension. All values are times 10^8, except for the three samples in core 9 at depths of 3.0, 4.0, and 5.0 cm.

The injection of starved cells of *K. pneumoniae* caused permeability reductions in the 400-mD cores (cores 5 to 9), with greater plugging when the cells were stimulated in situ with nutrients. The maximum permeability reduction achieved in core 5 was 4% with the injection of 630 PV of starved bacteria over 50 h. Core 5 was not stimulated by nutrients. Photomicrographs (Fig. 4A to E) of SEM data showed that the starved cells were located in nearly every section of core 5. The cells were a mixture of small rods and cocci, with little biofilm or slime observed around the cells.

Core 6 was treated with starved cells until the permeability was 18%, and then the cells were resuscitated with a continuous injection of SCM over 37 h. The initial addition of SCM caused the permeability to increase to 26%, but further additions supported microbial growth and the permeability decreased to 0.1%. As a consequence of nutrient stimulation, the cells formed rods, with the majority being up to 1.7 μm in length, and were associated with biofilm production (Fig. 4F to J).

The permeability of core 7 was reduced to 13% with starved cells; then the core was further flooded with 45 PV of SCM and locked in. Again, the permeability increased slightly on commencement of nutrient treatment. The nutrient flow was halted and the core was left soaking in SCM. After 12 days the bacterial growth caused the permeability of core 7 to decrease to 3.9%. Micrographs from SEM analysis demonstrated the growth and resuscitation of cells throughout the core and confluent biofilm production in every core section (Fig. 5A to D).

When it was established that SCM successfully resuscitated starved cells and caused plugging throughout the core, the response of the starved bacteria to another nutrient, 0.5 BHI, was investigated. Starved cultures of *K. pneumoniae* were injected into core 8, and the permeability of the core decreased to 15% before 0.5 BHI was injected through the core. The permeability increased to 21% but decreased to less than 0.5% after 10 h and 95 PV of nutrient. SEM data demonstrated that core 8 contained a skin plug at the core inlet (Fig. 5E). The 0.5 BHI allowed the cells to grow, and all were found to be rod shaped (length, between 1.5 and 1.8 μm) and produced biofilms (Fig. 5F to I).

Starved cultures (380 PV) reduced the permeability of core 9 to 16%. Locked-in 0.5 BHI for 12 h supported microbial growth and further reduced the permeability to 2.2%. The viable cell counts of core 9 demonstrated that most of the microorganisms were in the uppermost sections; this was confirmed by SEM (Fig. 6). The core inlet and sections from 1.0 cm down the core were completely colonized by bacteria, and some biofilm production was observed (Fig. 6A and B). Fewer microorganisms were observed at lower core sections (Fig. 6C to E).

When lower-permeability (200 mD) sandstone cores were used, the injection of 1,800 PV of starved *K. pneumoniae* failed to reduce the permeability of core 10 below 16%. SEM data showed that the cells were short rods or cocci (length, less than 1 μm) with no visible biofilm production (Fig. 7A to E). Most cells were located around the core inlet (Fig. 7A). However, when starved cells in core 11 were subsequently injected with 170 PV of SCM, the bacterial growth caused the permeability to decrease from 24 to 4%. Resuscitated cells were noted in most core sections (Fig. 7F to I), and both cell size and shape differed from those of the starved cells in core 10. Typically, the nutrient-stimulated cells were rod shaped, with lengths of up to 2 μm. Viable cell counts of core 11 were highest in the top core section and relatively constant in the lower core sections (Table 2).

Examination of cores 6 to 9 and 11 revealed that when the cores were plugged to between 15 and 25%, the cells were able to utilize either SCM or 0.5 BHI and reduce the permeability still further. In order to investigate whether the starved cells could grow and block the cores when they were infiltrated with fewer cells, three cores were prepared and injected with cells to permeabilities of between 40 and 80%. Core 12 was plugged to 40% with 340 PV of *K. pneumoniae* over 4.5 h. The injection of 415 PV of SCM allowed the cells to resuscitate, and growth reduced the permeability over 20 h. Core 13 required 930 PV of cells injected for 6 h to reduce the permeability to 60%. Continuous SCM injection (1,260 PV) resuscitated the cells, and the resultant microbial growth over 38 h reduced the permeability to 0.2%. Core 14 was injected for 2 h with 150 PV of *K. pneumoniae* until the permeability was 81%. Nutrient stimulation of the cells (1,160 PV) reduced the permeability to 1% over 33.5 h.

The viable cell counts of cores 12 and 13 demonstrated that the microorganisms were evenly distributed throughout both cores (Table 2). The nutrient supported growth and allowed the cells to proliferate in every core section. Growth was not limited to the skin plugging of the core inlet. The viable cell count of sections of core 14 showed a decline in cell numbers with increased distance from the core inlet, from 42.7×10^8 to 19.4×10^8 ml^{-1} (Table 2). SEM results of cores 12 to 14 confirmed that cell resuscitation in every core section was similar to that seen in core 6 (Fig. 4).

DISCUSSION

Natural environments, such as deep ocean waters or soil, are often deficient in growth-promoting nutrients, so bacteria became reduced in size as a physiological survival response to starvation (2, 5, 10, 16, 17). Conditions deep within the rock strata may similarly produce microorganisms that are reduced in size due to carbon deprivation. In this study, the produced water that passed through these underground regions picked up a mixed bacterial population and brought them to the surface under high pressure and flow, thus providing an opportunity to collect samples from the rock

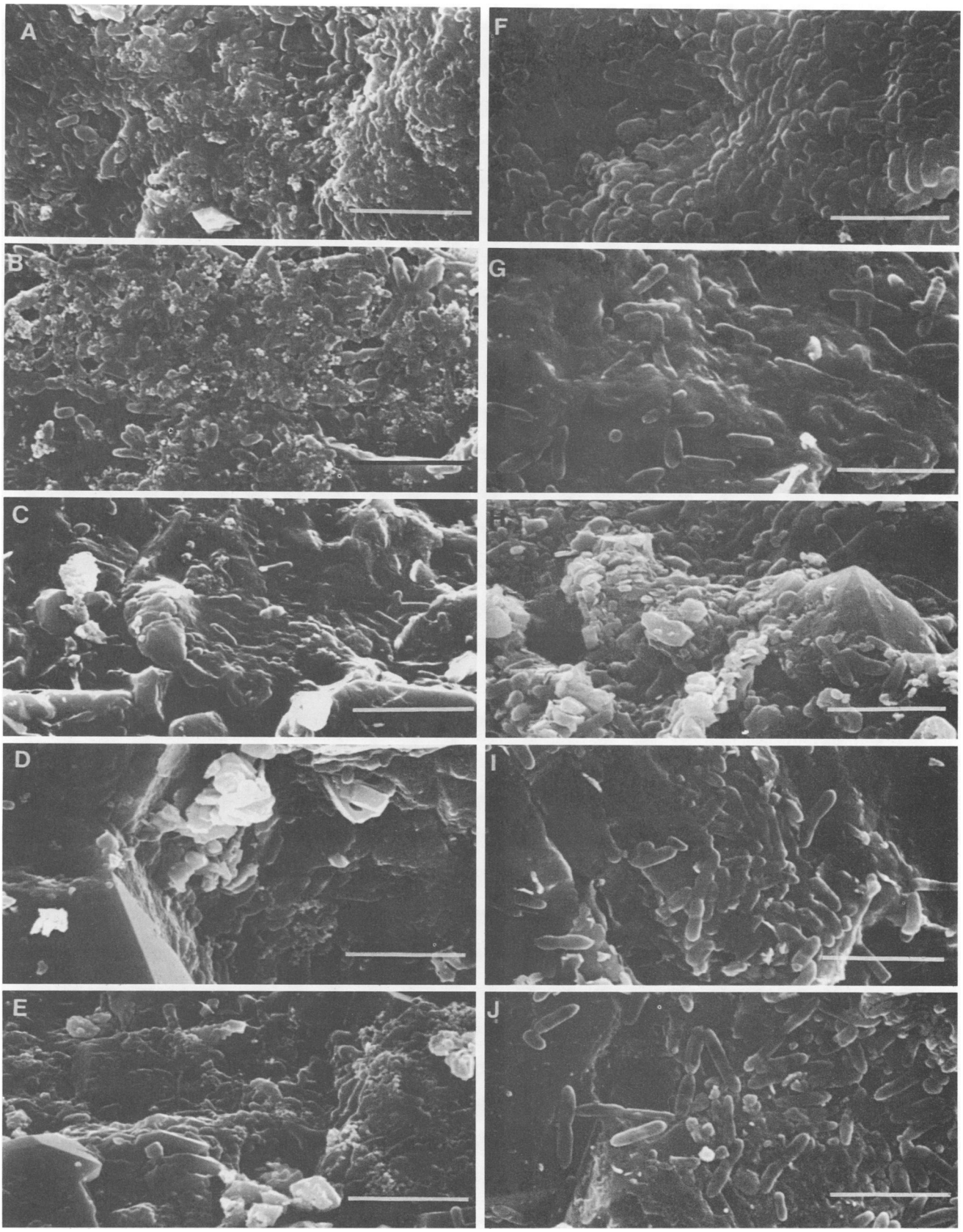

FIG. 3. Micrographs from SEM of sections of cores 3 (A to E) and 4 (F to J). Samples from core 3 were examined at 1.0 cm (A), 2.0 cm (B), 3.0 cm (C), 4.0 cm (D), and 5.0 cm (E). Samples from core 4 were examined at 1.0 cm (F), 2.0 cm (G), 3.0 cm (H), 4.0 cm (I), and 5.0 cm (J). Bars, 5.0 μm.

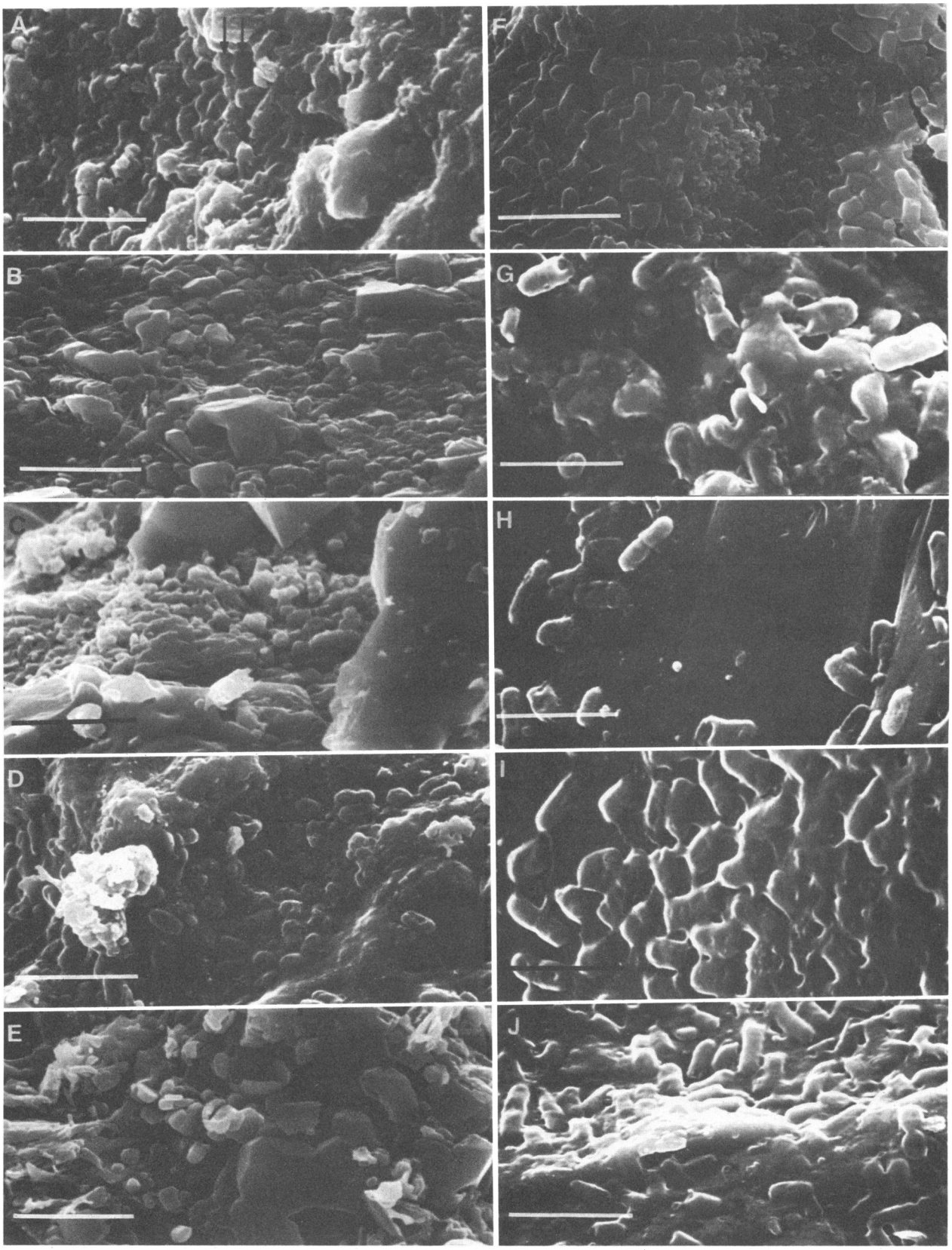

FIG. 4. Micrographs from SEM of sections of cores 5 (A to E) and 6 (F to J). Samples from core 5 were examined at 1.0 cm (A) (the arrows show some of the starved bacteria), 2.0 cm (B), 3.0 cm (C), 4.0 cm (D), and 5.0 cm (E). Samples from core 6 were examined at 1.0 cm (F), 2.0 cm (G), 3.0 cm (H), 4.0 cm (I), and 5.0 cm (J). Bars, 5.0 μm.

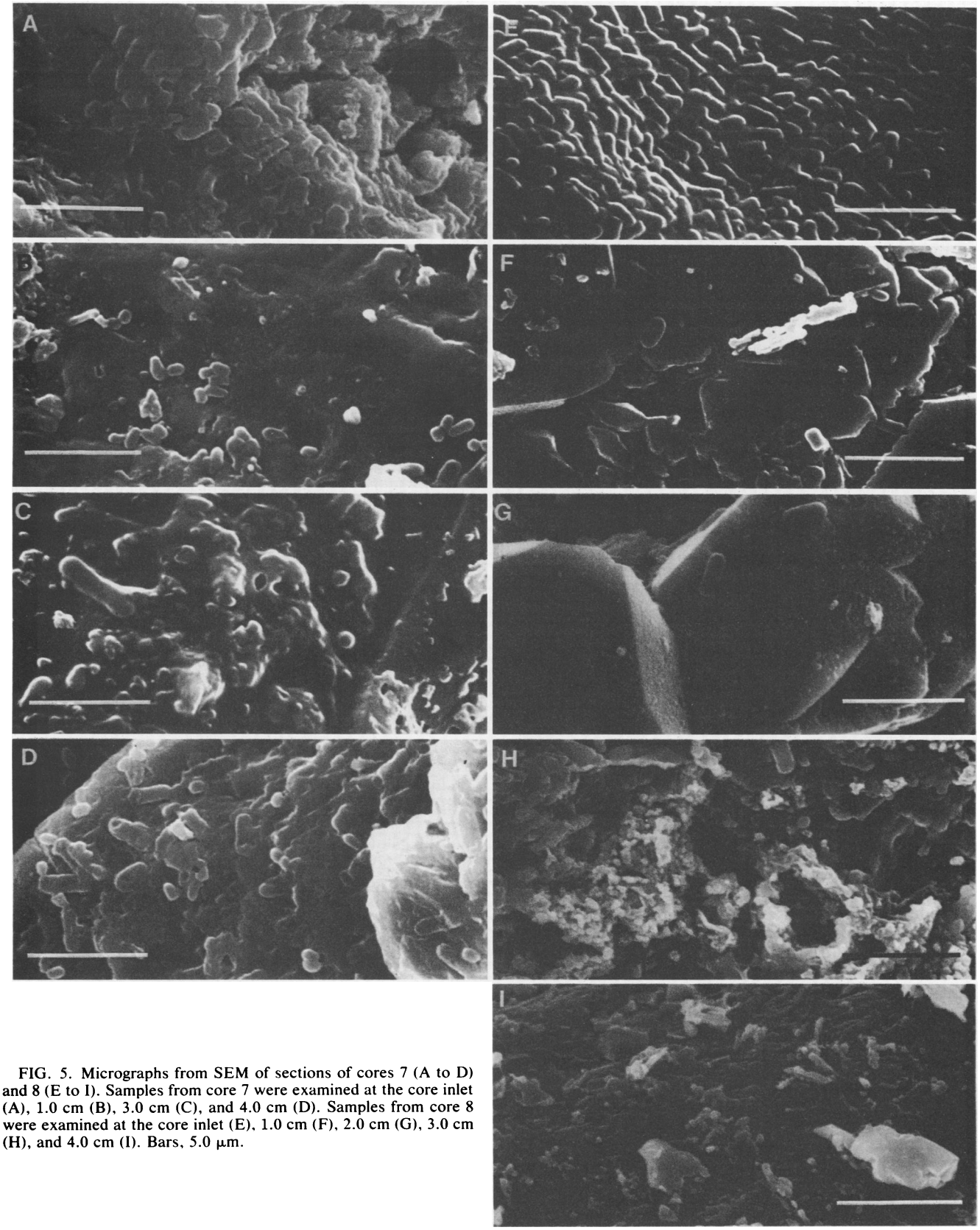

FIG. 5. Micrographs from SEM of sections of cores 7 (A to D) and 8 (E to I). Samples from core 7 were examined at the core inlet (A), 1.0 cm (B), 3.0 cm (C), and 4.0 cm (D). Samples from core 8 were examined at the core inlet (E), 1.0 cm (F), 2.0 cm (G), 3.0 cm (H), and 4.0 cm (I). Bars, 5.0 μm.

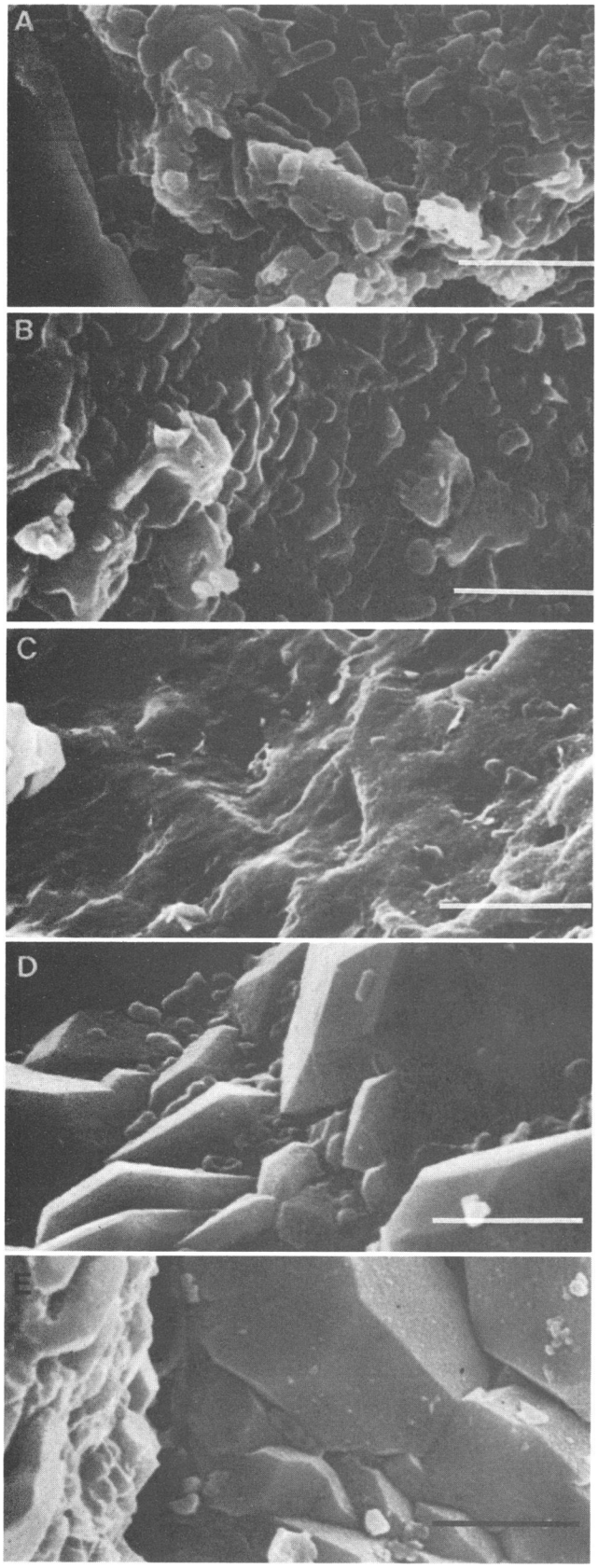

FIG. 6. Micrographs from SEM of core 9. Samples were examined at the core inlet (A), 1.0 cm (B), 2.0 cm (C), 3.0 cm (D), and 4.0 cm (E). Bars, 5.0 μm.

strata. When the larger microorganisms were filtered out of the produced water, the smaller remaining microorganisms were able to penetrate both the 200- and 400-mD cores. A combination of cell sizes (diameter, approximately 0.8 μm) and cell concentrations (approximately 10^7 ml^{-1}) caused the bacteria to collect at the top of the cores. With nutrient stimulation, the mixed bacterial populations reduced the permeability to less than 1%. The existence of indigenous small bacteria in the rock strata would reduce operational costs for selective plugging, as only nutrient injection to resuscitate the bacteria would be required to form deep plugging. However, results of this study demonstrated that the nutrient-stimulated small bacteria predominantly remain at the core inlet and do not form deep plugs. It was therefore necessary to starve bacteria down to specific reduced cell sizes and to inject them into the sandstone cores prior to nutrient flooding in order to obtain deep penetration and plugging.

Bacteria that are reduced in size as a consequence of cell starvation can be resuscitated to full size in liquid cultures when nutrients are added (1, 3, 10, 12, 17; Lappin-Scott et al., submitted). This study has demonstrated that conditions prevalent in rock matrices are permissive of bacterial growth and that only nutrient stimulation is required to resuscitate the starved bacteria and to permit growth. The resuscitation of starved *K. pneumoniae* in the sandstone cores followed a pattern similar to resuscitation in batch cultures (Lappin-Scott et al. submitted). That is, as the starved bacteria were injected with nutrients, the cell size and shape changed from small cocci to larger rods, and biofilm production commenced. The starved bacteria resuscitated at different rates when 0.5 BHI or SCM was injected through the cores, again confirming observations from parallel batch culture studies (Lappin-Scott et al., submitted). With the undefined 0.5 BHI medium, the bacteria returned to full size so rapidly that the growth of the starved cells located at the core inlet completely blocked the flow of 0.5 BHI through the core. This formed a skin plug of *K. pneumoniae* at the inlet, and the cells deeper in the core remained dormant in a starved state. Therefore, deep bacterial plugs were not formed in any of the cores treated with 0.5 BHI. However, when the defined medium SCM was injected through cores containing starved cells, the growth response was much slower. Consequently, there was no rapid growth of the bacteria at the core inlet, and this allowed all of the starved cells in the SCM-treated sandstone cores to receive and utilize nutrients and to grow, forming a deep bacterial plug throughout the core.

A comparison of SCM injected as a continuous flow (core 6) with a locked-in flow (core 7) showed that the former produced a core permeability of less than 1% within 37 h, whereas the permeability of the latter was reduced to 4% after 12 days. This demonstrated that locking in of the nutrients, which would be a cheaper process than a continuous nutrient flow, also reduced core permeability by bacterial plugging but that the process took longer. The quantity of injected starved cells can also be reduced to improve the cost-effectiveness of the operation. Some of the cores were injected with starved bacteria until the core permeabilities were reduced to less than 25% before nutrient stimulation. However, treatment of cores 12 to 14 demonstrated that by reducing the permeability to as little as 40 to 80% with starved bacteria, subsequent nutrient resuscitation further reduced the permeability to 1%. Reductions in core permeabilities caused by bacteria injected into glass bead cores are comparable (11, 15), but they are less representative of the

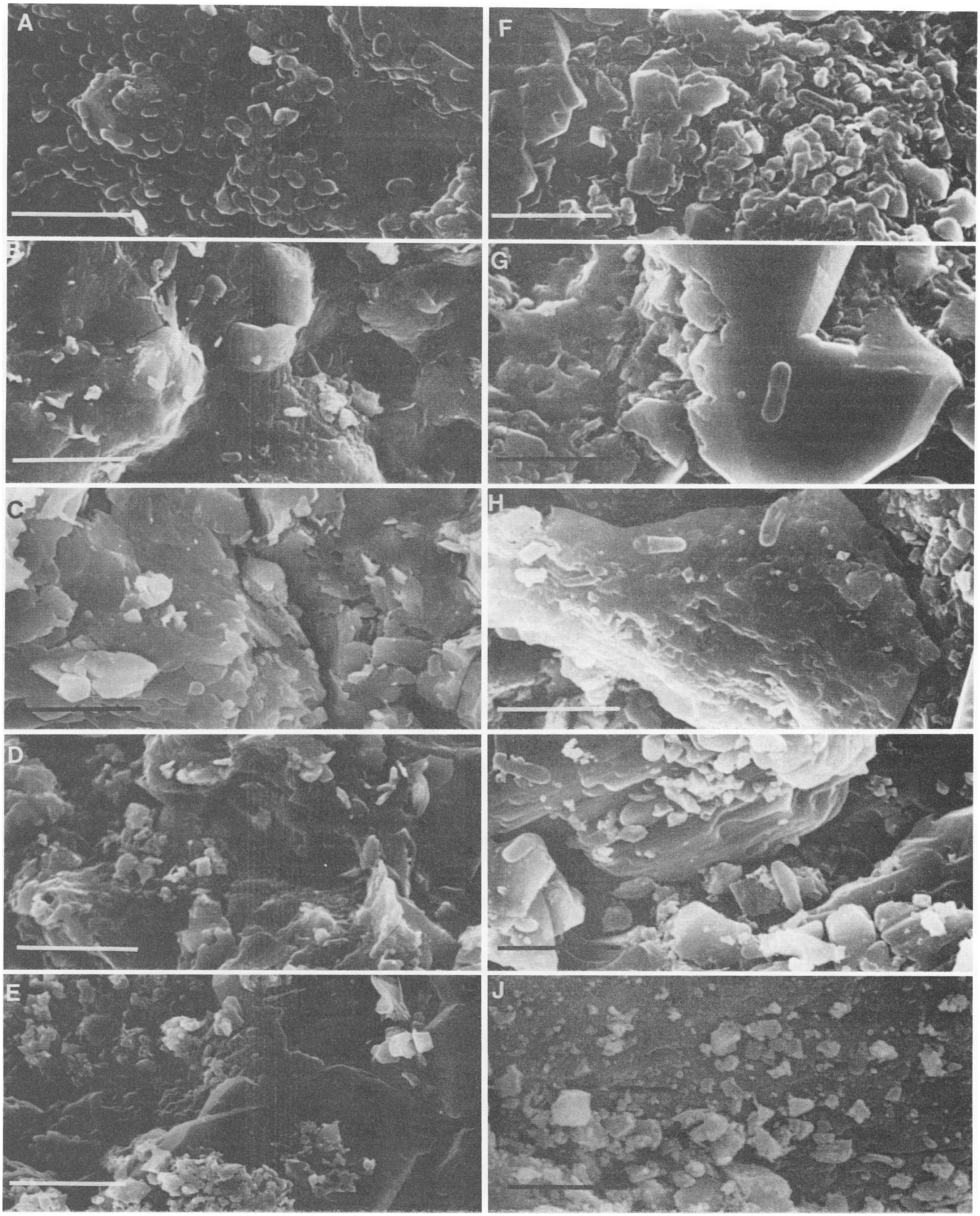

FIG. 7. Micrographs from SEM of sections of cores 10 (A to E) and 11 (F to J). Samples from core 10 were examined at the core inlet (A), 1.0 cm (B), 2.0 cm (C), 3.0 cm (D), and 5.0 cm (E). Samples from core 11 were examined at the core inlet (F), 1.0 cm (G), 2.0 cm (H), 3.0 cm (I), and 4.0 cm (J). Bars, 5.0 μm.

permeabilities commonly found in the underground rock strata than are those found in the sandstone cores.

Jenneman et al. (9) have considered that three conditions must be met to achieve successful in situ bacterial growth and core plugging: first, the cells must be transported throughout the rock stratum; second, nutrients must be transported for growth; and third, the organisms must be able to grow and reduce the permeability of the rock. Results of this study have demonstrated that starved bacteria meet all of these criteria when injected into 400- or 200-mD sandstone cores. Moreover, results of this study stress that a fourth factor must be included in order to achieve successful bacterial plugging. That is, in order to minimize the risks of skin plugging, nutrient packages must be developed that discourage rapid microbial growth and that encourage the formation of deeper bacterial plugs. In this manner, we consider that starved cells could be used to selectively plug high-permeability rock strata already drained of oil so that further recovery techniques can focus on the low-permeability strata that still contain oil.

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