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Bacterial transport through porous solids: interactions between *Micrococcus luteus* cells and sand particles

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SUMMARY

As part of an evaluation of microbial systems for potential application in enhanced oil recovery (EOR) in situ, the behaviour of bacteria within the labyrinths of porous structures was investigated. Sandpacks were utilised as models of reservoir formations. Using *Micrococcus luteus* (NCIB 8553) cells under non-growing conditions, the interaction between the bacteria and sand particles was investigated by a simple shake flask system. The attachment of bacterial cells to sand was time-dependent and reversible. With increasing density of cell suspensions, competitive effects on binding to sand were observed, indicating a possible interaction between two subpopulations of cells. Similar effects occurred when suspensions of *M. luteus* cells were pumped through sandpacks. Shake flask experiments indicated that the maximum binding of bacteria to sand was about 2×10^9 cells $\cdot$ g^{-1}, representing a total coverage of approximately 20% of the surface of sand particles by bacterial cells.

INTRODUCTION

There are several ways in which microbial systems may be of benefit in the enhancement of oil recovery [8,15]. Broadly speaking there are three potential classes of operation [8]: firstly, the injection of a purified microbial product such as a polysaccharide viscosifying agent; secondly, the stimulation of the natural microflora resident in the oil reservoir formation; and thirdly, the injection of selected bacteria with desirable characteristics [15]. The products synthesiscd by bacteria in situ which may aid the recovery of residual oil include carbon dioxide gas, solvents, biosurfactants and exopolysaccharides [8,15]. There have been many reports (e.g., Refs. 3, 9 and 18) of the successful isolation of bacterial strains capable of the biosynthesis of such compounds. Many oil reservoirs contain high-permeability regions, sometimes called thief zones, through which the waterflood will selectively flow, thus bypassing large pockets of oil. There is considerable interest in developing microbiological systems to plug such regions [18]. The behaviour of bacteria within the labyrinths of porous structures will be of considerable importance in assessing their

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potential for in situ microbial enhanced oil recovery (MEOR). This would include investigation of growth and productivity under environmental conditions and of the ability of such bacteria to pass through porous structures. It is essential for any in situ process where bacteria are injected into the oil reservoir that the cells do not plug the formation in the immediate area surrounding the injection well and that they are able to move with the waterflood. There may be occasions, however, when it would be desirable for bacteria to block part of the formation, for example in selective plugging of high-permeability regions. Thus a knowledge of the factors that may influence the passage of bacteria through porous structures will be of much importance.

The retention of bacteria within the labyrinths of porous solids such as oil reservoir rock will probably be due to a combination of events, including attachment and adhesion of cells to the solid phase, filtration and cell aggregation, Since the work of Zobell and Anderson [28] on the growth and productivity of bacteria in glass vessels of differing surface area to volume ratios there has been much interest in the attachment of bacteria to solid surfaces and the development of biofilms. The impact of surface colonisation has been discussed by many authors (e.g., Refs. 7, 22, 24 and 26) and there is an increasing awareness of the environmental and cell-associated factors and the physicochemical interactions involved [23] Biofilm production appears to be a two-phase process [26] involving the initial attachment of cells followed by growth on the solid surface. Exopolysaccharide production during the second phase effectively makes adhesion irreversible. The composition of the cell surface can have a major impact on attachment processes [1,6,19,23] and in some instances cells can have specific appendages, such as pili, to aid attachment [4]. There is an increasing interest in the adhesion of bacteria to non-water-wettable surfaces and the involvement of hydrophobic interactions [16,17,20,21]. With certain species of *Streptococcus* it has been discovered that cell-surface proteins are associated with cell hydrophobicity and coaggregation properties [13]. Interactions with hydrophobic surfaces could be of importance in the environment of oil reservoirs in respect to both the oil-water and liquid-solid interfaces. Zajic [27] discusses the hydrophobic effect and quotes a study in which 45% of reservoirs investigated were found to be water-wettable.

Investigations into bacterial transport through porous structures have been undertaken using a variety of conditions and several bacterial species [5,10–12,14]. Jang et al. [10] used two model systems in their investigation. Firstly, they pumped bacterial suspensions through Berea sandstone cores and monitored the discharge of cells in the effluent. The second system was configured to observe the migration of cells under stagnant conditions. They based their calculations on a deep filtration model and were able to obtain a filtration coefficient for each bacterial species investigated. Typically, in the pumping experiments they found that after one pore volume a plateau of cell output was obtained until a state of saturation was reached when the output cell density rose to equal that of the input. Their filtration coefficient was based on the plateau value. Investigations of migration under stagnant conditions [10,12] indicated that different species migrated at different rates. Jenneman et al. [14], also using Berea sandstone cores, concluded that bacterial transport through the cores was a complex process, and with bacterial cells suspended in brine they found that almost all cells were retained within the cores. During the course of their experiments they observed significant reduction in permeability. Shaw et al. [25] used a sintered glass bead core to model bacterial plugging. With cells under growing conditions they obtained a confluent biofilm which effectively reduced core permeability in excess of 99%. A direct comparison between the experiments performed by the different groups is not possible because of differences in their experimental approach.

The purpose of the current investigation was to relate cell attachment to sand particles under non-growth conditions to the retention of such cells within a sandpack and so determine the relative importance of the cell-pore wall interaction with respect to bacterial transport through porous structures. The bacterium *Micrococcus luteus* was chosen

for these investigations because it was considered that for in situ usage a spherical-shaped organism would be more appropriate, since it would be less likely to be entrapped within porous domains. However, similar investigations using gram-positive and gram-negative rods are currently being undertaken in our laboratory.

MATERIALS AND METHODS

Bacterial cultures

M. luteus (NCIB 8553) was grown in nutrient broth (Oxoid) at 28°C with shaking. The extent of culture growth was monitored by measuring OD_{675} using a Pye Unicam PU 8600 UV/VIS spectrophotometer. At OD_{675} = 0.4–0.5, cultures were in mid-exponential phase and cells were harvested by centrifugation (15 000 × *g*, 4°C, 10 min), washed and resuspended to the required density in K_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.4).

Sand binding assay

Acid-washed fine sand (40–100 mesh, obtained from BDH) was rinsed with phosphate buffer and then distilled water and finally dried at 150°C overnight. This rinsing protocol prevented the possibility of colloid formation during the binding assay which could have caused interference with optical density measurements. The binding of *M. luteus* cells to sand was assayed by adding 40 ml of cell suspension to a 150-ml glass conical flask containing a set weight of sand (1–10 g). Replicates and a series of control flasks, lacking sand or cells, were set up. All flasks were incubated at 28°C on an orbital shaker (shaking speed = 180 rpm). At various time intervals the shaker was momentarily stopped and the contents of the flask were allowed to settle. Aliquots of liquid were removed from each flask for optical density determination at 675 nm. The samples were then returned to the original flasks. Binding assays were performed on several occasions using various cell suspension densities and different amounts of sand. The extent of cell binding to sand was calculated by the drop in OD_{675} of the test flasks compared to that of the controls containing cells but no sand. Given that the size distribution of the sand used was 40–100 mesh it is possible to calculate the percentage of the surface area of sand that is covered by bound bacteria, assuming that the bacteria are evenly distributed across the sand particles (Table 3).

Reversibility of binding

Supernatant from flasks containing sand which had been incubated, with shaking, in the presence of cells suspended in phosphate buffer for a fixed period of time was decanted off and the sand was

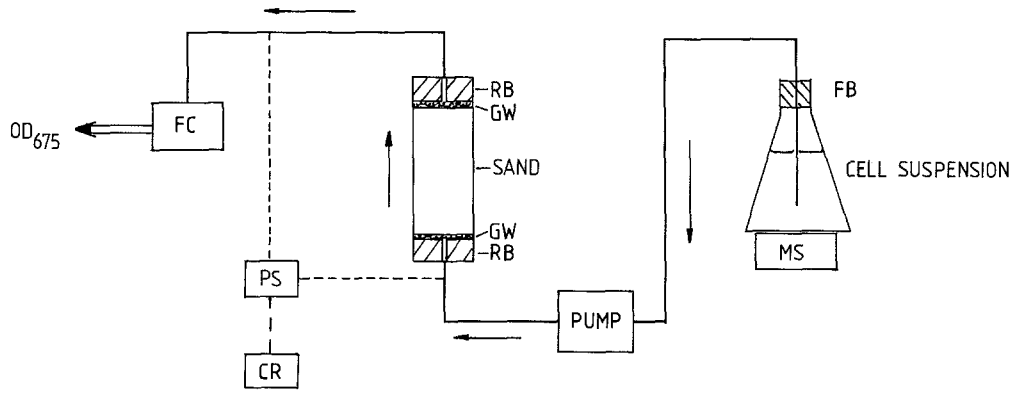

Fig. 1. Apparatus for investigation of bacterial transport through sandpacks. MS: magnetic stirrer; FB: plastic foam bung; PUMP: peristaltic pump; RB: rubber bung; GW: glass wool; SAND: 40–100 mesh; FC: fraction collector; PS and CR: optional differential pressure sensor and chart recorder. Sandpack is contained in glass tubing of 2.5 cm i.d., 13 cm long. Arrows indicate direction of flow of the cell suspension.

rinsed quickly with phosphate buffer to remove residual supernatant. 40 ml of buffer was then added to each of the flasks, which were then incubated with shaking (180 rpm, 28°C). The initial velocity of cell release from the sand was determined by the increase in OD_{675} over a period of 1 h.

Sandpacks

100 g quantities of acid-washed sand (40–100 mesh) were carefully packed into glass tubes (2.5 cm internal diameter) as shown in Fig. 1. Such sandpacks had a pore volume of 20 ml and a porosity of 35%. Sandpacks were flushed with several pore volumes of phosphate buffer prior to use. Cells suspended in phosphate buffer were pumped through sandpacks using a peristaltic pump (Fig. 1) at a flow rate of 1 ml · min^{-1} (3 pore volumes · h^{-1}). The effluent from the top of the sandpack (direction of flow being from bottom to top) was collected in 10-ml quantities by a Pharmacia FRAC-100 fraction collector. All experiments were performed at 28°C. The concentration of cells in the collected effluent samples was monitored by measuring the optical density at 675 nm. Sandpack runs were repeated on several occasions with cell suspensions of different densities. The input cell density was periodically checked by spectrophotometry to ensure that cell lysis was not occurring. Also, on one occasion, when the most concentrated cell suspension (OD_{675} = 4.0) was used, the viable count of the suspension was measured at the start and end of the experiment (t = 6 h). For samples with a high optical density (OD_{675} > 0.8) aliquots were diluted with phosphate buffer prior to the absorbance reading.

The permeability of representative sandpacks was determined by a falling head method assuming Darcian flow characteristics [2].

RESULTS AND DISCUSSION

The attachment of *M. luteus* cells to sand is time-dependent (Fig. 2). There is a rapid initial phase of binding followed by a second slower approach to an apparent plateau. Binding in these experiments is indicated by a decrease in the OD_{675} of the cell suspension. In the controls, where there was no sand present, there was little reduction in optical density, indicating that cells were not lysing, nor were they attaching in large quantities to the glass walls of the conical flasks. In a series of binding assays using cell suspensions with an OD_{675} of approximately 0.1 (equivalent to 3 × 10^7 cells · ml^{-1}, data not shown) and 1 g of sand per flask, an average decrease in optical density of 0.047 ± 0.006 (n = 8) was obtained. Thus only half the cells present in the suspension were attached to sand particles. One possible explanation of this observation is that cell attachment is reversible and that the plateau values obtained in binding assays represent an equilibrium position. Alternatively, it may be that the number of sites available for cell binding to the sand particles is limited and that saturation of such sites has occurred. Reversibility of attachment was demonstrated in a binding assay by replacing the cell suspension with phosphate buffer after incubation for 1 h (Fig. 3). Cells were released from the sand particles linearly on a time basis, with 22% detachment after 1 h. Since the conditions employed in the binding assays preclude the growth of bacteria during the course of the experiment, there is unlikely to be any synthesis of extracellular po-

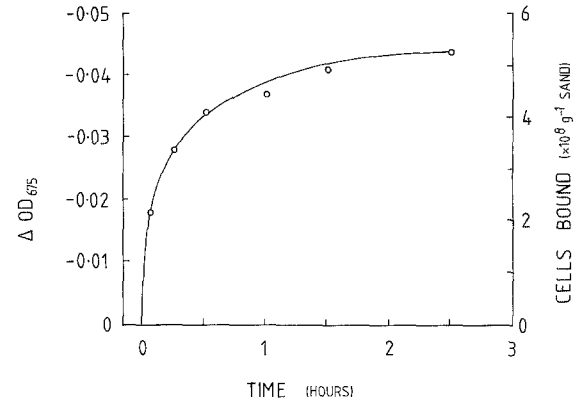

Fig. 2. Time course of attachment of *M. luteus* cells to sand. OD_{675} of cell suspension = 0.1 (3 × 10^7 cells · ml^{-1}); weight of sand per flask = 1 g. Extent of cell attachment to sand particles is indicated by the decrease in OD_{675}; the data presented are typical of those obtained in several binding assays using the same cell density. Shaking speed = 180 rpm.

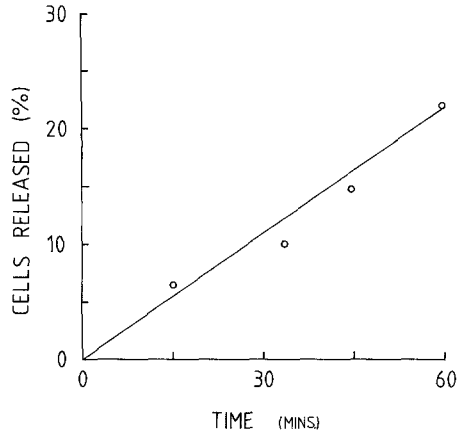

Fig. 3. Reversibility of cell binding. The release of cells from sand which had been previously shaken (180 rpm) in the presence of a cell suspension (OD_{675} = 0.4 (1.2 × 10^8 cells · ml^{-1}) for 1 h and then incubated, with shaking, in fresh phosphate buffer was determined by OD_{675} measurement. The data presented are the percentages of cells released from the sand compared to those originally bound and represent the means of three replicate flasks. Weight of sand per flask = 1 g.

lysaccharides which would facilitate irreversible adhesion and hence allow the growth of a biofilm [26]. It is known that the initial attachment of cells to surface is reversible [23,26] and that this can be

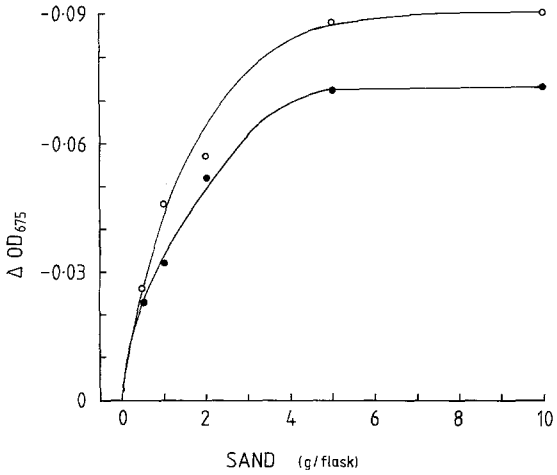

Fig. 4. Effect of increasing sand on the attachment of *M. luteus*. Binding assays using a cell suspension with an OD_{675} of 0.104 (3.1 × 10^7 cells · ml^{-1}) were performed with various quantities of sand (0.5–10 g). The extent of cell attachment is indicated by the decrease in OD_{675}. ●, t = 30 min; ○, t = 3 h.

mediated by shear forces. The shaking regime (180 rpm) used in these investigations is not excessive, so the implication is that the binding of the *M. luteus* cells to the sand particles is only moderate. The potential saturation of attachment sites was investigated by performing the binding assay with increasing amounts of sand but keeping the starting concentration of cells in suspension constant (Fig. 4). Using an initial cell suspension with OD_{675} = 0.104 (3.1 × 10^7 cells · ml^{-1}) there was an increase in the proportion of bacteria bound to sand particles as the amount of sand in the assay was increased from 0.5 g to 10 g. The increase in binding was not linear and tended towards a plateau so that the difference in cell binding in the presence of 5 and 10 g sand was minimal. In all instances the extent of binding followed a time course and reached a plateau after 2–3 h (data not shown). Approximately 90% of cells were bound in the presence of 10 g sand after 3 h of incubation. The data suggest that there may be a limitation in sites and that the plateau in binding observed (Fig. 2) may be due to saturation; however, if the reversibility of binding is considered (Fig. 3), the data obtained from increasing the amount of sand in the assay (Fig. 4) indicate that the equilibrium position is shifted in favour of attachment as an excess of sites becomes available. The effect of increasing cell concentration on attachment to sand was also investigated. Keeping the weight of sand constant (1 g) the cell density in the binding assay was increased up to 1.2 × 10^9 cells · ml^{-1}. The data obtained (Fig. 5) are complex. There appear to be three phases of attachment with increasing cell density. With cell suspensions with an OD_{675} of up to 0.5 (1.5 × 10^8 cells · ml^{-1}) there is a linear relationship between cells bound and cell density; this may represent a shift of equilibrium in favour of binding. However, as the OD_{675} of the cell suspension is increased to 2.0 (6.0 × 10^8 cells · ml^{-1}) the extent of binding is reduced to 40% of the maximum obtained with suspensions with OD_{675} = 0.5. Finally, with a further increase in cell density there is a trend towards increased binding. It might have been expected that as the cell density increases the extent of binding would plateau due to saturation of available sites. A reduction of bind-

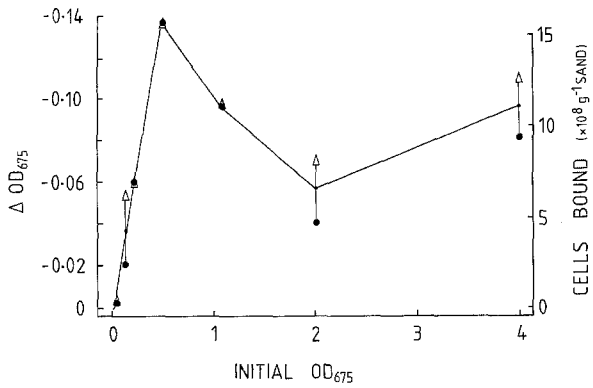

Fig. 5. Effect of increasing cell density on binding to sand. Binding assays with 1 g sand per flask were performed with different cell densities (up to OD_{675} g sand per flask were performed with different cell densities (up to OD_{675} = 4.0 (1.2 × 10^9 cells·ml^{-1})). The extent of cell attachment is indicated by the decrease in OD_{675}. The experiments were carried out on two separate occasions (shown as different symbols), each with replicates. The data presented are at t = 1 h.

ing indicates that there is an interference effect occurring, one possibility being that cells are binding to themselves in preference to sand (i.e., aggregation rather than attachment to sand) and another that in high concentration, cells tend to block the approach to the surface of the sand particles so that few come into close enough contact for stable attachment [23]. Neither of these would satisfactorily explain the increased binding associated with the third phase (Fig. 5). An alternative explanation is that within the suspension of *M. luteus* cells there are two subpopulations with differing affinities for binding to sand and that one blocks the attachment of the other. Evidence for two subpopulations with differing affinities was obtained by attempting a second binding assay using cells that had not attached in a first round of binding (Table 1). Only 25% attachment was obtained using these cells when compared to the original sample. It is known that the attachment of cells to surfaces depends on age and that in some instance cells in stationary phase do not attach [26]. Also, certain bacterial cells have different mechanisms for binding to different surfaces [13,17]. It is thus plausible that even in a culture harvested in exponential phase, as is the case in all the experiments described, not all cells will be identical because they are of different ages, and this could contribute to differences in binding affinity, perhaps due to changes in composition of the cell envelope. This aspect is being investigated further. In addition, although sand is a water-wett-

Table 1

Differential binding of *M. luteus* cells to sand

Treatments were as follows: A = original cell suspension; B = residual unbound cells from first round binding assay (A), harvested by centrifugation and resuspended in phosphate buffer to original cell density; and C = original cell suspension held at 28°C and used in binding assay at the same time as B. The weight of sand in the binding assay was 1 g/flask.

Treatment	OD_{675}[a] at start	Decrease in OD_{675} during binding assay (t = 1 h)
A	0.4	0.082 ± 0.002 (n = 6)
B	0.4	0.020 ± 0.001 (n = 3)
C	0.4	0.077 ± 0.005 (n = 3)

[a] OD_{675} = 0.4 is equivalent to 1.2 × 10^8 cells · ml^{-1}.

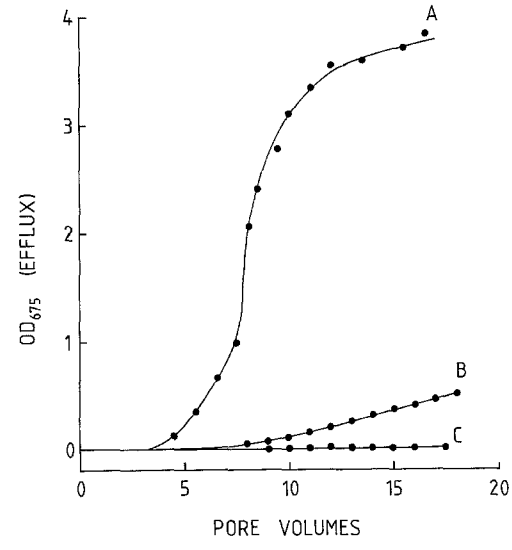

Fig. 6. Passage of *M. luteus* cells through sandpacks. OD_{675} of input cell suspension: A = 4.0, B = 2.0, C = 1.0. The cell density in the sandpack efflux is indicated by OD_{675} measurement. OD_{675} = 1.0 is equivalent to 3 × 10^8 cells · ml^{-1}. Experiments were repeated and gave identical results (for clarity, repeat data are not plotted). Weight of sand = 100 g per pack; flow rate = 3 pore volumes per h (1 ml · min^{-1}).

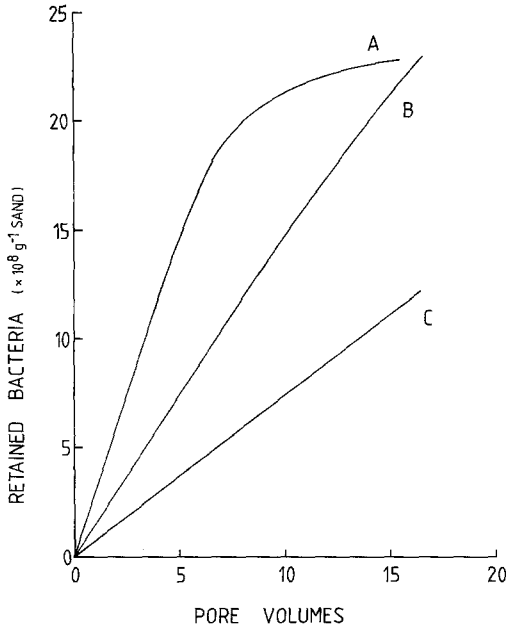

Fig. 7. Retention of bacterial cells in sandpacks. OD_{675} of input cell suspension: A = 4.0, B = 2.0, C = 1.0. OD_{675} = 1.0 is equivalent to 3×10^8 cells · ml^{-1}.

able material and is considered to have a net negative charge, it is possible that the surface of sand particles is not homogeneous and that there are domains with differing physicochemical characteristics that will differentially attract certain cell types.

The results of sandpack experiments are shown in Fig. 6. When cell suspensions with an OD_{675} of 1.0 (3×10^8 cells · ml^{-1}) were pumped through sandpacks there was very little release of cells in the effluent even after 18 pore volumes of suspension had been pumped through. However, as sandpack runs were repeated with cell suspensions of greater density there was release of cells as the available retention sites within the sandpacks became saturated. Only in the instances when cell suspensions with an OD_{675} of 4.0 (1.2×10^9 cells · ml^{-1}) were used did the cell density in the effluent eventually approach that of the inlet (when tested, it was found that *M. luteus* cells remained totally viable in suspension throughout the duration of sandpack runs). There is the possibility that some cells bound to the glass wool used in constructing the sandpacks. Under the conditions used in this investigation it is unlikely that such binding will be significant, especially since the amount of glass wool used was minimal. No obvious sign of extensive binding to the glass wool was seen. These sandpack data generally support the observations of Jenneman et al. [14] who found high retention of bacteria within sandstone cores when using relatively dilute input concentrations of cells. The plateau of cell output after the first pore volume of throughput as predicted by Jang et al. [10,11], on the basis of a deep filtration bed model, was not observed in these investigations. When the data obtained from the sandpack runs are transformed into cells retained (Fig. 7) it is apparent that an interference effect is occurring when the most concentrated cell suspensions are used. Although the initial rate of retention is linearly proportional to the input cell density (Table 2), there appears to be a trend to less overall retention of cells in instances when input suspensions with an OD_{675} of 4.0 are used. It is possible that this effect is related to that observed in the sand binding assays, although direct comparison is not

Table 2

Initial rate of retention of bacterial cells in sandpacks

OD_{675}[a] of input cell suspension	Initial rate of cell retention	
	$\times 10^6$ cells · g $sand^{-1}$ · min^{-1}	$\times 10^8$ cells · $pack^{-1}$ · $(pore\ volume)^{-1}$
4.0	15.0	3.0
2.0	7.4	1.5
1.0	3.8	0.8

[a] OD_{675} = 1.0 is equivalent to 3×10^8 cells · ml^{-1}.

Table 3

Attachment of *M. luteus* cells to sand particles

Size distribution of sand	40–100 mesh
Mid range	70 mesh
Average diameter (assuming spherical shape)	0.21 mm
Density (by displacement)	2.7 g · cm^{-3}
Particle surface area	1.4×10^{-7} m^2
Particle volume	4.9×10^{-12} m^3
Particle weight	1.3×10^{-5} g
No. of particles · g^{-1} sand	7.6×10^4
Total surface area · g^{-1} sand	1.0×10^{-2} m^2
	1.0×10^{10} μm^2
If approximately 2×10^9 bacteria bind per g sand and each bacterium has a diameter of 1 μm:	
Planar space occupied	2×10^9 μm^2
Available space occupied	20%

possible because sandpacks are an open system and the average residence time and shear effects will be different to those in the closed flask-based binding assay. With sandpack experiments using an input cell density of 1.2×10^9 cells · ml^{-1} ($OD_{675} = 4.0$) the retention of cells reaches approximately 2.3×10^{11} cells per sandpack (2.3×10^9 cells · g^{-1} sand). The maximum attachment of cells in the sand binding assay (Fig 5) was approximately 2×10^9 cells · g^{-1} sand; this represents a total coverage of 20% of the surface of sand particles by bacteria (Table 3). The binding of cells to sand and their retention within the sandpacks are of similar magnitude, suggesting that attachment to sand is probably, in this investigation, a major contribution to bacterial retention within the labyrinths of the porous sandpack structure. The average permeability of sandpacks constructed using 40–100 mesh sand was 17.0 ± 1.4 Darcies ($n = 3$), which is considerably higher than that of sandstone cores [10,11,14], thus some differences in the behaviour of bacteria in the model systems may be expected.

The data presented for both sand binding and sandpack experiments are complex and a full explanation of the issues raised may not be feasible at present. Attempts have been made to apply mathematical models used in the study of enzyme kinetics, ligand binding and enzyme inhibition to these data. It would appear that the data are too complex for a simple model to be applicable.

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