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Effect of Bacterial Extracellular Polymers on the Saturated Hydraulic Conductivity of Sand Columns

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Columns were packed with clean quartz sand, sterilized, and inoculated with different strains of bacteria, which multiplied within the sand at the expense of a continuous supply of fresh nutrient medium. The saturated hydraulic conductivity (HC_{sat}) of the sand was monitored over time. Among the four bacterial strains tested, one formed a capsule, one produced slime layers, and two did not produce any detectable exopolymers. The last two strains were nonmucoid variants of the first two. Only one strain, the slime producer, had a large impact on the HC_{sat}. The production of exopolymers had no effect on either cell multiplication within or movement through the sand columns. Therefore, the HC_{sat} reduction observed with the slime producer was tentatively attributed to the obstruction of flow channels with slime. Compared with the results with *Arthrobacter* sp. strain AK19 used in a previous study, there was a 100-fold increase in detachment from the solid substratum and movement through the sand of the strains used in this study. All strains induced severe clogging when they colonized the inlet chamber of the columns. Under these conditions, the inlet end was covered by a confluent mat with an extremely low HC_{sat}.

Biological clogging of waterlogged soils and aquifer materials has been an object of study for several decades because of its adverse effects in various applications, such as the disposal of wastewater (8, 12, 19, 27), groundwater recharge (2, 29, 33, 46), groundwater production (39, 40), or the in situ bioremediation of contaminated aquifer sediments (22). The microflora associated with this clogging process consists predominantly of bacteria (7, 19, 27, 35, 39), although fungi (30, 33) and protozoa (15, 30) may sometimes be involved in certain phases of the reduction of the saturated hydraulic conductivity (HC_{sat}).

Various mechanisms to account for the HC_{sat} losses observed in conjunction with microbial activities have been suggested. With few exceptions, they all rely on the assumption that the observed HC_{sat} reductions are associated with the production of a nonaqueous phase that decreases significantly the effective porosity (i.e., the fraction of the total volume that is available for water flow) of the soil or aquifer material. Microbes in subsurface environments and sediments can produce solid, gas, and gel phases. The solid phase may be the cells themselves or precipitates of by-products of their metabolism, e.g., iron sulfide or iron hydroxides (39, 40). The gas phase consists of poorly soluble gaseous compounds such as dinitrogen or methane (21, 29). Finally, the gel phase typically consists of hydrated extracellular polymers (or exopolymers). These exopolymers, commonly polysaccharidic in nature (6, 23), occur in two basic forms. As a capsule, the exopolymers are intimately associated with the cell surface and may be covalently bound, whereas in the form of slime they are only loosely associated with the cell surface (45).

The idea that bacterial exopolymers can block, or at least hinder, water flow in natural porous media was suggested as early as in the 1950s (24). In later years, supportive evidence was obtained by a number of researchers (2, 8, 27, 38), who found a positive correlation between the degree of clogging and the accumulation of exopolymers. Other authors (15, 17), however, observed no relationship between these two parameters or even an inverse relationship, i.e., a clogging intensity inversely proportional to the polysaccharide content (36). These results suggest that exopolymer production is not necessary to achieve significant clogging (41). However, it may be sufficient to give rise to large reductions of HC_{sat} under specific conditions. The clogging of artificial porous media inoculated with pure cultures indeed becomes comparatively more pronounced when the percolating medium is such that it stimulates the production of exopolymers (16, 41).

In order to be able to control or, in some cases, to increase the extent of the clogging by bacteria in subsurface environments, more detailed information on the various mechanisms listed above is required than is currently available. In particular, a fundamental understanding is needed of the exact role played by exopolymers in the clogging process, of the factors that influence their clogging efficiency, and of whether their morphology has an effect on their ability to clog soils or aquifer materials.

The objective of the study reported in the present article was to determine whether the production of bacterial exopolymers can, in and of itself, significantly reduce the HC_{sat} of sand columns. Two bacterial isolates which produced different types of exopolymers were used in the experiments. The reductions of the HC_{sat} by these two isolates were compared with those obtained with nonmucoid mutant strains.

MATERIALS AND METHODS

Strains and medium. The four strains used in this study were subclones of isolate B0693 (DOE Subsurface Microbial Culture Collection, Florida State University, Tallahassee), which was isolated in 1986 from a sample obtained at the Savannah River site drilling (site P24) at a depth of 259 m.

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The four strains were obtained from this isolate as the result of a two-step procedure.

In the first step, we isolated two subclones on the basis of the morphological features of the colonies they produced on agar. These two subclones differed with respect to three physiological tests of the API Rapid NFT physiological testing system (Analytab Products, Plainview, N.Y.): the subclone SLI hydrolyzed esculin, produced gelatinase, and grew on phenylacetate; the subclone CAP did not exhibit any of these three properties. Negative staining with China ink revealed that the SLI cells produced extensive slime layers when grown in the nutrient medium described below, whereas the CAP cells were encapsulated.

In the second step, nonmucoid variants, SLI^- and CAP^-, were isolated from the strains SLI and CAP, respectively. Use was made of the fact that cells not associated with exopolymers should have a higher sedimentation velocity. Centrifuge tubes were filled with a suspension of either SLI or CAP cells up to a mark at 8 cm and centrifuged for 8 min at 2,000 × g. On the basis of Stokes' law for the sedimentation velocity of spherical particles, we estimated that these conditions should be appropriate for all the cells not associated with exopolymers to settle, while leaving in suspension most of the cells that were bound to exopolymers. The procedure was repeated 10 times, by discarding the supernatant and resuspending the pellet up to the mark at each step. Numerous nonmucoid variants were isolated from plates streaked with the final pellets. Negative staining with China ink indicated that these variants did not produce exopolymers when grown in the nutrient medium described below. The physiological characteristics of the nonmucoid variant strains tested with the API Rapid NFT were identical to those of their respective parental strains. None of the four strains was motile, and, when tested with the API Rapid NFT, none could ferment glucose or use nitrate as an electron acceptor.

The nutrient medium used for the percolation experiments contained (in milligrams per liter of deionized water) glucose (20), KNO_3 (0.7), $MgSO_4 \cdot 7H_2O$ (0.7), $CaCl_2 \cdot 2H_2O$ (14.3), $Na_2HPO_4 \cdot 7H_2O$ (165), KH_2PO_4 (80), ferric EDTA (4), yeast extract (0.36), and Casamino Acids (0.36), in addition to 0.25 ml of a trace element stock solution (26). The pH was adjusted to 6.8. Except for $MgSO_4 \cdot 7H_2O$, the phosphate buffer, and the trace elements, which were all autoclaved with the bulk water, all nutrients were filter sterilized.

Flow system. The flow system is represented schematically in Fig. 1. The nutrient solution, held aseptically in 10-liter Pyrex bottles, was continuously pumped (0.53 ml min^{-1}) through columns packed with sand. Each column consisted of an acrylic cylinder (length, 4 cm; inside diameter, 2.6 cm) capped at both ends with headpieces manufactured with a conical chamber to ensure an axial flow within the sand. Rubber O-rings were used to provide a good seal at the contact between columns and headpieces. The sand was confined within the cylinder by 53-μm-opening nylon meshes that were glued at each end of the cylinder. The quartz sand (63- to 125-μm particle size) had been washed with sodium acetate (pH 5) to remove carbonates; with hydrogen peroxide (6%) to remove organic matter; and with a mixture of sodium citrate, sodium bicarbonate, and sodium hydrosulfite to remove iron oxides. It was further autoclaved for 90 min shortly before assemblage of the columns. Once packed in the column, the sand had a porosity (volume of voids divided by the total volume) of 0.39 ± 0.01. The residence time of the solution in the sand was about 15 min. Except for the columns, all parts in contact with the flowing solution were

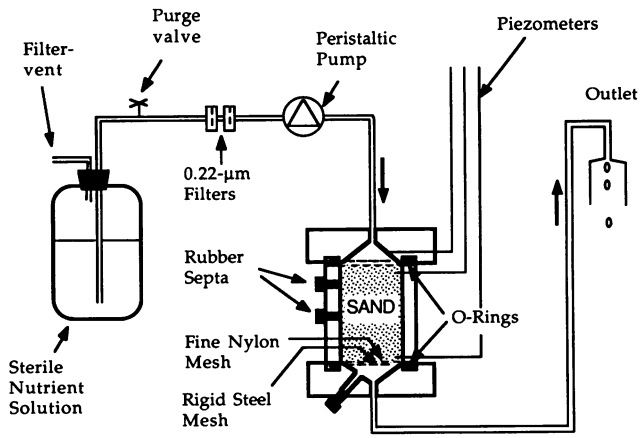

FIG. 1. Schematic diagram of the flow system (not to scale).

autoclaved and assembled aseptically in a laminar flow hood. Once completely assembled, the columns were sterilized by overnight exposure to ethylene oxide. Two filters (0.22-μm pore size) were inserted in series between the bottles with the nutrient solution and the peristaltic pump to prevent any accidental contaminant from reaching the columns. Contamination was checked regularly by measuring the glucose concentration upstream from the columns. When contaminants were detected, all parts that had been in contact with the contaminant were immediately replaced with aseptic ones.

The sand was slowly saturated from beneath, and a steady upward flow of boiled, autoclaved water was maintained for 1 week until the entrapped air was removed. The columns were then inverted to establish a downward flow, and the nutrient solution was substituted for water. The columns were inoculated by injecting 5 ml of a 20-h-old culture through a rubber septum. In two separate experiments, this septum was located at 2- and 0.8-cm depths, respectively. The inoculum, grown in a 2-fold-concentrated version of the nutrient solution described above, had a cell density of (2.6 ± 0.4) × 10^7 cells ml^{-1}. Immediately after inoculation, the flow was stopped for 2 h to foster cell attachment. The whole flow system was kept at 25.0 ± 1.0°C in a constant-temperature room.

HC_{sat} measurement. The HC_{sat} can be calculated from a measurement of the hydraulic head gradient by using Darcy's law (18): $Q = A\ HC_{sat}\ (\Delta H)/L$, where Q is the flow rate (0.53 ml min^{-1}), A is the cross section of the column (5.3 cm^2), HC_{sat} is measured in centimeters per minute, H (centimeters) is the hydraulic head (water potential per unit weight), and L (centimeters) is the distance between the two points where H is measured. The head difference between two points, ΔH, is the difference in elevations of water columns held in small tubings, called piezometers, connected to these points at one end and open to the atmosphere at the other end. When, as a result of severe clogging, the pressure inside a column exceeded ca. 1.5 m of water column, the piezometers were connected to Hg manometers. Piezometers, in duplicates at each depth, protruded horizontally by about 3 mm inside the sand column and were capped with nylon mesh to prevent the sand from creeping in. They were inserted at three locations in the permeameters: in the inlet chamber just above the inlet mesh, at 3 mm below the inlet mesh, and at 36 mm depth just above the outlet mesh (Fig. 1). Thus, the HC_{sat} could be calculated for the top layer

comprising the inlet mesh and the first 3 mm of sand by using the data from the first and second pairs of piezometers and for the bulk of the sand between 3 and 36 mm by using the data from the second and third pairs of piezometers. The two H measurements from duplicate piezometers at any given depth rarely differed by more than 2 mm. H values were measured daily. In order to facilitate the comparison of treatments, HC_{sat} values for each column were divided by the initial value, $HC_{sat\ (t=0)}$, so that the adimensional conductivity ratio, $[HC_{sat}/HC_{sat\ (t=0)}]$, was equal to 1 at the time of inoculation. The average value of $HC_{sat\ (t=0)}$ was 0.38 ± 0.07 cm min^{-1}.

Cell counts and glucose assay. A rubber septum was connected to the outlet chamber of the columns to allow effluent sampling directly underneath the outlet mesh. At regular time intervals, a 1.1-ml sample of effluent was slowly withdrawn with a syringe whose needle had been sterilized by flaming. A droplet was transferred into the counting chamber of a hemacytometer for a direct microscopic determination of cell density. One milliliter of the sample was filtered (0.22-μm-pore-size filter) to remove the cells. The filtrate was assayed for glucose by the method of Dubois et al. (13), by using glucose standards for calibration. In addition to glucose, this method also assays any dissolved carbohydrate by-product of the bacterial metabolism.

Biomass assay. At the end of a percolation run, a cork borer (inside diameter, 1 cm) was used to sample the columns. Duplicate samples were obtained for each depth interval (5 mm thick), mixed, and assayed colorimetrically for lipid-bound phosphates (14). The lipid-bound phosphate concentrations were converted into cell densities by using a conversion factor that had been determined for batch cultures in their stationary phase of growth (2 to 4 days). Cell numbers were determined by direct counts (see above). The conversion factor, averaged over all four strains, was 0.60 ± 0.06 nmol of lipid-bound phosphates per 10^8 cells. The values of this conversion factor for the four strains were not statistically different. Use of an average cell volume of 0.48 ± 0.05 μm^3 (determined by light microscopy in the same cultures; again, there was no significant difference among strains) and a value of 1.1 g cm^{-3} for the cell mass density (20) allowed the cell densities to be also expressed on a mass basis (milligrams [wet weight] of biomass per cubic centimeter of bulk sand).

SEM. The sampling and mounting methods used in the experiments have been described in detail elsewhere (42). Only a rapid overview of the technique is presented here. Upon disassembling the columns, small cores (length, 8 mm) were taken at different depths by inserting a small cylinder made of a copper sheet (thickness, 75 μm) rolled up around a rod (diameter, 3.5 mm) and soldered at the joint. This cylinder was then capped at each end by securing a fine nylon mesh by means of a flexible silicone tubing. The cylinders containing the samples were rinsed for 1 to 2 h in phosphate buffer (same pH and ionic strength as the nutrient solution) and fixed for 90 min with 2.5% glutaraldehyde–50 mM lysine–50 mM cacodylate buffer (CB; pH 7.4). The lysine and glutaraldehyde were mixed immediately before use. This particular mixture has been found to improve the preservation of bacterial exopolymers for scanning electron microscopy (SEM) (43). After one rinse in CB (30 min), fixation was pursued in 2.5% glutaraldehyde–CB at 4°C for 1 to 2 days. After three rinses in CB, the cells were progressively dehydrated in a graded series of ethanol (10, 25, 50, 75, 90, and 100% three times for 20 min each) and, without any delay, critical point dried with CO_2 as the transitional fluid. The dried cores were partially extruded by using a small piston, and sequentially exposed surfaces were mounted on SEM stubs covered with a piece of adhesive tape. The stubs were coated with a thin layer of Au-Pd alloy and viewed with a JEOL JSM-35CF (AMRAY 1000A for Fig. 8) SEM operated at 10 kV. All glutaraldehyde solutions were freshly prepared from a frozen stock that had been shipped on dry ice (Sigma Chemical Co., St. Louis, Mo.).

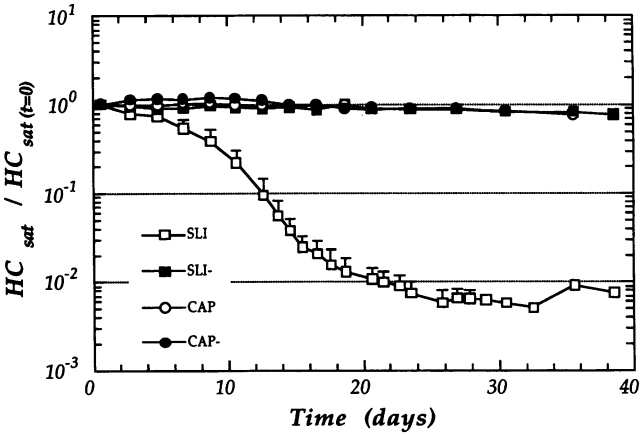

FIG. 2. Time change of the HC_{sat} in columns inoculated at a 2-cm depth with a slime-producing strain (SLI), with an encapsulated strain (CAP), or with their respective nonmucoid variants (SLI^- and CAP^-). Each point represents the mean value from two experiments, and bars indicate the standard deviation (they do not appear when hidden by the symbol).

RESULTS

Of the four bacterial strains tested, only the slime producer, SLI, had a substantial effect on the HC_{sat} of the sand (Fig. 2). This variation among strains does not appear related to population densities in the columns since the glucose consumption rate was very similar in the different columns (Fig. 3A). Separate experiments in batch cultures (in a 25- or 100-fold-more-concentrated version of the medium than was used in the column experiments) showed that there was no significant difference among strains with respect to the yield coefficient for glucose (approximately 10^6 cells per μg of glucose), the rate of glucose uptake by nongrowing cells (5.2 ± 0.6 μg of glucose per 10^{10} cells per min), or the maximum specific growth rate (0.41 ± 0.04 h^{-1}). The cumulated numbers of cells washed out from the columns, calculated on the basis of cell densities in the column effluents (Fig. 3B), were not significantly different among strains, according to the F test ($P = 0.05$).

The HC_{sat} loss observed with the SLI strain could not be attributed to an increase of the percolate viscosity. Indeed, when measured after 37 days of flow, the viscosity of the effluents was found to be identical to that of pure water.

After 38 days of flow, the biomass distributions in the different columns were determined (Fig. 4). The biomass distributions showed a marked peak somewhat upstream from the point of inoculation (2 cm deep), except in the case of the nonencapsulated variant (CAP^-), which accumulated at the inlet. The total amount of biomass accumulated in the columns was not significantly different among the four strains, according to the F test ($P = 0.05$). The average value was (12.1 ± 2.4) × 10^{10} cells per column ($n = 8$).

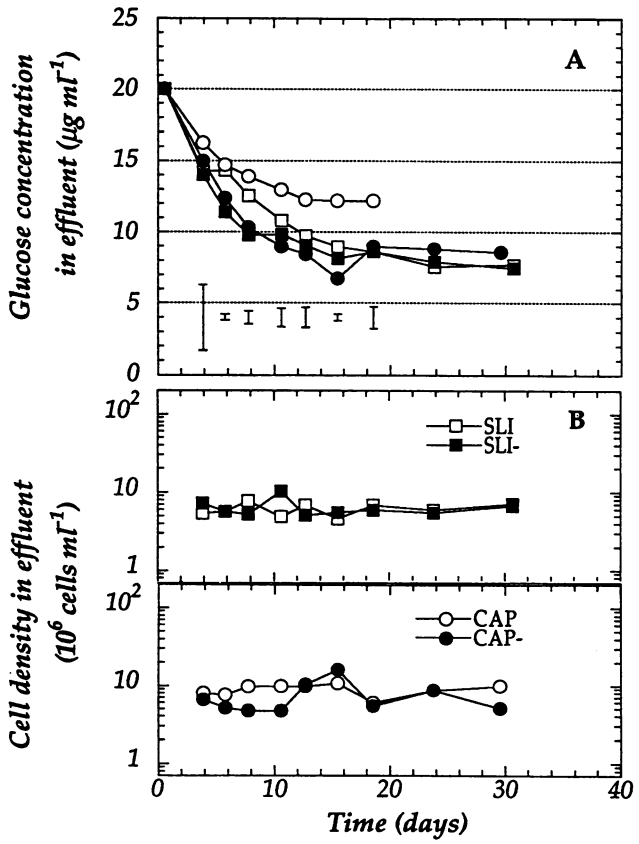

FIG. 3. Time change of glucose concentration (A) and cell density (B) in effluents of columns inoculated with a slime-producing strain (SLI), with an encapsulated strain (CAP), or with their respective nonmucoid variants (SLI$^-$ and CAP$^-$). Each point represents the mean value from two experiments, and bars indicate the least significant difference (P = 0.05).

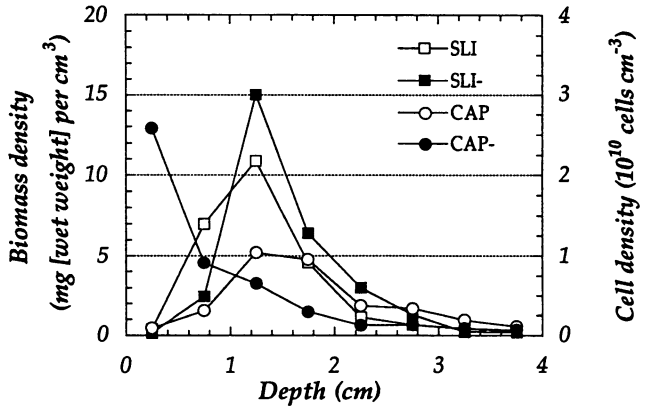

FIG. 4. Biomass distribution in columns inoculated at a 2-cm depth with a slime-producing strain (SLI), with an encapsulated strain (CAP), or with their respective nonmucoid variants (SLI$^-$ and CAP$^-$). Each point represents the mean value from two experiments.

The production of polymeric extracellular material (exopolymers) by the SLI strain inside the column was demonstrated by direct observation of samples taken after 38 days of flow (Fig. 5A and B). No exopolymers were detected inside the columns inoculated with the nonmucoid variant (Fig. 6). Whereas copious amounts of exopolymers surrounded SLI cells in colonies that developed at a depth of 16 mm (Fig. 5B), almost none were visible in colonies at 23 mm (Fig. 5C). After 37 days of flow, dilutions of the effluent were plated on agar supplemented with the percolation medium (concentrated eightfold). In the case of the column harboring SLI cells, a large fraction of the colonies (61 and 64% for the two replicate columns) were nonmucoid. Because the nonmucoid colonies from the duplicate columns showed identical characteristic features and because the cells within these colonies had the exact same shape and size as the SLI cells, an accidental contamination was unlikely.

Very few, but large, colonies of the capsulated strain, CAP, were detected on the sand particles (Fig. 7A). The cells were buried within a matrix of exopolymers (Fig. 7B). Often these colonies were concentrated on specific sand particles, on which 5 to 20 colonies were usually seen. An X-ray analysis (energy-dispersive spectroscopy) did not reveal any difference in elemental composition between colonized and uncolonized sand particles, a single Si peak being observed in both cases. It seemed, however, that the surface of colonized sand particles was always much more irregular and pitted than that of uncolonized sand grains, which had smooth surfaces in general.

In a previous series of experiments, the inocula had been injected at a shallower depth (8 mm), which resulted in colonization of the inlet chamber of the column. As a result, mats composed of bacteria and their associated exopolymers (strains SLI and CAP) or bacteria alone (strains SLI$^-$ and CAP$^-$) coated the entire surface of the inlet mesh after several weeks of flow (Fig. 8A). Contrary to what happened when they did not colonize the inlet chamber, all four strains built up a considerable resistance to flow when they formed mats (Fig. 9). An interesting observation was that HC_{sat} losses were greater in the columns that had been inoculated with the nonmucoid variants (strains SLI$^-$ and CAP$^-$) compared with the losses caused by their respective mucoid parental strains. However, this should be taken with caution since only one treatment (strain CAP) was duplicated over the entire course of the experiment (three columns became contaminated and were discarded). The cell density of mats formed by the CAP$^-$ strain seems greater than that of mats formed by CAP cells (Fig. 8B and C). For the latter, the intercellular spaces appear partially filled with condensed exopolymers.

The HC_{sat} ratios plotted in Fig. 9 are averages for the entire length of the columns and are therefore directly comparable to the HC_{sat} ratios plotted in Fig. 2. However, in the series of experiments resulting in the HC_{sat} ratios plotted in Fig. 9, the HC_{sat} in fact decreased only in the region between the inlet chamber and a depth of 3 mm, remaining constant in regions deeper than 3 mm. Thus the HC_{sat} values for the top layer, comprising the mat and the top 3 mm of sand, were about 10 times smaller than the values plotted in Fig. 9. For example, in the case of the top layer of the column inoculated with the CAP$^-$ strain, HC_{sat} was equal to 2.3×10^{-6} cm min^{-1} 40 days after the initiation of mat formation.

DISCUSSION

The production of slime by a bacterial population growing within a sand column seems to have a very important impact on the HC_{sat} of the sand, since the nonmucoid variant strain,

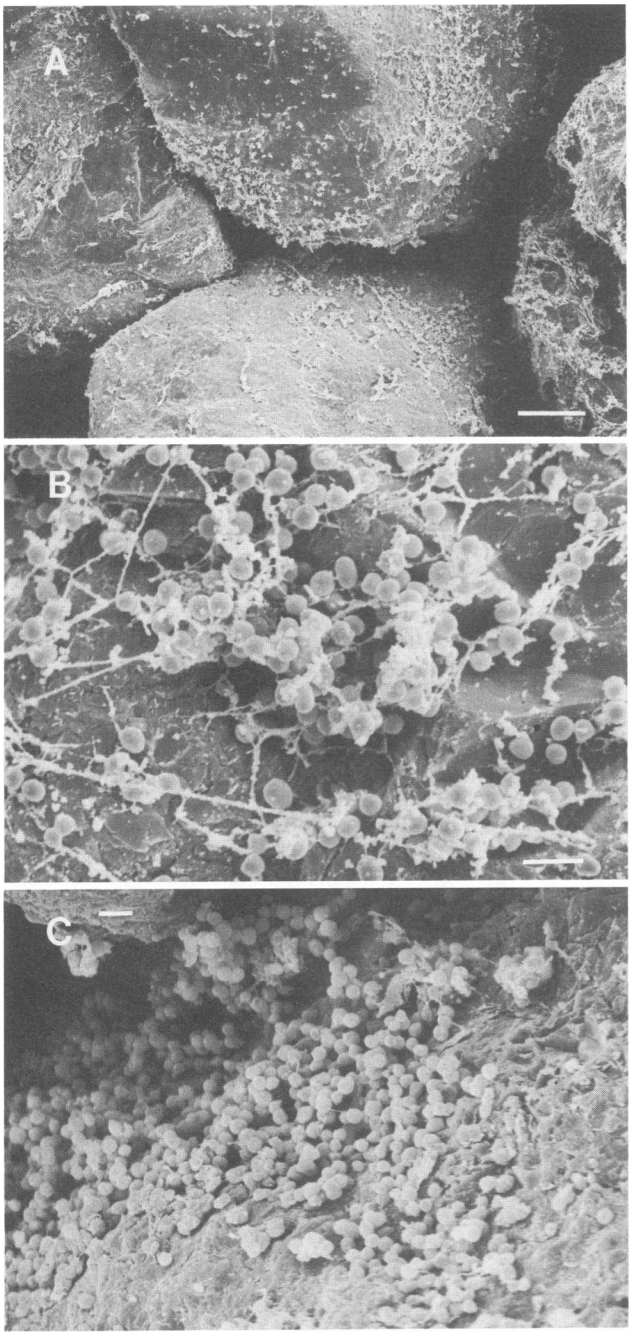

FIG. 5. SEM micrographs of sand grains colonized by the slime-producing strain, SLI. (A and B) At a 16-mm depth, bacteria were entangled in a meshwork of long fibrillar material. Bars, 20 (A) and 2 (B) μm. (C) At a 23-mm depth, exopolymers were almost entirely absent. Bar, 2 μm.

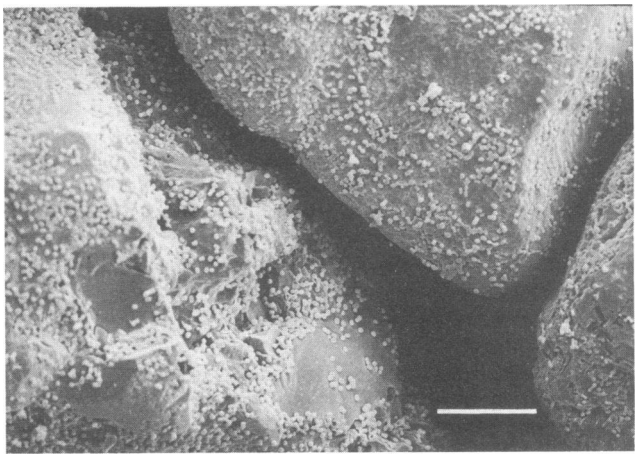

FIG. 6. SEM micrograph of sand grains colonized by the non-slimy variant, SLI^-, at a 16-mm depth.

SLI^-, had a negligible effect on the HC_{sat}, unlike its parental strain, the slime-producing SLI strain. The severe clogging observed with the latter strain was attributed to the production of slime and not to a greater accumulation of cells, because the cell densities inside the various columns were not significantly different at the end of the experiments. Slime excretion could affect the HC_{sat} by different means: it could increase the viscosity of the percolating solution, it could enhance cell adhesion and retention, it could increase the frictional resistance at the solid-liquid interfaces, or it could decrease the effective porosity, i.e., the space available for the transmission of the liquid phase. It is apparent that the two first mechanisms were insignificant in this study, because no increase of the viscosity was detected and because biomass densities were not higher in the case of the SLI strain (Fig. 4). It has been reported that the growth of filamentous microorganisms (25, 31) and the production of bacterial slimes (9) in the biofilms attached on the inside wall of water-conducting pipes can cause important pressure losses. In those studies, the resistance to flow was much greater than what would have been expected on the basis of the decreased cross section area available for flow as a result of biofilm growth and was attributed rather to increased frictional resistance (31). In porous media, however, the work by Characklis et al. (10) involving a slime-producing *Pseudomonas aeruginosa* strain indicated that the increased roughness (frictional resistance) of the particles was probably insignificant and that the biofilms increased hydraulic resistance essentially by reducing the cross section area for flow. In the present study, however, it is not clear that the amount of slime that is visible in Fig. 5A and B would be sufficient to decrease the effective porosity to the extent that a 100-fold decrease of HC_{sat} would ensue. Of course, during the preparation for electron microscopy, slimy materials can be extracted (11) and undergo considerable shrinkage (4, 43). The volume occupied by the slime in Fig. 5A and B may therefore be significantly less than what it really was in the sand. Interestingly, the production of cell-bound exopolymers by the capsulated strain CAP, unlike the free exopolymers produced by the strain SLI, had no detectable effect on the HC_{sat}.

The total number of cells, both retained in the columns and washed out with the effluent, could be calculated from Fig. 3B and 4 and was found to be strain independent according to the F test ($P = 0.05$), with an average of $(31.1 \pm 1.1) \times 10^{10}$ cells ($n = 8$). Assuming that all the nitrogen supplied with the nutrient solution (including the nitrogen provided with the yeast extract and Casamino Acids but not with FeEDTA) was consumed by the cells growing inside the columns, the nitrogen content of the bacterial protoplasm would be 0.030 ± 0.002 pg of N per μm^3 of biovolume ($n = 8$). This value is very close to that reported by Nagata and

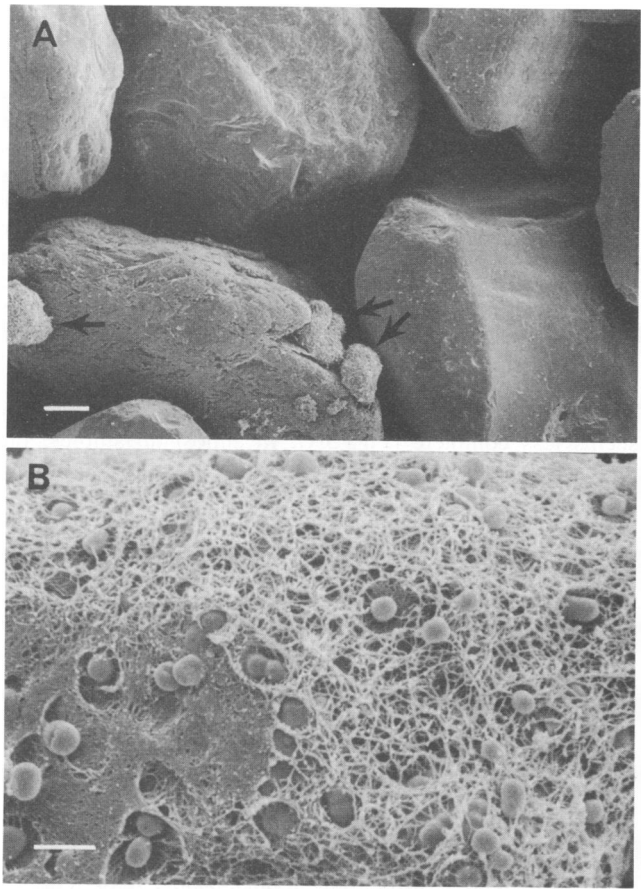

FIG. 7. SEM micrographs of sand grains colonized by the encapsulated strain CAP at a 16-mm depth. (A) Microcolonies (arrows) attached to sand grains with a rough surface. Bar, 20 μm. (B) Cells buried in an extensive matrix of exopolymers. Bar, 2 μm.

Watanabe (28) for freshwater bacterial assemblages (0.027 pg of N per μm^3). It seems, therefore, likely that all of the nitrogen supplied to the columns was completely consumed at a very early stage during the percolation experiments; otherwise the actual nitrogen content of the bacteria would be lower than the calculated value, which seems unlikely because Nagata and Watanabe (28) observed a fairly constant N content over a wide range of nutritional conditions. The same argument can be used to show that cell decay was probably negligible. When growth is restricted by the supply of a limiting nutrient, the total increase in biomass (combining the biomass retained inside the column and the cumulated mass of washed-out cells) is controlled by the supply of this nutrient (1). Since the rate of supply of N to the columns was constant and the cell density in the column effluents was more or less constant after 4 days of flow (Fig. 3B), the biomass retained within the sand was, therefore, probably increasing more or less linearly after 4 days of flow.

The total number of cells retained within the sand, estimated from Fig. 4, was $(12.1 \pm 2.4) \times 10^{10}$ cells per column (average of the four strains), while the cumulated number of washed-out cells (estimated from Fig. 3B) was $(19.5 \pm 2.9) \times 10^{10}$ cells per column (average of the four strains). Thus the proportion of cells that remained attached to the sand (38%) was smaller than that of the washed-out cells (62%). These proportions should have remained constant through time if,

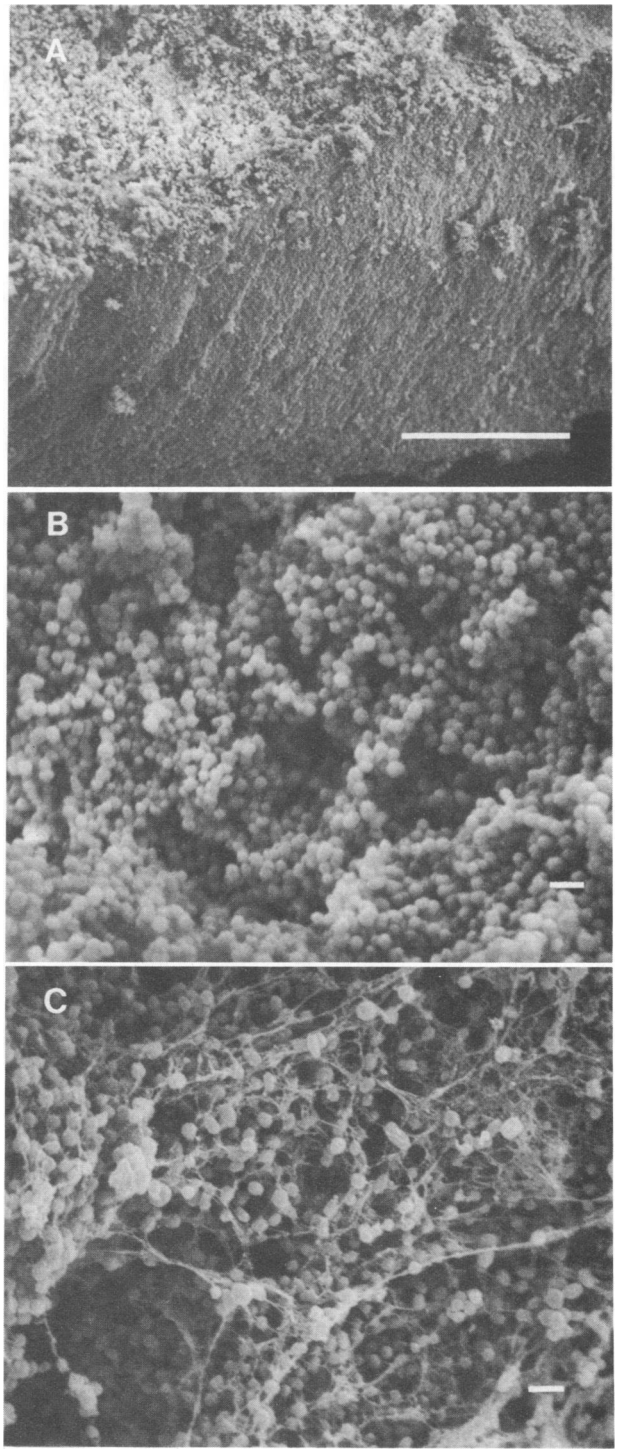

FIG. 8. SEM micrographs of bacterial mats sampled on the inlet end of columns inoculated at a 0.8-cm depth. (A) Vertical cross section of a mat consisting of CAP^- cells. The top surface of the mat is visible on the left upper part of the micrograph. Bar, 100 μm. (B) Higher magnification of the top surface of the mat shown in panel A. Bar, 2 μm. (C) Midsection of a mat consisting of exopolymer-producing bacteria (strain CAP), where the cells appear less densely packed and are entangled in a matrix of long fibrillar material. Bar, 2 μm.

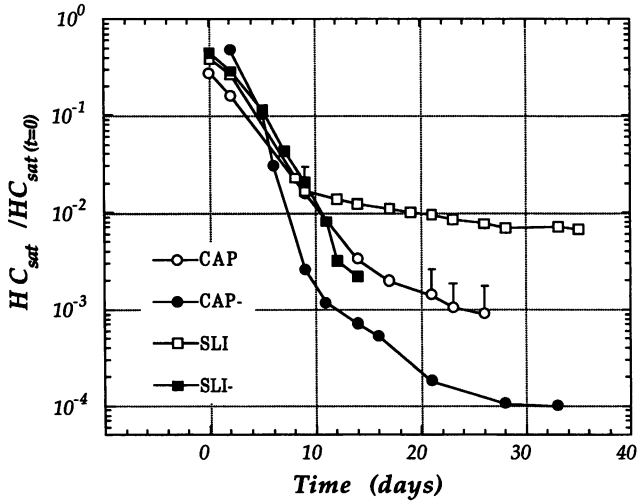

FIG. 9. Time change of the HC_{sat} in columns inoculated at a 0.8-cm depth with a slime-producing strain (SLI), with an encapsulated strain (CAP), or with their respective nonmucoid variants (SLI^- and CAP^-). Each point represents the mean value from two experiments for the CAP treatment and single observations for the other treatments. Bars represent the standard deviation. The origin of the x axis corresponds to the initiation of clogging in the 0- to 3-mm layer caused by an extensive bacterial mat (a variable lag phase of 10 to 20 days elapsed between the inoculation time and mat formation).

as proposed earlier, the biomass attached to the sand increased linearly, as did the cumulated washed-out biomass. Another way to analyze such data involves the concept of removal rate, which can be defined as the number of cells detached per unit of surface area of substratum per unit of time. The removal rate has been found to be a first-order function of the cell density when the cells form a biofilm on a flat surface (3). In the present study, the removal rate was more or less constant between days 3.9 and 31 (Fig. 3B) while the biomass must have increased substantially (about eightfold if the attached biomass indeed increased linearly). This observation suggests that at least some properties of biofilms do not apply to certain bacteria growing in columns packed with a fine sand.

After several weeks of flow, more than 60% of the cells in the effluent of the columns inoculated with the SLI strain had lost the ability to produce slime. Since it was estimated that the cells in the effluent accounted for more than 60% of the cells newly formed within the columns, nonmucoid cells should therefore have accounted for at least 36% of the biomass production in the columns inoculated with the mucoid, slime-producing SLI strain. This phenomenon could have resulted from the proliferation of a small subpopulation of SLI^- cells initially present in the SLI inoculum. Alternatively, it could have resulted from the loss of the capacity to produce slime during the incubation. At least in the absence of sand, we noticed that the production of slime was a stable characteristic of the SLI strain. Indeed, in a first, unsuccessful attempt to isolate spontaneous SLI^- mutant strains, an SLI culture was maintained in the exponential phase of growth for 8 days by transferring a small volume of culture to fresh nutrient medium every 2 days (slime production was stimulated during that experiment by using a concentrated version of the high C/N ratio nutrient medium described above). All colonies growing on streak plates made from the last culture tube were mucoid. The presence of sand in the column experiments could conceivably promote instability in slime production. However, we have observed that the addition of sand to batch cultures either enhances exopolymer production or has no effect on polymer production, depending on the strain. It appears therefore plausible that the large number of SLI^- cells detected after several weeks of incubation in the effluents of the columns inoculated with the SLI strain resulted from the proliferation of SLI^- cells initially present in the inoculum.

A possible mechanism that could account for the selective pressure in favor of SLI^- mutant cells in the columns inoculated with SLI cells would be an increased adsorption rate when the cell envelope is not surrounded by slime. It can be seen (Fig. 6A) that SLI^- cells colonize the sand particles uniformly, whereas the coverage by SLI cells seems less complete (Fig. 5A). In almost all studies, the presence of a bacterial capsule or slime has been shown to decrease the surface hydrophobicity (34), thereby decreasing the extent of adhesion when it is the result of hydrophobic interactions. A recent study by Stenström (37) has shown that cell surface hydrophobicity was the most important factor affecting bacterial adhesion to quartz sand particles. Stenström found a high correlation between the adhesion of various bacterial strains to quartz particles and the cell surface hydrophobicity (estimated by hydrophobic interaction chromatography). Antiadhesive properties of bacterial exopolymers for strains interacting with various types of substrata have been demonstrated (5, 23, 32, 44).

Whereas the nonexopolymer-producing strains used in this study did not cause substantial clogging when inlet chamber colonization was avoided, at least one other bacterium, *Arthrobacter* sp. strain AK19, has been reported to cause drastic HC_{sat} reductions in the absence of exopolymer production (41). The transport properties of these different strains may have played a role in controlling their impact on the HC_{sat}. Indeed, all conditions being equal (flow rate, sand, and nutrient medium), the cell densities in the effluent streams of columns inoculated with *Arthrobacter* sp. strain AK19 remained in the range of 10^4 to 10^5 cells ml^{-1} (data not published), about 100 times lower than the values obtained with the strains used in the present study. For the latter strains, approximately 60% of the newly formed cells were washed out from the columns, while in the case of *Arthrobacter* sp. strain AK19, a negligible fraction of the cells were washed out. As a consequence, given similar growth rates and yield coefficients, the increase in the biomass retained within the column and the associated HC_{sat} reduction are likely to proceed at a faster pace with *Arthrobacter* sp. strain AK19 than with the strains used in the present study. A possible explanation for the low washout rate of *Arthrobacter* cells is that, upon cell division, the daughter cells remained attached and eventually formed large aggregates of cells that were easily trapped at pore constrictions (41).

Although three of the four strains tested had a negligible effect on the HC_{sat} when they were prevented from colonizing the inlet chamber (Fig. 2), they all induced a high resistance to flow when they multiplied on the mesh confining the sand at the inlet end of the columns (Fig. 9). The cell densities measured just underneath the mesh in the 0- to 5-mm layer were, except in the case of the SLI strain, always smaller than 10 mg (wet weight) per cm^3, the threshold density at which the HC_{sat} started to decline substantially in columns inoculated with *Arthrobacter* sp. strain AK19 (41). Therefore, the very large head losses measured across the top layer, comprising the bacterial mat and the first 3 mm of

sand, must have taken place principally, if not exclusively, within the structure of the mats (at least for the nonmucoid strains, SLI^- and CAP^-, for which this threshold value could apply). This consideration enables us to estimate the HC_{sat} value of the mats with Darcy's law, since mat thicknesses could be approximated on SEM micrographs. In the case of the CAP^- strain, the mat thickness was about 270 μm (estimated from a micrograph similar to the one shown in Fig. 8A, with the mat section running parallel to the plane of the picture), the head loss was 230 kPa (ΔH = 24 m of water), and the flow rate was 0.1 ml min^{-1} (at that pressure, the peristaltic pump failed to deliver 0.53 ml min^{-1}), yielding an HC_{sat} value of 2.1×10^{-7} cm min^{-1}. This extremely low HC_{sat} value is more than 10 times lower than that of clayey soils whose HC_{sat} values range between 6×10^{-3} and 6×10^{-6} cm min^{-1} (18).

In conclusion, the key result of the study reported in this article was the observation that, under conditions such that the cell density was too low to have an effect on the HC_{sat} of the sand, the production of exopolymers by bacteria may drastically reduce the HC_{sat}. The exopolymers affected the HC_{sat} only when they were produced in the form of loose slime layers; cell-bound capsular exopolymers had no significant effect on the HC_{sat}. This study also showed that clogging of the columns occurred considerably faster and was much more pronounced when the bacteria were allowed to multiply in the inlet chambers. Under those conditions, all strains formed a virtually impervious mat on the inlet mesh.

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