

45. THE POTENTIAL FOR BACTERIA GROWTH IN DEEP SEDIMENT LAYERS OF THE JAPAN SEA, HOLE 798B — LEG 128¹

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ABSTRACT

Sediment whole-round cores from between 0.15 and 425 meters below seafloor (mbsf) were obtained, using special techniques to prevent external contamination and maintain anaerobic conditions, to determine the potential for bacterial growth. Samples were taken from the centers of whole-round cores and handled under strict aseptic conditions to prevent contamination and then incubated anaerobically with 1 g/dm³ yeast extract at 15° C. Bacteria were present in all samples prior to incubation and their numbers decreased logarithmically with depth from 9.3×10^8 /g at 0.15 mbsf to 1.7×10^7 /g at 425 mbsf. Direct evidence of *in-situ* growth, as shown by dividing cells, was found in the upper sediments but not below 10 mbsf.

Growth was stimulated in all samples except at 9.75 mbsf. Numbers of bacteria increased with time, showing typical growth curves. The frequency of dividing and divided cells (FDDC) increased, and FDDC values were maximal in the midlogarithmic growth phase, as were bacterial productivity estimates calculated from ³H-thymidine incorporation. The absence of growth at 9.75 mbsf may indicate that these bacteria were changing to dormant forms and were under considerable metabolic stress, resulting in substrate accelerated death when a carbon source was added.

Bacteria in deep sediments, below 9.75 mbsf, appeared to be largely dormant but were able to grow quickly (within 12 to 96 hr) when incubated with a growth substrate. Extrapolation of the logarithmic growth phase to zero time gave estimates of bacterial viabilities between 0.02% and 35%. These were probably unrealistically high, and considerably higher than those found by other workers using selective media. There was no evidence for contamination of these deep sediment layers by the more active surface sediments during coring. The bacterial distributions and their subsequent growth were consistent with a more comprehensive study of bacterial populations and activity at the same site by Cragg and others. Together, these data demonstrate that bacterial populations can remain viable for very long periods of time, presumably by slowly metabolizing increasingly recalcitrant organic compounds, but can rapidly grow when a suitable growth substrate is supplied.

INTRODUCTION

Bacteria are intimately involved in many of the global geochemical cycles (Jørgensen 1983). Geochemical data such as chemical changes in pore water, gas production, isotopic evidence, and the modification of organic complexes such as kerogen (Krumbein, 1983) all indicate that bacterial diagenetic processes continue to considerable depths within sediments. This is supported by the recent direct observation of significant bacterial populations within sediments down to 80 mbsf in the Peru margin, with corresponding profiles of viable bacteria and rates of bacterial activity (Cragg et al., 1990; Parkes et al., 1990). Similar profiles, but to greater depths, have also been found in sediments from the Japan Sea (Cragg et al., this vol.). These data are consistent with, and considerably extend other, more limited data on the presence and activity of bacteria in deep sediment layers (Oremland et al., 1982; Belyaev and Ivanov, 1983; Bianchi, 1986).

Extensive investigations of deep aquifers have also demonstrated the presence of viable bacteria and low levels of bacterial activity, which suggests that the presence of bacteria far below the surface is a common situation (Erlich and Ghiorso, 1989; Balkwill et al., 1989; Chapelle and Lovley, 1990; Fredrickson et al., 1991). The presence of intact bacteria in sediment layers in excess of 1 m.y. old (Cragg et al., 1990) raises important questions concerning the metabolic status of these bacteria. Although previous work (Cragg et al., 1990) has shown that a portion of the bacterial population in deep sediments could be cultured, this was in rich, selective media with long incubation times, and the resulting populations represented a very small percentage of the total bacterial population. Additional information

could be obtained regarding the metabolic status of *in-situ* populations if growth could be stimulated under more realistic conditions, such as a nonselective medium, coupled with short incubation times. Such experiments were conducted with sediments from the Japan Sea (Leg 128, Hole 798B) at five depth intervals as part of a comprehensive study of the distribution and activity of bacteria within these sediments (Cragg et al., this volume).

The aim of these experiments was to address the following questions: are the majority of bacteria within deep sediment layers alive or dead, actively growing or dormant, can they respond to added substrate and grow and if so, how fast? Such knowledge is vital to determining the role of bacteria in deep sediments and their contribution to the continual diagenesis of organic matter and subsequent oil and fossil formation.

MATERIALS AND METHODS

Sample Handling

Full details of the shipboard sample handling and initial laboratory procedures are described in Cragg et al., (this vol.). In the laboratory one 5-cm³ subcore was taken using a sterile 5-mL syringe, from which the luer fitting had been removed, from five whole-round cores (Table 1). Sampling was from near the center of the core to avoid possible contamination from the inside walls of the core liner (Cragg et al., this vol.) and performed under a stream of sterile (0.2 μm gas filter) oxygen-free nitrogen (OFN). The syringe was sealed with a sterile butyl rubber "Suba-Seal" (Wm. Freeman and Co., Barnsley, U.K.) and placed in an anaerobic jar for immediate transfer to an anaerobic cabinet (Forma Scientific, Ohio, U.S.A.). A 1-cm³ sample was inoculated into a crimp-top serum vial containing 99 mL of sterile Widdels marine medium modified as a diluent for viable heterotrophs (Cragg et al., 1990) and enriched with 1 g/dm³ yeast extract. The vials were sealed, removed from the anaerobic cabinet, and incubated at 15° C (mean down-hole temperature), in an orbital incubator at 200 RPM for up to 10 days.

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Table 1. Sample designation and sediment depth (mbsf).

Sample	Designation	Depth (mbsf)
	127-798B-	
1	1H-1, 0.15-0.20	0.15-0.20
2	1H-1, 1.15-1.20	1.15-1.20
3	2H-1, 0.35-0.40	9.75-9.80
4	9H-3, 0.95-1.0	78.85-78.90
5	45X-3, 0.50-0.55	425.00-425.05

Direct Counts and Frequencies of Dividing and Divided Cells

Slurry samples (0.5 mL) were aseptically removed from the serum vials at time zero, to give counts from unincubated samples, and thereafter at 12-hourly intervals, using a sterile, preflushed (OFN) syringe and 19G hypodermic needle. These were fixed in filter sterilized (0.2 μm) 2% formaldehyde made up in deionized water. Direct counts of acridine orange stained bacteria were performed using epifluorescence microscopy (Fry, 1990), ensuring that no more than 70% of the field of view was covered by sediment particles (Getliff and Fry, 1990). The number of bacteria recorded on sediment particles were doubled to account for hidden bacteria (Goulder, 1977) and at least 400 cells (Fry, 1990), or 100 fields of view counted. Where possible a minimum of 30 dividing and divided bacteria were also counted. Dividing cells were classified as bacteria showing a clear invagination, and recently divided bacteria as closely adjacent but identical cells. The latter are in the final stages of division and probably did not have separate cell walls (Fry, 1990). Dividing bacteria were counted as one cell in both the total count and number of divided cells, and divided cells were counted as two cells in both categories. The FDDC was calculated as follows:

$$\text{FDDC} = \frac{\text{dividing} + \text{divided cells}}{\text{total count}} \times 100$$

Cell volumes of bacteria were measured using TV image analysis (Getliff and Fry, 1989, 1990). A conversion factor of 308 fg carbon/ μm^3 was used to estimate biomass (Fry, 1988). All direct count and biomass data are expressed per gram of dry sediment.

Incorporation of ^3H -Thymidine

Incorporation of radiolabeled thymidine (^3H -methyl thymidine, specific activity 80 Ci/mmol; Amersham International, Amersham, U.K.) into bacterial DNA was used to estimate rates of DNA replication and calculate rates of bacterial growth and productivity (Fuhrman and Azam, 1980; Moriarty, 1986; Moriarty, 1990). Radiotracer preparation was as described by Cragg et al. (this volume). The initial, time zero, samples (2 mL aliquots in triplicate) were taken immediately after the slurries were prepared using sterile 2 mL syringes preflushed with OFN. Five further samples were taken at time intervals between 12 and 96 hr (Table 2) depending on the anticipated metabolic activity of the bacteria. Each subsample was incubated with 5 μCi of ^3H -thymidine, injected into the slurry through the luer fitting of the syringe and the syringe was then sealed with a hypodermic needle pushed into a rubber bung. The syringe contents were mixed by gentle hand agitation and each subsample incubated at room temperature with occasional mixing for different times (Table 2) to confirm the linear uptake of thymidine (Fig. 1; Moriarty, 1986). As bacterial growth was to be stimulated it was anticipated that relatively short incubation periods would be required (Getliff, 1990); consequently incubation times of between 1 and 15 min were used. Actual incubation times were varied, those for the potentially least-active layers

Table 2. Sampling times for bacterial productivity estimates and length of incubation with ^3H -thymidine.

Sample depth (mbsf)	Sampling times, hours (^3H -thymidine incubation times, min)						
0.15	0 (0.1,3)	12 (0.1,3)	24 (0.1,3)	48 (0.1,3)	84 (0.1,3)	120 (0.1,3)	168 (0.1,3)
1.15	0 (0.2,6)	24 (0.2,6)	36 (0.2,6)	60 (0.2,6)	96 (0.2,6)	132 (0.2,6)	192 (0.2,6)
9.75	0 (0.3,9)	48 (0.3,9)	60 (0.2,6)	84 (0.2,6)	120 (0.2,6)	168 (0.2,6)	192 (0.2,6)
78.85	0 (0.4,12)	72 (0.4,12)	84 (0.3,9)	108 (0.2,6)	144 (0.2,6)	180 (0.2,6)	192 (0.2,6)
425.0	0 (0.5,15)	96 (0.4,12)	108 (0.4,12)	132 (0.3,9)	168 (0.2,6)	192 (0.2,6)	192 (0.2,6)

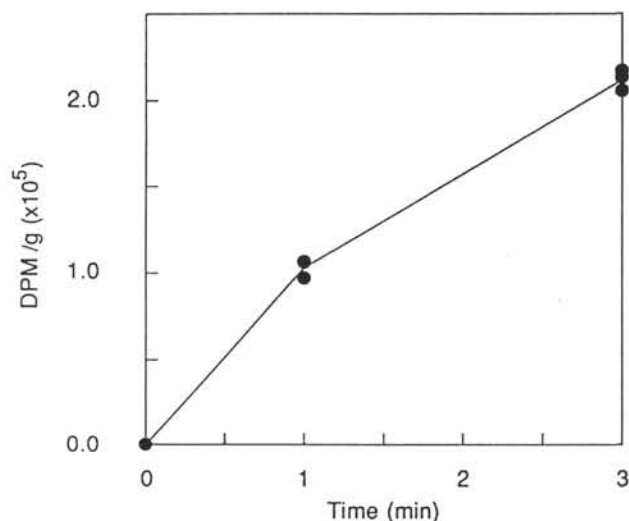


Figure 1. Sediment samples from 0.15 mbsf after 48 hr incubation with 1 g/dm^3 yeast extract; ^3H -thymidine incorporation into the DNA fraction at 1 and 3 min incubation with ^3H -thymidine after subtraction of the time zero control.

were initially long and then decreased toward the end of the experiment when the bacteria were growing rapidly (Table 2). Incubations were terminated by expelling the syringe contents into 5 mL of 10% trichloroacetic acid (TCA) at 4°C. Time zero controls were included to allow for nonspecific adsorption of the radioisotope. Labeled DNA was extracted using a protocol based on the methods of Karl (1982), Craven and Karl (1984), and Carmen et al., (1988) and fully described in Cragg et al., (this vol.). The aqueous labeled DNA fraction was counted in 10 mL of "Instagel" scintillation fluid (Canberra Packard, Caversham, U.K.) using an LKB Wallac 1219 liquid scintillation counter (Pharmacia LKB, Milton Keynes, U.K.). Bacterial productivity ($\mu\text{gC}/\text{g}\cdot\text{h}$) was calculated from the rate of thymidine incorporation obtained from the middle of the three incubation times (after subtraction of the time zero values) combined with the bacterial biomass and a conversion factor of 2.0×10^{18} bacteria produced per mole of thymidine incorporated into DNA (Moriarty, 1986).

RESULTS

Direct Counts

Bacteria were present in all samples of unincubated sediment (Fig. 2A) and showed a highly significant ($p < 0.001$) logarithmic decrease from $9.3 \times 10^8/\text{g}$ at 0.15 mbsf to $1.7 \times 10^7/\text{g}$ at 425 mbsf. This distribution is similar to that found by Cragg et al. (1990), and Parkes et al. (1990) for deep sediments from the Peru Margin and in samples from the Japan Sea (Cragg et al., this volume). Dividing cells and filamentous bacteria were only detected down to 9.75 mbsf, and hence their distribution was similar to the total AODC, showing decreasing populations with increasing sediment depth (Fig. 2).

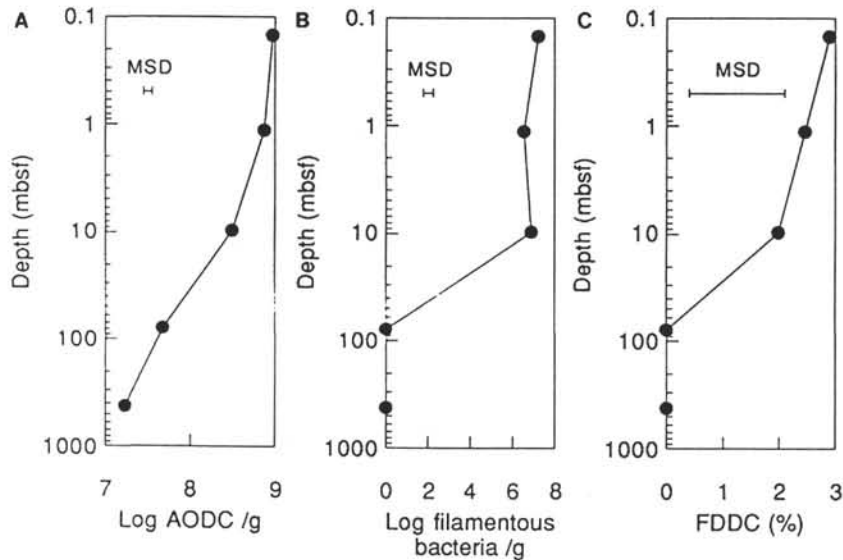


Figure 2. Depth distribution of (A) total numbers of bacteria (AODC), (B) numbers of filamentous bacteria, and (C) frequency of dividing and divided cells (FDDC) from Hole 798B. MSD = minimum significant difference (Sokal and Rohlf, 1981).

Growth Experiments

Bacteria in slurries from all depths, except 9.75 mbsf grew to similar population sizes ($2.12 \times 10^9 - 3.73 \times 10^9/g$) and had in general clear lag, logarithmic, and stationary growth phases (Fig. 3A). Neither growth rate nor the length of the lag phase varied consistently with depth (Table 3). Extrapolation of the logarithmic growth phase to time zero gave an estimate of the number of culturable bacteria in the sediment inoculum (Alexander and Mikulski, 1961), and hence an estimate of bacterial viability. These decreased with depth and ranged from 35% to 0.02% in the shallowest and deepest sediment layers respectively (Table 3). Filamentous bacteria were culturable in slurries from samples at 0.15 and 1.15 mbsf (Fig. 3B), with the deeper sample exhibiting a slower growth rate. In contrast, at 9.75 mbsf numbers of filamentous bacteria actually decreased during incubation (Fig. 3B). The FDDC increased during incubation (Fig. 3C), generally reaching a maximum during the mid-logarithmic growth phase (Figs. 3A and 3C) and then decreased during stationary phase. Maximum FDDC values were similarly high in the slurries from 1.15, 78.85, and 425 mbsf (Table 3, average 14.8%).

³H-Thymidine Incorporation

Except for samples from 9.75 mbsf, thymidine incorporation was not detectable at the start of the experiment and it was not possible to estimate initial bacterial productivities (an approximation of *in-situ* bacterial productivity). ³H-thymidine incorporation did occur however, when the bacteria were grown with added yeast extract (Fig. 3D). Bacterial growth in slurries from 0.15, 1.15, and 425 mbsf reached a maximum productivity in the late-log or early-stationary growth phase (Figs. 3A and 3D) that generally corresponded with the peaks in FDDC (Fig. 3C). Maximum productivity at 1.15 mbsf ($464 \mu\text{gC/g}\cdot\text{h}$) was higher than that for other samples ($15.9-22.4 \mu\text{gC/g}\cdot\text{h}$; Table 3). This difference may be due to the growth of large filamentous organisms in this sample. Maximum productivity for the bacterial population from 78.85 mbsf occurred much later than both the end of lag phase and the peak in dividing cells. All of the growth parameters for the 9.75 mbsf sample decreased or remained constant (Figs. 3A and 3D).

DISCUSSION

The bacterial depth profile observed here strongly correlates ($p < 0.01$) with the distributions found by Cragg et al. (this volume) in a more comprehensive set of samples from the Japan Sea. Their data clearly demonstrate the presence of bacteria to below 500 mbsf. The bacterial profile also correlates ($p < 0.05$) with the depth distribution of bacteria in samples from the Peru Margin down to 80 mbsf (Cragg et al., 1990; Parkes et al., 1990). The lowest numbers of bacteria ($1.7 \times 10^7/g$) were at 425 mbsf, approximately 2% of the population at 0.15 mbsf ($9.3 \times 10^8/g$), and as such constitute a substantial bacterial population. As the presence of microorganisms in pristine deep subsurface environments is still controversial (Sinclair and Ghorse, 1989), the possibility that these bacteria are contaminants must be considered. The most likely sources of contamination are mixing or smearing of surface sediment with high bacterial populations along the inside of the core tube and hence into deeper sediments, or contamination of the core during drilling. Piston coring was used to take samples ahead of the drill bit, and contamination from the core sides was prevented by taking samples only from the center of whole-round cores. In addition, direct counts across a similar core showed no indication of contamination, even near the sides of the core (unpublished data). Cragg et al. (this volume) discuss fully the potential contamination problems with regard to their more comprehensive data on bacterial distributions obtained for the same core, and they conclude that there was no evidence of contamination of the deeper sediments from the more active surface layers.

The absence of contamination is more crucial for the growth experiments, as even a few opportunistic bacteria introduced during sampling and subsequent handling could quickly outgrow the natural population. These experiments were conducted under identical conditions in a rich medium, and hence would be expected to select for contaminants with similar growth characteristics and cell morphologies. This would result in very similar growth curves; however, each of these were quite distinct (Fig. 3A). Although differences in growth curves could be generated by different amounts of contamination, this would not account for the observed differences in maximum specific growth rate or cell volume (Table 3). Such characteristics would

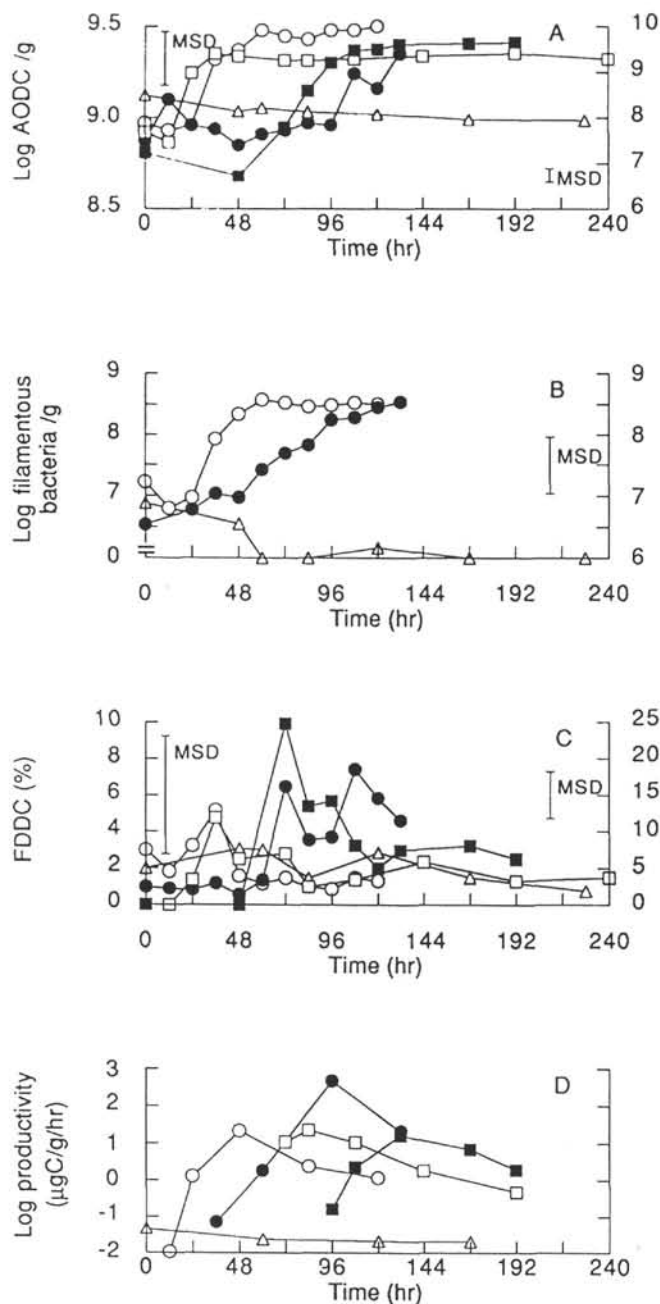


Figure 3. Growth of bacteria vs. time in slurries from five different sediment depths in Hole 798B: 0.15 mbsf (○), 1.15 mbsf (●), 9.75 mbsf (△), 78.85 mbsf (□), and 425 mbsf (■). (A) AODC; samples from 0.15 and 1.15 mbsf on the 8.5–9.5 bacteria/g scale, samples from 9.75, 78.85, and 425 mbsf on the 6–10 bacteria/g scale. (B) filamentous bacteria. (C) FDDC; samples from 0.15 and 9.75 mbsf on the 0%–10% scale and samples from 1.15, 78.85, and 425 mbsf on the 0%–25% scale. (D) productivity. MSD = minimum significant difference (Sokal and Rohlf, 1981).

require contamination by different types of bacteria, which seems very unlikely considering the rigorous aseptic handling conditions used throughout the experiment.

Principal-component analysis of the characteristics of the bacterial populations (Table 3 and Fig. 4), established two principal axes that explained 98% of the data variation, with all the enrichments well separated (Fig. 4), further demonstrating that the growth curves were characteristically different. These considerations, together with the ab-

Table 3. Principal features of five bacterial growth experiments (Figs. 3A–3D).

	0.15 mbsf	1.15 mbsf	9.75 mbsf	78.85 mbsf	425.0 mbsf
Length of log phase (h)	12	36	a	24	48
Maximum specific growth rate (h ⁻¹)	0.087	0.052	-0.007	0.24	0.15
Culturable cells ^b (%)	34.7	20.1	a	3.7	0.02
Maximum FDDC (%)	5.2	8.5	3.0	11.8	14.2
Maximum productivity (µgC/g · h) ^c	21.4	464.0	0.05	22.4	15.9
Time of maximum productivity (h)	48	96	a	84	132
Length of lag phase (h)	24	96	a	12	48
Change in biomass ^d (µgC/g)	1.44	1.33	-0.52	2.19	3.01
Mean AODC during stationary phase × 10 ⁹ (/g)	2.96	1.78	a	2.12	3.73
Maximum cell volume (µm ³) during log phase	1.98	5.67	0.34	0.49	0.47

^aNo result as no growth occurred.

^bPercentage culturable cells estimated from the initial total count and extrapolation of logarithmic growth phase to zero time (Alexander and Mikulski, 1961).

^c³H-thymidine incorporation.

^dChange in biomass = mean biomass during stationary phase minus mean biomass during lag phase.

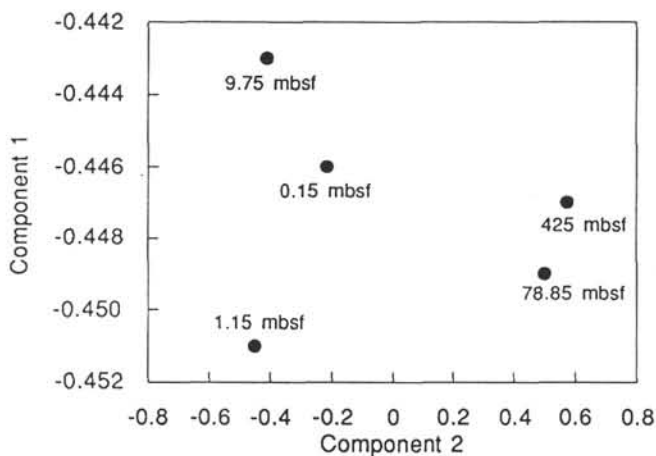


Figure 4. Principal-component analysis (explaining 98% of the variability) of the main features of five growth curves (Table 3) based on six independent growth parameters from Table 3.

sence of growth at 9.75 mbsf (Fig. 3), provide clear evidence that the bacterial populations that developed in the enrichments were indeed from the original sediment and not due to external contamination.

The close relationship between log growth phase and peak FDDC together with that of stationary phase and low FDDC (Figs. 3A and 3C) in incubated samples clearly demonstrates that within these slurries FDDC can be used as an index of growth, although existing algorithms cannot be used to predict actual bacterial growth rates in these samples (Newell and Fallon, 1982; Getliff, 1990). Hence dividing cells in the top three samples indicate the presence of actively

growing bacteria in the shallower sediment layers. FDDC values in the top sediment sample (2.9% at 0.15 mbsf; Fig. 2C) were similar to those in sediments from the Peru Margins (4.9% at 1.5 mbsf; Cragg et al., 1990). It is unlikely that as sediment depth increased, bacterial growth stopped in mid-division and dividing cells merely survived with increasing depth (Fig. 2), and there is no reason why dividing cells would be preserved any differently from single bacteria. Active bacteria in aquatic environments respond to substrate limitation by dividing to form smaller bacteria (Morita, 1985), typical of the stationary growth phase (Stanier et al., 1980), and bacteria in deep sediment layers may respond in a similar manner.

Although ^3H -thymidine incorporation could not be detected at time zero, subsequent incorporation and hence productivity changes during incubation corresponded with, and confirmed, the growth and FDDC data. Maximum productivities coincide in general with mid-log phase and peak FDDC times (Fig. 3). Recent criticism of the use of ^3H -thymidine in oligotrophic, carbon limited, environments (Brittain and Karl, 1990) is not applicable to these experiments, as the method is being used under conditions where it accurately estimates bacterial productivity (Carman et al., 1988), a well-mixed, actively growing bacterial population with excess substrate.

If we consider the age of the deeper sediments studied (about 4 Ma; Ingle, Suyehiro, von Breymann et al., 1990) the bacteria cultured from these sediments show a remarkably short lag phase of between 12 and 48 hr (78.85 and 425.0 mbsf, respectively) and this was reflected in the very high estimates of numbers of viable bacteria (Table 3). We appreciate that the accuracy of the technique of estimating bacterial viabilities by extrapolating back to zero time has not been established for natural bacterial populations, and the very high viabilities (0.02%–35%; Table 3) may be largely attributed to this technique. In the absence, however, of any other technique such as direct microscopic estimation of viability, without incubation, these results are the only estimates that can be obtained and should provide a relative index of viability. Viability, for example, decreases sharply with increasing sediment depth. This observed decrease and the absence of dividing cells at 78.85 and 425 mbsf indicates that the proportion of moribund, viable but nonculturable dormant bacteria probably increases with sediment depth. However, the results clearly demonstrate the presence of culturable bacteria even in the deepest samples (Figs. 2 and 3). Cragg et al. (this volume) using most probable number counts and highly selective media, reported much lower *in-situ* viabilities for the same core (0.00004%–0.57%) and the “true” viability must fall somewhere between the two estimates of viability.

The response of the bacteria at 9.75 mbsf was very different from all the other samples; although there was thymidine incorporation in the unincubated sample there was no subsequent growth (Fig. 3). The inability to stimulate bacterial growth at 9.75 mbsf was surprising, but closer examination of the FDDC data for these samples (Fig. 3C) does show some slightly higher values, suggesting that the bacteria did initially attempt to grow but were unsuccessful. A speculative suggestion for this apparently contradictory data is that these bacteria might be in the process of changing to a low-activity, semi-dormant population and were under considerable metabolic stress. This could have resulted in substrate accelerated death (Postgate and Hunter, 1964) when they were exposed to a large amount of a rich carbon source, such as yeast extract. Although this explanation is contentious, as bacteria would be expected to be adapted to substrate limitation much nearer the sediment surface, it is consistent with the results of Cragg et al. (this volume) that the bacterial population below about 9 mbsf decreases much less rapidly than that above 6 mbsf, and hence bacteria in deeper sediments may be metabolically more adapted to survival under conditions of low concentrations of available organic carbon. Thus in contrast to those at 9.75 mbsf, bacteria in deeper sediments (78.85 and 425 mbsf) may be fully adjusted to low metabolic activity and able to respond, by growth, to new energy sources

added either externally (Fig. 3) or from within the sediment (Cragg et al., this volume).

SUMMARY

Bacteria were present in deep sediment layers down to a depth of 425 mbsf. The presence of dividing cells in sediments above 9.75 mbsf gave direct evidence of microbial growth in the upper layers. Due to the short incubation times used, it was not possible to estimate bacterial growth in unincubated sediments by ^3H -thymidine incorporation. Rapid bacterial growth occurred during anaerobic incubation of sediments from 0.15, 1.15, 78.85, and 425 mbsf with yeast extract. Growth did not occur in sediment from 9.75 mbsf. Viabilities (0.02%–35%) were estimated by extrapolation of the logarithmic growth phase to zero time. Although these viabilities decreased rapidly with depth they seemed unrealistically high, and were much higher than those obtained by growth in selective media (Cragg et al., this volume). A high proportion of the bacteria studied appeared to be dormant, particularly in the deeper sediment layers. But despite this the bacteria had a surprisingly high potential for growth, especially as the deepest sediments studied were in excess of 4 m.y. old. Although this growth potential seems surprising, bacterial populations and viability were shown to increase (Cragg et al., this volume) in intact sediment from deep layers within this core, including samples from below 375 mbsf. In addition, increased bacterial populations have also been observed in intact sediments from the Peru Margin (Parkes et al., 1990) where bacteria have responded to a brine incursion by increasing biomass and activity in sediments at 80 mbsf and approximately 1 m.y. old. Similarly, culturable bacteria have been isolated from deep aquifers down to 467 m (Fredrickson et al., 1991) thought to be the most oligotrophic environment that has active microbial metabolism (Chapelle and Lovley, 1990). Together, these data demonstrate that bacterial populations can remain viable for very long periods of time, presumably by slowly metabolizing increasingly recalcitrant organic compounds, but can rapidly grow when a suitable growth substrate is supplied.

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