Starvation and penetration of bacteria in soils and rocks

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Summary. The soil and subsurface strata are low nutrient environments and their bacterial inhabitants must adopt starvation responses to survive. These responses include the formation of dormant, viable cells which, although reduced in cell size and volume, are able to respond to any improvement in nutrient availability. Starved bacteria are able to survive for extended periods without nutrients and their reduced size allows them to disperse deeply within rocks and soils greatly improving their penetration. These combined factors may increase opportunities for bacteria to reach a deep waste disposal site.

Key words. Bacterial starvation; ultramicrobacteria; bacterial survival; transport of bacteria.

Starvation responses of bacteria

Microbiologists first investigated bacterial growth using pure cultures and rich laboratory media. The growth pattern was classified into lag, exponential and stationary phases to be followed by cell death once the media was depleted. However, studies commencing in the 1920's began to report the survival and persistence of bacteria in the absence of carbon, energy or other essential growth nutrients[73]. During this starvation survival bacteria survive for indefinite periods and adopt specific starvation responses. These responses include that when growth ceased, due to nutrient depletion, a proportion of the bacterial population entered a dormant, viable, starved state and remained in that state until favourable growth conditions were restored (fig. 1).

The microflora of deep soils and rocks was not investigated until recently as it was considered that microorganisms could not survive below the top soil layers or within porous rocks. It is now more fully appreciated that subsurface environments contain a diverse microbial population[52]. Many natural environments, including soils and rocks, have poor or unevenly distributed areas of nutrients and are considered to be oligotrophic so that starvation is the normal state of their microinhabitants. In these environments growth nutrients are scarce, available for a limited period or non-existent. These microhabitats may differ in nutrient status to adjacent habitats. Therefore, some specific responses must occur that allow some bacteria to survive in such areas of differing nutrient status for more than 30 years[32].

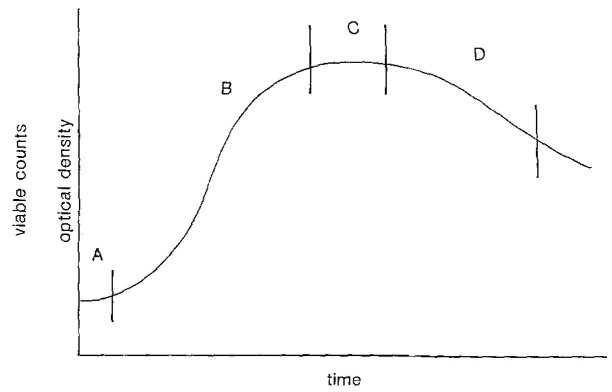

Figure 1. Growth and starvation survival of bacteria. The growth curve is broken into A, the lag phase, B, the exponential growth phase, and C, the stationary phase which occurs when all available growth nutrients have been utilized. During the fourth phase, D, the viable cell counts decline and the culture absorbance decreases. This is usually attributed to cell death. However, frequently the cells are not dead and can survive in a starved, dormant, viable state for an indefinite period.

There are three methods to examine the effects of low nutrient habitats on bacteria: 1) direct observation of the environment; 2) the incubation of environmental samples in the absence of all energy sources; and 3) the incubation of environmental samples in nutrient conditions from the particular environment, for example, sterile soil, filtered seawater or filtered river water. To date there have been few reports of the direct observation of bacteria in soils and rocks because of the difficulties involved in such operations[4,6,12]. Consequently it has been necessary to base descriptions of bacterial starvation responses from both habitats largely on laboratory studies. This review describes the basic starvation responses, focusing on those that influence bacterial persistence, penetration and dispersal through soils and rocks.

Physiological starvation responses

An understanding of how bacteria change in response to nutrient limitation will improve our knowledge of how they behave in nature[21]. Clark[13] estimated that 75 % of bacteria from some soils do not form spores and questioned how bacteria survived the low nutrient conditions. It was apparent from studies of the 1960's that bacteria could survive in low nutrient environments[32] and studies since then have concentrated on the mechanisms and adaptations of the starved cells. Harrison and Lawrence[22] considered that the bacteria in the portion of the population that survived starvation were different from those that died and classed them as mutants.

Bacteria survive starvation conditions by altering their cell physiology to produce a viable structure that is considered to be analogous to spore or cyst formation[74]. There are many terms for these starved forms and all focus on the reduction in cell size rather than other physiological changes to the cell, for example, ultramicrobac-

Summary of reported starvation responses with some examples

Starvation response	Environment/ Organism studied	Reference
1) Formation of periplasmic spaces	Soil	4
	Arthrobacter crystallopoites	11
	Marine isolate S14	45
	V. cholerae	25
	Marine *Vibrio* sp. ANT 300	54
	K. pneumoniae	38
2) Reduction in cell size	Soil	4
	Soil	6
	Marine bacteria	2
	Estuarine water	26
	Marine bacteria	27
	Estuarine water	43
	Seawater	70
	Ocean waters	67
	Marine *Vibrio* sp. ANT 300	54
	K. pneumoniae from oilwell waters	38
3) Protein degradation	Marine isolate 41	33
	E. coli K12 & *Salmonella typhimurium*	61
	Coryneform spp.	10
	A. aerogenes	58
	E. coli	63
	Brevibacterium linens	9
	V. cholerae	25
4) Reduction in endogenous respiration rate	Marine bacteria	27
	Coryneform spp.	10
	A. crystallopoites	19
	Marine *Vibrio* sp. ANT 300	55
5) Reduction in RNA levels	*B. linens*	9
	E. coli	63
	Peptococcus prevotii	48
	Corynebacterium spp.	10
	A. aerogenes	58
	Arthrobacter spp.	64
	Zymomonas spp.	16
6) DNA level stable or increased	*Arthrobacter* spp.	64
	Zymomonas spp.	16
	A. aerogenes	58
	B. linens	9
7) Reduction in microbial diversity in a habitat	Soil, subsurface sediments & aquifers	8
8) Changes in levels of carbohydrates	Marine isolate 95A	33
	Cellulomonas sp.	65
	V. cholerae	25
	K. pneumoniae from oilwell waters	44
	A. crystallopoites	11
9) Production of specific antigens	Gram-negative rod S14	1
10) Decrease in adenylate energy charge	*P. prevoti*	48
11) Decrease in ATP	*E. coli* K12	69
12) Changes in fatty acid profiles	*V. cholerae*	20
13) Changes in culture viability		Numerous reports

teria (UMB[70]), dwarf cells[27] or filterable bacteria[3,57]. The starved state is reversible and the bacteria resuscitate and return to the cell physiology prior to starvation once nutrients are restored[5,43,54,70].

Many of the starvation responses reported in the literature are summarized in the table and include a few examples of each response. Starvation survival responses are not confined to a few genera or only associated with bacteria isolated from a particular habitat. For example, reduction in cell size during starvation has been observed by bacteria obtained from environments as varied as soil, marine and estuarine waters (table). Frequently more than one response is observed, for example Hood et al.[25] reported that cell plasmolysis, protein degradation and changes in the levels of carbohydrate all occurred during starvation of *Vibrio cholerae* (table). Similar responses have also been reported during the starvation of anaerobes[16,48,60].

Some general observations of the physiological changes occurring during starvation include the utilization of non-essential intracellular materials to meet the energy requirements of the cell[15,17,56,64]. The reduction of the endogeneous metabolic rate by as much as a factor of 80 ensures that the degradation of cellular components occurs at a slow rate[19]. This uses the reserves more slowly and in turn increases the life of the cell[10]. Intracellular carbohydrate storage compounds are also utilized to help meet the energy requirements. Protein and RNA are partially, not totally degraded, suggesting that some residual protein and RNA are probably essential to the survival of the cell. DNA levels either remained at a constant level or increased during starvation. The cytoplasmic membrane, substrate transport and transport proteins may also play a role in increasing starvation survival[21,24,37,47,60].

A proportion of the population survives and remains viable during starvation. The four most common reported viability responses are summarized in figure 2[2,49,50]. They include that "(a) the cells increase in number and then decline to a constant number, (b) the cell number remains constant, (c) the cells increase in number and

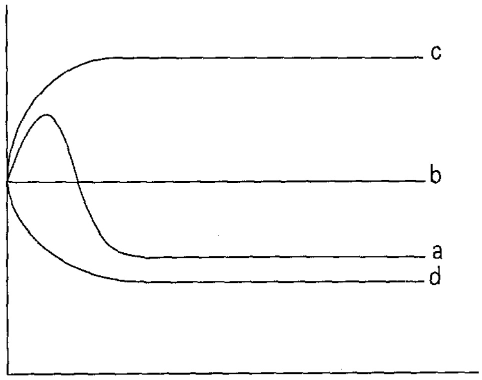

Figure 2. A summary of the four most common culture viability responses during starvation[2,49,50]. See text for details.

then remain constant, and (d) the cells increase in number to a constant level"[50]. Several studies have demonstrated that a proportion of the population is viable after more than a year without nutrients[26, 53, 55].

The possibility exists that cryptic growth may support the surviving bacterial population. Cryptic growth, first observed by Winslow and Falk[73] and defined by Ryan[62], involves the living proportion of a population utilising dead bacteria as a nutrient supply to enhance their survival. Such cannibalism was not considered to support starved bacteria by several investigators as no DNA or cell lysis products were detected in the starvation media[9, 10, 19]. Thomas and Batt[68] considered that cryptic growth did not account for the survival of *Streptococcus lactis* ML 3 during 20 h starvation as ML 3 had specific nutritional requirements which were unlikely to be met by cryptic growth. However, Postgate and Hunter[58] reported that cryptic growth occurred to support the survival of *Aerobacter aerogenes* after 60–70 h starvation.

All of the above responses produce a dormant, viable, starved form which permit the bacterial cell to survive for long periods of time in soils and rocks. Several of the starvation responses not only increase survival but also greatly influence the penetration and dispersal of bacteria in these habitats and these will now be considered in detail.

Changes in cell morphology and adhesion

At least two starvation responses are considered to affect the penetration and dispersal of bacteria through porous matrices such as rocks and soils[39]. These are, the reduction in cell size and the change in adhesion abilities of starved cells. The reduction in cell size to form UMB and the change in cell shape from rods to cocci are widespread responses exhibited by bacteria from a range of environments[4, 27, 38, 67]. However, unlike the degradation of cellular components described above, no explanations of why the starved bacteria forms UMB are currently available. The cell contents shrink away from the cell wall leaving distinct periplasmic spaces and electron dense cytoplasmic masses to form a cell much reduced in size when compared to a vegetative cell. Reduction in cell size from over 2.0 µm in length down to 0.5 µm or less have been observed during laboratory starvation procedures[2, 38, 54]. Bacteria isolated from low nutrient environments are similarly reduced in size compared with laboratory grown cultures[26, 43, 67, 70]. However, not all starved bacteria produce UMB. Boylen and Pate[11] reported no change in cell morphology after an 8-week starvation regime of *Arthrobacter crystallopoites* and Boylen and Mulks[10] did not observe any change in cell dimension during starvation of Coryneform bacteria.

Data from our laboratory differed from those of others with respect to changes in cell adhesion and production of exopolysaccharides during starvation[18, 34–36, 39, 44, 46]. Marshall[18, 34] and Kjelleberg[35, 36] demonstrated that bacteria, isolated from high nutrient environments, increased polymer production[18] and increased adhesion rates during short term starvation[39]. During incubation in the presence of a carbon source *Vibrio* DW1 did not adhere, however increased adhesion rates were reported in the absence of a carbon source[18]. Similarly, increased adhesion occurred when Leptospires were starved[34]. Production of cell surface lipopolysaccharide-containing materials increased during bacterial starvation[18, 46]. Our work with *Klebsiella pneumoniae* isolated from low nutrient oilwell waters demonstrated that a reduction in extracellular polysaccharide production occurred during starvation[44]. The exopolysaccharide production decreased from 53.3 µg/ml to 4.3 µg/ml during 9 weeks starvation and fewer fimbriae were observed around the cell wall[44]. A comparison of starved and growing cells demonstrated the presence of extracellular slimes that were present on the latter and absent on the former[39]. The adhesive qualities of growing cells and their absence in starved cells assists the former to adhere to rock and soil particles more readily than starving cells.

Penetration of bacteria through rocks and soils

Studies of starvation survival of bacteria have largely been confined to aqueous and soil environments whereas studies of the penetration, dispersal and transport of bacteria have been mostly confined to that within rocks. The latter have been undertaken for the oil industry as such data are critical in evaluating the movement of both beneficial and deleterious bacteria within the underground strata. Therefore, much of the data of direct relevance to bacterial penetration and survival in rocks is located in oil-related literature.

Both rocks and soil can be regarded as porous matrices supporting diverse microflora and permitting bacterial transport via a network of fluid channels. The following discussion will be based on bacterial penetration of rocks as little such literature is available on soils. However, the broad principles can also be applied to soils.

Bacteria within rocks

Rock is not solid but contains many openings or pores. Such rock is termed porous or is said to have porosity. The greater the porosity of the rock the larger the fluid volume it can hold within its pores. Rock porosity varies from below 5 % in tightly cemented sandstone to over 30 % in unconsolidated sands. The rock pores often interconnect to form channels. Such channels allow underground waters to flow through the rock and give it permeability, that is fluid movement within the rock. Bacteria living in such underground waters can thus be transported through the rock via these channels. The environment is low in nutrients but the channelling via rock pores may bring fresh nutrients from soil or an above ground water source.

The ability of bacteria to travel via the pore spaces and penetrate through rock was first reported by Myers and McCready[51]. The authors demonstrated that injected radiolabelled *Serratia marcescens* ATCC 274 could pass through 36.2 cm cores of Berea sandstone and 7.6 cm cores of limestone. Prior to this study the presence of microorganisms in ancient rocks had been attributed to prehistoric invasion and not bacterial penetration[40–42]. Indeed, Lipman[41] believed that the microorganisms must have been trapped within rocks and coal as he considered that the pore spaces were too small to allow bacterial transport. Since these early studies many investigations of the subsurface microflora have been undertaken. Representatives of most major groups of microorganisms have been isolated either from rocks or from water collected from rocks[52]. Beloin et al.[7] confirmed the presence of microorganisms in rocks and deep soils but noted that the biomass and microbial diversity decreased with increasing depth. This investigation also linked microbial activity to sediment type, that is, sandy soil supported higher microbial activity whereas clay type sediment supported lower activity.

Factors affecting transport of bacteria through porous matrices

Factors affecting the transport of bacteria through rocks include the distribution of the pores and the pore throat sizes[29, 31, 71, 72], permeability[30, 31], the physical and chemical properties of rock[7], bacterial size[39, 44], shape[39, 44], bacterial adhesion to rocks and soil[28, 66], gas production by bacteria[31], cell charge and cell motility[31]. The narrowest dimension within the pore space, termed the pore throat size (fig. 3), is an important parameter in terms of bacterial penetration through rocks[31, 71, 72]. The throat must be of a certain size in relation to the size of the bacteria to allow penetration. For example, if the throats are of a comparable size to the bacteria the throats are 'jammed' with bacteria and this prevents bacterial penetration[29]. Other researchers considered that the pore throat size must be at least double the size of the cells for them to pass through[71, 72].

Rock is not homologous in structure and has areas of differing permeability. The water channels flow preferentially through the areas of least resistance, that is through the higher permeability zones[14]. The permeability of rock therefore affects the dispersal of bacteria and transport in rocks as they are carried preferentially to certain zones. The injection of live bacteria into rock cores is usually accompanied by a decrease in permeability of the rock[30] attributed to bacterial growth. Jenneman et al.[31] were able to calculate the rate of bacterial penetration of motile strains of *Bacillus* and *Enterobacter aerogenes* and a nonmotile strain of *K. pneumoniae* through nutrient saturated sandstone cores. The rates were reported to be independent of permeability where the rocks were above 100 millidarcys, however below this permeability penetration was limited.

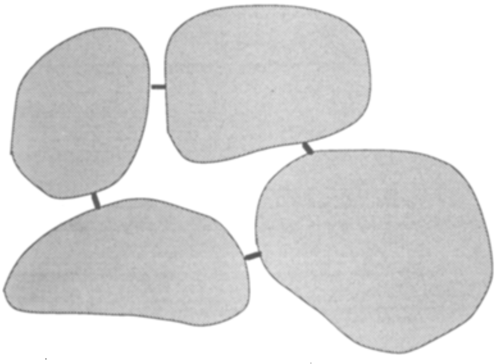

Figure 3. Particles of rock showing the pore throat sizes (black bridging lines) through which bacteria must penetrate.

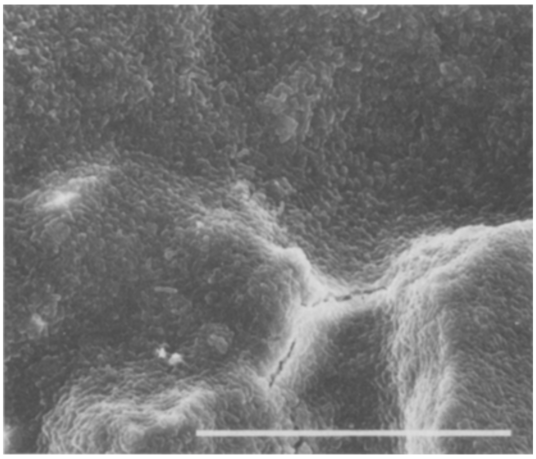

Figure 4. Scanning electron micrograph showing confluent bacterial growth at the inlet of a sandstone core. The growth has completely sealed the core and prevented bacteria from penetrating deeper into the rock. The scale bar represents 50 μm.

Some of the early studies on the transport of bacteria injected dead bacteria into rocks[23, 59]. In such instances bacteria were treated as inert particles and have been reported to cause a jamming effect at the inlet of rock cores when injected at high concentrations[23]. However, such studies are now considered to be misleading because it is known that live bacteria do not act like inert particles[28]. Live bacteria are more damaging in terms of blocking up pore spaces by their production of exopolysaccharides and adhesion to rocks. Work in our laboratories demonstrated that bacteria grow and completely block up model rock cores by a combination of exopolysaccharide production, bacterial adhesion and cell biomass[66]. This effectively halts bacterial penetration (fig. 4).

Many of the factors affecting penetration have been studied by Mike J. McInerney and his colleagues at the University of Oklahoma. In particular, this research group has studied the effects of cell motility, gas production and chemostaxis on penetration[31] (and Reynolds et al., personal communication). Jenneman et al.[31] considered that bacterial gas production and motility could assist transport and increase the penetration rate through rocks. Reynolds et al. (pers. commun.) have shown that motile strains of *Escherichia coli* penetrated faster through sandpacked cores than nonmotile mutants. Gas production improved the penetration abilities of the nonmotile mutants. However, chemotaxis was not considered to be involved in penetration.

UMB formation by isolates from water within rocks has been shown to be a universal response [38, 39] (and Lappin-Scott, Cusack and Costerton, unpublished observation). The combined effects of a reduction in cell size, change in cell shape and less exopolysaccharide production all permitted starved bacteria to penetrate and disperse throughout rock cores [39]. However, full-size vegetative cells were not able to penetrate, instead they blocked the core inlets (fig. 5) [39, 44]. As bacteria in soils and rocks probably exist predominantly as starved cells they may penetrate further than vegetative cells.

In conclusion, bacteria can survive in low nutrient environments for much longer than is generally appreciated. Changes in cell size, shape and exopolysaccharide production during such starvation increases the ability of the starved cells to penetrate and disperse deeply throughout such environments. This combination of increased survival and penetration abilities may allow the bacteria to reach deep disposal sites.

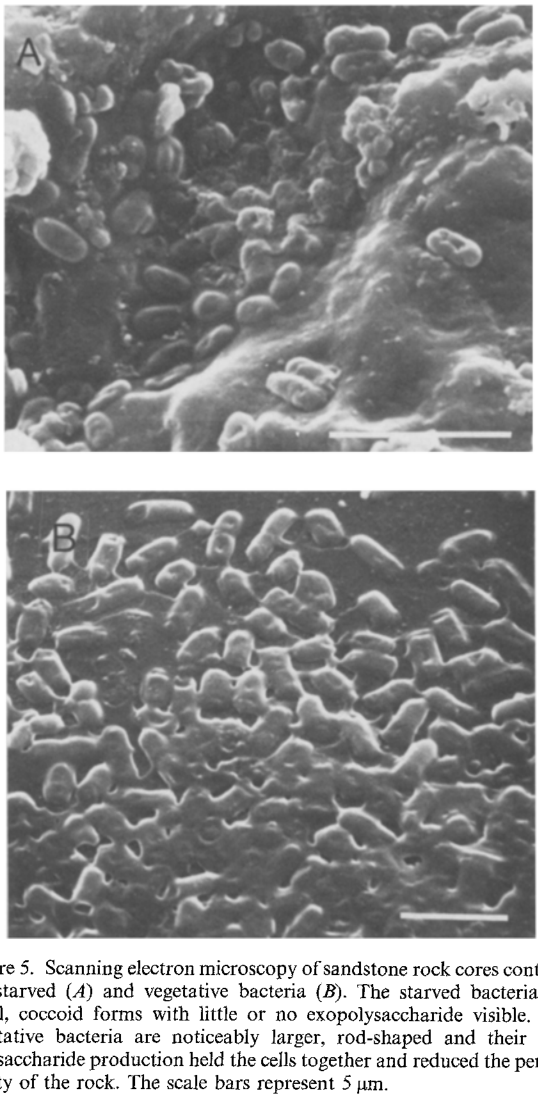

Figure 5. Scanning electron microscopy of sandstone rock cores containing starved (*A*) and vegetative bacteria (*B*). The starved bacteria are small, coccoid forms with little or no exopolysaccharide visible. The vegetative bacteria are noticeably larger, rod-shaped and their exopolysaccharide production held the cells together and reduced the permeability of the rock. The scale bars represent 5 μm.

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