PHYSIOLOGICAL AND ECOLOGICAL STUDIES
OF MANNITOL UTILIZING MARINE BACTERIA

By

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Submitted for the degree of Doctor of Philosophy,

University of Cape Town

August 1985
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Assoc. Prof. F.T. Robb
Department of Microbiology
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ABSTRACT

Bacteria were isolated from the kelp beds on the West Coast of South Africa. Strains isolated from the water column and kelp fronds were classified as *Pseudomonas*, *Vibrio*, *Acinetobacter* and *Flavobacterium* species. Bacterial diversity in adjacent kelp dominated habitats was examined using numerical analysis, and it was found that nearshore and offshore isolates were similar, whereas bacteria isolated from beached kelp and interstitial waters were dissimilar from them and from each other. Changes in numbers of bacteria able to form colonies on plates were monitored during upwelling and downwelling conditions.

The mannitol transport systems of a *Pseudomonas* and *Vibrio* strain were examined in some detail. The *Vibrio* had a constitutive mannitol phosphotransferase system (PTS), and this was confirmed using various mannitol mutants that were isolated. The *Pseudomonas* had an inducible, non PTS mannitol transport system.

The starvation survival responses of various isolates were tested. A proportion of the cells in cultures of the nearshore kelp bed bacteria remained viable for long periods without exogenous energy. By comparison, *Escherichia coli* tested under similar conditions lost viability rapidly. Uptake systems, and the potential for respiring mannitol, glucose, glutamate, aspartate and alanine were maintained by the marine isolates during starvation. The kinetic constants (Km values) for mannitol, glucose and glutamate transport in a *Vibrio* strain were measured during nutrient deprivation. Induction of the mannitol transport system in a *Pseudomonas* strain after a period of starvation was observed. Mannitol
negative mutants were compared in co-culture with mannitol utilizing isogenic *Vibrio* strains. The mannitol transport deficiency did not alter the specific survival rate unless mannitol was introduced into the culture flasks, when selection for wild type mannitol positive strains occurred.

The bacterial strain collections contained a high frequency of isolates that produced laminarinase, alginase and other hydrolytic enzymes. These degradative enzymes may be important in the kelp bed ecosystem, where a large fraction of the primary production is recycled via a detrital pathway.

Bacteria were isolated from the crystalline style and gut of a filter feeder in the kelp beds, the mussel, *Choromytilus meridionalis*. Some of the style bacteria produced a lysozyme like factor (LLF) that lysed marine bacterial cells. The LLF of one isolate was examined with respect to its spectrum of activity against various bacteria. It was inactive against several terrestrial bacterial strains, but active against the majority of marine bacteria. The spectrum of hydrolytic enzymes of the gut and style bacteria was determined, and their ecological significance is discussed.
I would like to thank my supervisor, Professor Frank Robb, for all the advice and encouragement he has given me during the course of this study. I am grateful to my colleagues, Muffy Seiderer and Dave Muir, as well as other members of the Microbiology and Zoology Departments of U.C.T., for their help and discussions. I am most grateful to my family and friends for all the support I have received from them. Thanks are also due to Gail Gilfillan who typed this thesis. This work was supported by funds from the Benguela Ecology Programme of the S.A. National Committee for Oceanographic Research.
Bacteria play a vital role in marine ecosystems such as estuaries, and coastal, offshore and deep waters. Historically, bacteria were studied primarily using plate counts and light microscopy, but these methods have been found to be inadequate for gathering ecological data necessary to calculate factors such as bacterial biomass and production. Marine microbiology has thus diversified into two main streams:

1. The study of marine bacterial assemblages as a step in the planktonic food chain, in terms of biomass, production and interactions with other trophic levels. These studies are usually carried out using the total heterogeneous populations found in seawater, and do not involve isolation of bacteria.

2. The study of individual bacterial species with respect to their physiology and adaptions to the marine environment.

The nature of these investigations excludes field studies, as cultural techniques have to be employed. A large percentage of marine bacteria cannot be isolated using conventional techniques, thus these strains have not been studied individually. However, valuable extrapolations can be made from laboratory studies of individual species to the field situation.
The work presented in this thesis falls primarily into this second category of study.

The kelp beds off the West Coast of South Africa constitute a nearshore marine environment. The area has been studied extensively during the last decade (Field et al., 1977; Field et al., 1980 a, b; Mazure and Field, 1980; Field et al., 1981; Jarman and Carter, 1981; Newell, 1981; Linley and Field, 1982; Koop et al., 1982 a, b). It is an area of high productivity, as is found in other kelp bed systems, salt marshes and swamplands (Newell, 1979). General features of such ecosystems are:

1. High primary production;
2. Export of organic material to adjacent coastal waters;
3. Few herbivores;
4. Many filter feeders.

Thus it appears that secondary production is not driven by energy from primary production in a typical plant-herbivore-carnivore food chain (Newell, 1981) but includes a detritus food chain employing a microbial loop (Azam et al., 1983). Components of this loop include bacteria, flagellates, ciliates and other microzooplankton.

Fenchel and Jørgensen (1977) have proposed 3 properties of bacteria that can explain their dominating role as primary decomposers in ecosystems:
1. Bacteria can utilize dissolved organic substrates at low concentrations, and also assimilate dissolved inorganic nutrients such as nitrate and phosphate. These properties are due in part to the small size and thus large surface to volume ratio of bacteria.

2. They can decompose nutrient poor plant tissue and hydrolyse structural plant compounds.

3. Many forms have developed efficient systems of anaerobic metabolism.

The environmental regime that bacteria in the kelp beds encounter is subject to rapid changes due to the phenomenon of upwelling that occurs on the Southern African West Coast. During upwelling, strong south easterly offshore winds cause movement of surface waters from nearshore to offshore regions, and influx of colder water (less than 12°C). The upwelled water (generally 9 - 10°C) is rich in NO₃, P and Si (Field et al., 1980b), but is relatively poor in phytoplankton (Carter, 1982). Onshore winds reverse the pattern, and warmer waters (14 - 16°C), rich in phytoplankton but poor in inorganic nutrients are encountered onshore. It has been calculated that the kelp bed water column may be turned over or flushed out 3 to 7 times daily during an upwelling cycle (Field et al., 1981).
During heavy wave action, fragmentation of kelp plants occurs, resulting in large quantities of dissolved and particulate organic matter (DOM and POM respectively) being released into the water column. It has been shown that standing stock of DOM is related primarily to swell height, rather than winds and upwelling conditions (Field et al., 1981). If fragmentation occurs during periods of upwelling, large quantities of DOM and POM are exported from the kelp beds. Koop et al., (1982a) have shown that bacteria may also be transported from the kelp beds to the open ocean during periods of active upwelling.

Heterotrophic utilization of kelp-derived mucilage (DOM) and fragmented particles (POM) depends to a large extent on bacterial action (Linley et al., 1981; Lucas et al., 1981; Stuart et al., 1981). Newell et al., (1980) carried out a detailed analysis of the soluble organic components of the two main kelp species in the South African kelp beds - Ecklonia maxima (Osbeck) and Laminaria pallida (Grev.) J. Agr. They found that mannitol dominates the sugars and polyols, and laminarin and alginates occur as structural components. Analysis of protein hydrolysate showed that the dominant amino acids are glutamate, aspartate and alanine, all of which occur in the side chains of mucopolypeptides. These are thus the principle soluble substrates that bacteria in the kelp beds encounter.

Lucas et al., (1981) have shown in experiments incubating kelp mucilage with natural seawater as a source of bacteria, that 50% of the mannitol is utilized within 48 h, whereas alginates plus other sugars reach 50% of
their initial concentration after 6 to 10 days (d). This differential utilization of kelp products also applies to particulate matter, which reaches a 50% utilization level only after 10 d (Stuart et al., 1981).

The work on the role of bacteria in the South African kelp beds has concentrated on studies of biomass and production (Field et al., 1980b; Linley and Field, 1982) and seasonal variations in bacteria associated with kelp fronds (Mazure, 1978; Mazure and Field, 1980). Succession in the microbial communities associated with kelp debris, which includes factors such as predation by flagellates and ciliates, has been investigated by Linley et al., (1981), and Stuart et al., (1981). However, little is known about the population structures that constitute the bacterial assemblages involved in these processes. In the studies of bacterial utilization of kelp-derived substrates mentioned earlier, only heterogenous populations have been used, and nothing is known of individual species responsible.

The potential of kelp bed bacteria to adjust to variable growth substrate levels has not been evaluated. Stevenson (1978) formulated the hypothesis that many bacteria deal with various changes (for example, in nutrient levels) in the aquatic environment by entering into a state of dormancy. An adaption of this kind may be important for the bacteria in this study, as they encounter nearshore, relatively high substrate levels, and offshore conditions where nutrients are scarce.
This thesis is a synthesis of data accumulated about individual bacterial strains found in the kelp bed. Chapter 2 deals with the changes in bacterial numbers during upwelling and downwelling cycles, and describes strains isolated from various habitats occurring within and near the kelp bed. Mannitol uptake and utilization systems of two dominant strains are compared in Chapter 3. The response of some isolates to starvation are examined (Chapter 4), in order to determine levels of survival during this potential environmental stress. Chapter 5 deals with the utilization of kelp products such as laminarin, alginate, as well as degradation of other complex substrates such as chitin and cellulose by various bacterial strains. Possible interactions between bacteria and a dominant filter feeder, the black mussel, *Choromytilus meridionalis*, are reported in Chapter 6. Finally, a general overview of all the data is presented in Chapter 7.
BACTERIA IN A KELP-DOMINATED ECOSYSTEM

SUMMARY

Bacterial numbers during upwelling-downwelling cycles were monitored at two sites on the West Coast of South Africa. Culturable bacteria (determined by plating) constituted only a fraction of the total bacterial population (determined by acridine orange direct counting). Colony counts on agar plates containing low nutrient levels reached a maximum 3 - 6 days after upwelling events. Bacterial isolates from adjacent kelp dominated habitats were examined. Nearshore and offshore isolates were similar, whereas bacteria isolated from beached kelp and interstitial waters were dissimilar from them and from each other. Classification of bacteria isolated from the water column and kelp fronds showed that Pseudomonas, Vibrio, Acinetobacter and Flavobacterium strains were present in the kelp beds.
2.1 INTRODUCTION

Various techniques can be utilized for studying the bacteria in a particular ecosystem, in order to gather information on bacterial numbers, biomass, population structures, microbial activity, and investigations of particular species and their adaptations to their environment. Analysis of bacterial assemblages by direct count using microscopy is limited, as bacteria can be differentiated between only in terms of size and shape (large rods, small cocci, and so on). The technique of microautoradiography (Zimmermann and Meyer-Reil, 1974; Tabor and Neihof, 1982) allows the distinction to be made between bacteria active in the uptake of the particular radiolabelled substrate being employed in the experiment, and those 'inactive' bacteria that are unable to transport that substrate.

It has long been recognized in marine and freshwater ecosystems that a discrepancy exists between numbers of bacteria obtained by direct-counting (DC) using a microscope, and by plating to obtain colony forming units (cfu), as the cfu may represent less than 1% of the DC numbers. This difference in numbers may be attributed to the presence of bacteria called 'oligotrophs' (Kuznetsov et al., 1979; Poindexter, 1981), or 'ultramicrobacteria' (Torrella and Morita, 1981; MacDonnell and Hood, 1982). These bacteria only grow and divide at very low nutrient concentrations. Some form microcolonies, detectable only by microscopy (Torrella and
Morita, 1981), others grow for several cell divisions, and the remainder fail to grow at all on the solid media utilized, even if low nutrient levels are employed.

The other type of bacteria that increase in size and show a high growth rate on a rich nutrient agar surface have been termed 'copiotrophic' (Poindexter, 1981) or 'eutrophic' (Kuznetsov et al., 1979). These two types of bacteria will be further discussed in Chapter 4.

Thus bacteria that can be enumerated as cfu represent a subpopulation of the total bacterial community present in the ecosystem. It is not possible to specify to what extent the characteristics of culturable bacteria reflect those of the bacterial assemblage as a whole.

Previous studies of kelp bed bacteria have consisted of enumeration of total numbers by acridine orange direct counting (AODC), and biomass estimates, calculated by using the numbers obtained and volume estimates from scanning electron microscopy, (Field et al., 1980b; Stuart et al., 1981; Linley-and Field, 1982). The changes in bacterial numbers during the varied environmental conditions encountered during upwelling cycles have been followed using AODC (Field et al., 1980b).

In this chapter, two studies are described that were undertaken to
follow changes in culturable bacteria in the kelp bed water column during upwelling cycles. These cycles are easily monitored, as newly upwelled water can be recognized by its low temperature. The first study was a comparison of AODC and plating techniques in following changes in bacterial numbers, and the later study involved plating on high and low nutrient media.

Two methods may be used to select bacterial isolates for characterization after plating. In order to determine the dominant culturable bacterial species and their relative proportions in a population, random samples of colonies are isolated and studied. A different approach involves isolating all the morphologically distinct colony types. The resulting 'strain collection' will yield no information about population structures, but rarer bacterial strains may be represented.

The first approach was used in work carried out in conjunction with D. Muir and K. Koop, in a study on the distribution and some properties of bacteria sampled from various kelp-dominated habitats (Davis et al., 1983). Koop et al., (1982a) had suggested that there were specialized nearshore bacteria that were mannitolytic as an adaptive response to the high mannitol concentrations encountered in the kelp bed. The question thus arose whether different populations of bacteria were maintained in the adjacent habitats encountered in and near the kelp bed. The results described in this chapter explore this possibility with respect to the culturable bacteria.
The final section of this chapter is a description of the following strain collections:

1. Kelp bed water column bacteria, and

2. Bacteria isolated off kelp fronds.

It was from these collections that isolates were chosen for the work described in the following chapters.

2.2 MATERIALS AND METHODS

Sampling sites

The study sites were all in the Benguela upwelling region, on the west coast of the Cape Peninsula. All samples were taken during summer (November to February). Samples for the first upwelling cycle experiment were collected from a rocky intertidal reef at Bloubergstrand (33° 48' S; 18° 27' E). Oudekraal (33° 59' S; 18° 21' E) was the major study site, and samples taken there include those for the second upwelling cycle experiment, and for the strain collections. The investigation of bacteria occurring in adjacent habitats was carried out at Kommetjie (34° 08' S; 18° 18' E).
Media and maintenance of bacteria

Seawater broth (SWB - see Appendix) was used where a liquid growth medium was required. Agar was added to SWB to make SWA plates. 1/5 SWA plates contained SWB diluted 1 : 4 with seawater. Strains were isolated and restreaked to purity on SWA plates, and subsequently stored on slants of SWA.

Sampling methods

Water samples were collected in sterile glass bottles. Glutaraldehyde (5% final concentration) was used to fix samples immediately for AODC. Samples for plating were processed within 3 h.

Samples were collected from 4 adjacent habitats for population studies:

1. A nearshore water column sample was taken at the surface within the Kommetjie kelp bed.

2. An offshore water column sample was taken at the surface 12 km west of Kommetjie.

3. A sample of liquified degraded kelp fluids was taken
underneath beached kelp.

4. A sample of interstitial water was taken from sand on an adjacent exposed sandy beach.

Strain collections were set up in various ways. In order to isolate water column bacteria, samples of seawater from Oudekraal were incubated at 10°C with 0.5 g.l⁻¹ sterilized powdered kelp detritus. This had been prepared by grinding freeze-dried tips of the fronds of *Laminaria pallida*, sieving to obtain particles 43 - 63 μm in diameter, followed by sterilization for 24 h under UV light (Stuart *et al.*, 1981). Samples of the water were plated each day during incubation with the detritus.

A strain collection of bacteria off the fronds of kelp plants was obtained as follows: 2 X 2 cm² strips of the kelp *Ecklonia maxima*, from the tip, middle and base of the frond were sampled in a sterile manner at Oudekraal. These strips were incubated in 10 ml sterile seawater at 22°C for 16 h, and samples of the water then plated out. Isolates from both this and the water column collection were restreaked to purity.

**Determination of bacteria cell counts**

Total cell count was determined by the AODC method of Hobbie *et al.*
(1977), as modified by Linley et al., (1981). 5 ml sample was stained with an equal volume of acridine orange (10 mg.1⁻¹). After 10 min, the sample was filtered onto 0.22 µm Nuclepore filters that had been stained for at least 2 h to reduce background fluorescence, with 2 g.1⁻¹ Irgalan Black in 2% acetic acid, and rinsed with water. The Nuclepore filters were supported on Millipore pads (AP 1004751) during filtration, and 1% Photoflo was used as a surfactant on the filter. The filters were viewed at a magnification of 1000 X with a Zeiss microscope fitted with an epifluorescence condenser, and non-fluorescent immersion oil was used. Where necessary, samples were diluted (before staining) with 0.22 µm filtered sterile seawater, so that there were approximately 50 bacteria visible per field. 10 fields were counted per sample. Bacterial numbers (N) were calculated using the expression:

\[
N = \frac{S \times 10^6 \times n}{s \times V} \text{ cells ml}^{-1}
\]

where 
- \( S \) = working surface area of the filter (mm²);
- \( s \) = surface area of the field of observation (µm²);
- \( V \) = volume of sample filtered (ml);
- \( n \) = average number of cells per field.

Plate counts were obtained by plating appropriate dilutions of seawater onto SWA or 1/5 SWA, called high nutrient (HN) and low
nutrient (LN) agar respectively. The plates were incubated at 22°C for at least 1 week before counting the colonies, in order to allow the very small colonies to develop.

Characteristics of strains

The following properties were tested:

1. Gram reaction;
2. Morphology (rods and cocci);
3. Motility (hanging drop method);
4. Flagellation (Leifson's flagellar stain);
5. Oxidase (oxidase reagent);
6. Catalase (3% H₂O₂);
7. Agarolytic activity (Gran's test - Hodgson and Chater, 1981);
8. Fermentation of mannitol, glucose, mannose and glycerol.

Fermentation was assessed by growing the cells for 16 h in a liquid medium containing the carbohydrate. A drop of the indicator phenol red was added. A yellow colour indicated the presence of acid, whereas orange and red indicated slight and no fermentation. All fermentation tests were carried out under aerobic conditions and repeated in an anaerobic jar perfused with high purity nitrogen. Strains that gave an orange colour under aerobic conditions, always
gave a red (negative) result under anaerobic conditions. This test gave the same results as tests using Leifson's MOF medium.

Tests 1, 3, 7 and 8 were carried out on the strains isolated from 4 adjacent habitats, with mannitol and glucose being used as fermentation substrates. Bacteria in the strain collections were tested for all the properties (1 - 8). This allowed a tentative classification of the strains (Sieburth, 1979; Fukami et al., 1981).

2.3 RESULTS

Sampling at Bloubergstrand

Figure 1 presents the results of the preliminary study to monitor changes in total bacterial numbers and culturable bacteria ('viable' counts - VC) during upwelling cycles. Total counts (AODC) varied according to the water temperature. A decrease in temperature was accompanied by a decrease in total bacterial numbers, and vice versa. A summary of 2 upwelling - downwelling cycle 'peaks' is presented in Table 1.
FIGURE 1: Sampling experiment carried out at Bloubergstrand during March, 1983. Water temperature (■) was measured as an indicator of upwelling events, which are labelled with arrows. Total and viable bacterial cell counts (TC and VC) were determined by AODC and plating respectively. Plateability (□) was calculated as follows: VC/TC.
TABLE 1

Bacterial numbers and plateability during 2 upwelling-downwelling 'peaks' in the sampling experiment carried out at Bloubergstrand. Total numbers were determined by AODC (Linley et al., 1981), and viable count (VC) by plating on SWA. Plateability is the ratio VC : AODC expressed as a percentage.

<table>
<thead>
<tr>
<th>Day</th>
<th>Temperature (°C)</th>
<th>Condition</th>
<th>Viable count</th>
<th>Total numbers</th>
<th>Plateability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>15.0</td>
<td>Downwelling</td>
<td>5.4 x 10^3</td>
<td>2.9 x 10^6</td>
<td>0.18</td>
</tr>
<tr>
<td>6</td>
<td>11.5</td>
<td>Upwelling</td>
<td>6.6 x 10^3</td>
<td>2.0 x 10^6</td>
<td>0.33</td>
</tr>
<tr>
<td>9</td>
<td>13.7</td>
<td>Downwelling</td>
<td>5.5 x 10^3</td>
<td>5.1 x 10^6</td>
<td>0.11</td>
</tr>
<tr>
<td>19</td>
<td>10.0</td>
<td>Upwelling</td>
<td>1.9 x 10^3</td>
<td>2.3 x 10^6</td>
<td>0.08</td>
</tr>
</tbody>
</table>

No correlation between plate counts and variations in temperature was discerned. The bacteria from this sampling site in both upwelled and downwelled water showed an extremely low plateability.

Sampling at Oudekraal

Figure 2 shows the results of a two month sampling programme, where different plating was carried out on HN and LN agar. With the exception of 2 samples, the numbers of bacteria on LN agar were uniformly higher than those on HN agar. Furthermore, although no clear pattern of changes in bacterial numbers determined on HN agar in response to upwelling cycles could be distinguished (similar to the Bloubergstrand experiment - Figure 1), numbers on LN agar appeared to cycle in response to temperature changes. Four upwellings were recorded where the temperature fell to 10°C or
FIGURE 2: Sampling experiment carried out at Oudekraal during Nov. - Dec. 1984. Upwelling events (1 - 6) are labeled with arrows. Viable cell counts were determined using low nutrient agar (▲-▲) and high nutrient agar (●-..●).
below (labelled 1, 2, 4, 6) and 2 at 11°C (marked 3,5). On LN agar, bacterial numbers reached a maximum after 4, 6, 3, 3, 9 and 3 days for upwellings 1 - 6 respectively. During upwelling 3 and 4, there was a period of continuous low water temperatures.

**Cell counts of bacteria from adjacent habitats**

In the collaborative study carried out on bacteria in adjacent kelp-dominated habitats, it was found that interstitial waters and beached kelp contained far higher total cell counts than the nearshore and offshore samples (Table 2).

Similarly, the fraction of plateable bacteria out of the total number was nearly an order of magnitude greater. Nearshore and offshore samples contained relatively high numbers of bacteria smaller than 0.45 μm (approximately half), that were unable to form colonies.

**Characteristics of bacteria from adjacent habitats**

The characteristics of the strains isolated by random sampling from four marine habitats are presented in Table 3. Water column bacteria (nearshore and offshore) were all Gram negative, but a few gram positive strains were isolated in the onshore collections. Motility was a common feature of the bacteria in all habitats.
TABLE 2

Total bacterial counts compared with numbers of colony forming bacteria in samples representing the habitats examined in this study.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total cell count (cells ml⁻¹)</th>
<th>Colony forming bacteria (ml⁻¹)</th>
<th>Plateable bacteria (% of total count)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nearshore</td>
<td>(^{a}1.4 \times 10^5)</td>
<td>(3.6 \times 10^3)</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>(^{b}3.9 \times 10^4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Offshore</td>
<td>(^{a}3.5 \times 10^5)</td>
<td>(8.0 \times 10^3)</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>(^{b}1.7 \times 10^5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Interstitial</td>
<td>(^{a}7.5 \times 10^7)</td>
<td>(1.3 \times 10^7)</td>
<td>17.0</td>
</tr>
<tr>
<td>Beached kelp</td>
<td>(^{a}2.3 \times 10^9)</td>
<td>(3.2 \times 10^8)</td>
<td>13.0</td>
</tr>
</tbody>
</table>

\(^{a}\) Acridine orange direct counting of whole sample

\(^{b}\) Acridine orange direct counting of 0.45 \(\mu\)m filtrate

\(^*\) Bacteria plated on high nutrient agar (SWA) plates
TABLE 3

Summary of properties of bacterial populations from the nearshore water of a kelp bed, from water more than 4.5 km offshore, from the interstitial water of an adjacent exposed sandy beach, and from degrading, beached kelp plants. Data represent numbers of bacterial strains exhibiting the properties tested; percentage of the total number of strains within each population is also shown.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Near-shore</td>
</tr>
<tr>
<td>Gram negative</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>Motile</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>97.5%</td>
</tr>
<tr>
<td>Fermenters</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>62.5%</td>
</tr>
<tr>
<td>Agar digestion</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>20%</td>
</tr>
</tbody>
</table>
except in the beached kelp strains. The ability to produce fermentation end products from utilization of the sugars mannitol and glucose was present in most of the strains from beached kelp, whereas bacteria from interstitial waters were predominantly non-fermenters. There were slightly more fermenters than nonfermenters in both the nearshore and offshore strain collections. Agar digestion was not a common feature of all 4 groups of bacteria.

Table 4 presents the fermentation spectra of the facultative anaerobes mentioned in Table 3. The beached kelp sample contained the highest number of fermenters, most of which fermented mannitol and not glucose. Both sugars were fermented by the majority of facultative anaerobes in the nearshore and offshore populations.

Strain collections

All of the isolates, whose classification is summarized in Table 5, were Gram negative. Of the 20 strains characterized from the water column collection, 16 were nonfermenters of mannitol, glucose and mannose, and were motile. These were identified as members of the Pseudomonas-Alcaligenes group, according to the determinative schemes of Fukami et al. (1981), and Sieburth (1979). Another non-fermentative isolate was nonmotile, and thus was classified as an Acinetobacter strain. Two yellow pigmented agarolytic strains from the water column fell into the nonfermentative Flavobacterium group, of which none were isolated off kelp fronds. Eight of the ten strains characterized after isolation from tip, middle and base of the kelp fronds were fermentative Vibrio types, and there was one Acinetobacter.
TABLE 4

Properties of facultative anaerobes from the nearshore water of a kelp bed, from water more than 4.5 km offshore, from the interstitial water of an adjacent sandy beach, and from degrading, beached kelp plants. Data represent the number of bacterial strains exhibiting the properties tested, expressed as a percentage of the facultative anaerobes in each sample.

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Near-shore</td>
</tr>
<tr>
<td>Mannitol</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>Glucose</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>76%</td>
</tr>
<tr>
<td>Mannitol only</td>
<td>6</td>
</tr>
<tr>
<td>Glucose only</td>
<td>0</td>
</tr>
<tr>
<td>Both substrates</td>
<td>19</td>
</tr>
</tbody>
</table>
TABLE 5

A summary of the classification of the bacterial strains in the collections isolated from the kelp bed water column and from kelp plants at Oudekraal. Isolates were classified according to the determinative schemes of Sieburth (1979) and Fukami et al., (1981).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of isolates</th>
<th>Water column</th>
<th>Kelp plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas, Alcaligenes</td>
<td>16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vibrio</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
2.4 DISCUSSION

The preliminary sampling experiment carried out at Bloubergstrand aimed to discover whether there was a variation in plateable bacterial numbers similar to the variation in direct (total) counts that had been reported previously (Field et al., 1980b). No correlation was found between colony forming bacteria or % plateability, and total counts. However, the sampling period was very short, and plating was only carried out on high nutrient plates.

The bacterial numbers obtained by AODC for downwelling (2.9 - 5.1 X 10^6 cells.ml^-1) were similar to those obtained by Linley (2-3 X 10^6 cells.ml^-1, reported in Seiderer et al., 1984). There was a difference of almost an order of magnitude between Linley's and these results for upwelling periods (4 X 10^5 and 2.0 - 2.3 X 10^6 cells.ml^-1 respectively). A lower total cell count was obtained in this study on the populations from different habitats, where 1.4 X 10^5 cells.ml^-1 were counted in the nearshore region. These discrepancies may arise from the fact that samples were taken from various sites, where there may be differences in cell numbers. Various workers carrying out AODC may get differing results - it was noted during this study that extremely small 'cocci' lost their fluorescence and became 'non-visible' in a short time, which may lead to underestimates of bacterial numbers.
Approximately $10^3$ cells ml$^{-1}$ were able to form colonies in samples from various sites. Great variability is to be expected in these enumerations, as many factors influence the plateability of these marine bacteria.

Field et al. (1980) reported periods of low bacterial counts (AODC) corresponding to active upwelling conditions, as well as peaks in numbers during periods when the water was warmer. These peaks lagged 1 day behind peaks in POM concentration. In the sampling experiment carried out at Oudekraal described here, the peaks in bacterial numbers on LN agar plates occurred when water temperatures were warmer. It may be preferable to correlate an increase in cultural bacteria with the foregoing upwelling, rather than with the increase in temperature that occurs after upwelling. Upwelled and downwelled water can be differentiated by their salinities. It has been found (Verheye, pers. comm.), that warmer, apparently downwelled water occasionally has a salinity characteristic of upwelled water, indicating that it is actually water from an upwelling event that has been warmed on reaching the surface. These warmer water temperatures are not necessarily a good indication of downwelling.

Fluctuating total and culturable bacterial numbers may result from either or both of 2 causes, as follows:

1. Physical and biological factors responsible for changes in bacterial numbers (for example, dilution during upwelling, and predation) may vary in intensity.
2. Alternatively, the activity of the bacteria may increase or decrease, resulting in variation in net production and colony forming ability.

Some evidence that these factors do play a role in the ecosystem is available. Field et al. (1980b) have reported that vertical mixing of the water column does occur. Predator-prey interactions have been described by Linley et al. (1981), who found that a decrease in bacterial biomass was dependent on the appearance of flagellate and ciliate populations. Finally, changes in available substrates may influence bacterial activity – for example, mannitol is the primary sugar liberated during kelp fragmentation, whereas glucose is released during phytoplankton blooms. Fukami et al. (1981) have shown that bacteria respond to the changing nutrient status of contained natural seawater, resulting in a succession of bacterial populations. This could presumably entail changes in colony forming ability.

As there are no physical boundaries between nearshore and offshore waters, a certain degree of mixing of the corresponding populations must occur. However, characterization of onshore and offshore strain collections indicated that different populations are maintained in each habitat (Koop et al., 1982a). The nearshore region is kelp-dominated, in contrast to the offshore region, where phytoplankton communities flourish. This difference is reflected in the hydrolytic properties of the bacteria found in both habitats, with mannitol and glucose fermentation found inshore and
offshore respectively, although some strains utilize both sugars. A higher percentage of agarolytic strains occurs nearshore, which suggests that agar-like polysaccharides may be a carbon source in the inshore region, although the presence of agar has not been noted in these waters. Apart from this, the nearshore and offshore groups are very similar to one another.

The lowest number of fermentative bacteria in the 4 habitats examined was found in the interstitial collection. Water movement through the upper 30 cm of sand is rapid and anoxia is rare. In contrast, the population of bacteria isolated from beached kelp has the highest number of facultative anaerobes, probably in response to the anaerobic environment that rotting kelp clumps generate.

Data analysis of strain characteristics was carried out by D. Muir and K. Koop (as described in Davis et al., 1983), to obtain a measure of similarity between the populations. It was found that the kelp bed and offshore water column bacteria showed the most similarity (56%). The other two strain collections were distinctly different from these open water communities, as well as from each other. The bacteria isolated from beached kelp were most dissimilar to the isolates from the other habitats, showing only a 3% similarity to them. (The Bray-Curtis measure of dissimilarity was used.)
Classification of the isolates in the strain collections of bacteria from the nearshore water column and off kelp plants show that the genera *Pseudomonas* and *Vibrio* are well represented, with *Flavobacterium* and *Acinetobacter* also being present. The presence of these bacteria in similar environments is well documented. For example, Fukami *et al.* (1981) described their presence in surface waters of Aburatsubo Inlet in Japan. The bacteria associated with the fronds of *Laminaria longicruris* found off the coast of Nova Scotia were investigated by Laycock (1974), who found *Vibrio* and *Pseudomonas* to be the predominant genera of culturable bacteria.

Thus culturable marine bacteria in the water column and on kelp surfaces are predominantly Gram negative and motile. As Sieburth (1979) has noted, the Gram negative cell envelope is well adapted for life in the low nutrient aqueous marine environment. Its degradative enzymes are usually retained in a highly protective association within the cell wall. This is in contrast to Gram positive cells that release extracellular enzymes, which would be 'wasteful' in the dilute aqueous marine environment. Possession of a periplasmic space, containing binding proteins, between the outer and inner cell membranes, allows Gram negative bacteria to concentrate substrates such as sugars and amino acids in the space before transport into the cell. The periplasmic compartment may also serve as an area of recapture for substrates that have 'leaked' from the cell. Motility, if it is coupled with chemotaxis, is also an advantageous characteristic, as it enables bacteria to position themselves in favourable microclimates.
The taxonomy of marine *Vibrio*, *Pseudomonas* and related strains has been subjected to many changes over the years. Members of the genera *Beneckea* and *Achromobacter* have been reclassified as *Vibrio*. Various techniques have been used in attempts to group strains and elucidate relationships between them, for example, 5S rRNA sequence similarity (MacDonell and Colwell, 1984), immunological comparisons of glutamine synthetase and superoxide dismutase (Baumann and Baumann, 1980; Baumann et al., 1980), and the G+C content of DNA (Citarella and Colwell, 1970). For the purposes of this study, it was felt to be sufficient to classify the fermentative motile strains as members of the genus *Vibrio*, and the oxidative motile strains as *Pseudomonas*.

Finally, a mention must be made of the bacteria, found in great numbers in seawater, that are recalcitrant to plating, and thus do not usually appear in strain collections. A study of the bacteria from a Gulf Coast estuary able to pass through a 0.2 μm filter was made by MacDonnell and Hood (1982). It is generally believed that the very small 'ultramicrobacteria' are the cells unable to grow on agar. By subjecting the ultramicrobacteria to an initially low but increasing nutrient regime, growth of colonies on agar was achieved. Phenotypic characterization classed the bacteria as *Vibrio*, *Aeromonas*, *Pseudomonas* and *Alcaligenes*, which are also the genera found among the easily culturable bacteria. Similar
experiments performed previously by Tabor et al., (1981) showed that strains of *Alcaligenes, Flavobacterium, Pseudomonas* and *Vibrio* were among those passing through a 0.45 μm filter. These isolates, from deep sea water samples, did not have to be cultured on gradually increasing nutrient levels. It is probable, however, that only a fraction of the ultramicrobacteria have actually been isolated and described in these studies.

Thus, culturable and perhaps some of the 'nonculturable' bacteria in and near the kelp beds fall into conventional genera. It appears that they use their high degree of genetic flexibility to adapt effectively to the physical conditions and substrates available to them.
MANNITOL UPTAKE AND UTILIZATION BY KELP BED BACTERIA

SUMMARY

The mannitol transport systems of a *Pseudomonas* and *Vibrio* strain were examined in some detail. The fermentative *Vibrio* strain had a constitutive mannitol phosphotransferase system (PTS), and a mannitol-1-phosphate dehydrogenase. Strains mutated in various mannitol transport and utilization functions were isolated after treatment of the parent *Vibrio* strain with nitrosoguanidine. The oxidative *Pseudomonas* strain had an inducible, non PTS mannitol transport system, and a mannitol dehydrogenase. The significance of oxidative or fermentative utilization of the sugar is discussed.
3.1 INTRODUCTION

Mannitol, the primary photosynthate of kelp plants, comprises 77% of the free sugars and polyols released into the water column during fragmentation of the macrophytes (Newell et al., 1980). It is also the most abundant hexitol found in nature. As demonstrated in Chapter 2, a high proportion of the bacteria isolated from the kelp bed is able to utilize mannitol as a carbon source. The bacteria may be biased towards mannitol as the predominant, and therefore the 'preferred' carbohydrate in the kelp bed, rather than glucose.

Rapid, efficient and preferential utilization of mannitol by kelp bed bacteria has been demonstrated in flask experiments by Lucas et al. (1981). These workers showed that when kelp mucilage was incubated with seawater, more than 50% of the initial concentration (360 - 385 µg.ml⁻¹ = 2 mM) was utilized within 48 h, with a corresponding increase in bacterial biomass.

The first step in the utilization of a nutrient is the transport of the substrate into the cell. Mannitol transport systems that function in kelp bed bacteria are likely to be active (concentrative), as passive mechanisms are uncommon for carbohydrates other than glycerol, and inappropriate in environments where
relatively low sugar concentrations exist. Active transport systems may be conveniently classified according to the source of energy coupled to the process (Dills et al., 1980). Energy utilized may be chemical, as in the ATP-powered binding protein systems, or electrochemical gradients may be used, as in proton symport, or sodium cotransport. A different energy resource is utilized in the group translocation process, where phosphoenolpyruvate (PEP) fuels a phosphotransferase system (PTS), and phosphorylated sugar is the product of transport.

Mannitol transport in various genera of bacteria has been found to fall into two of these categories of active transport, namely the binding protein and phosphotransferase systems. A summary of all the mannitol transport systems described to date is presented in Table 6. Binding proteins, found in the periplasmic space of the Gram negative cell, have not been shown to have any enzymatic activity, and are only 'active' in forming complexes with their associated substrates, before these are transported across the inner membrane (Rosen, 1978). The group of proteins forming a PTS, functions both as an enzymatic complex, namely, a kinase, and in a transport capacity (Saier et al., 1976).

Bacterial genera that have a PTS which phosphorylates mannitol during transport into the cell, possess a soluble mannitol-1-phosphate (Man-I-P) dehydrogenase, which catalyzes the following reaction:
TABLE 6
Distribution of mannitol uptake systems in bacteria.

<table>
<thead>
<tr>
<th>Type of system</th>
<th>Genus</th>
<th>Gram stain reaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Periplasmic mannitol binding protein, mannitol dehydrogenase (inducible)</td>
<td>Pseudomonas</td>
<td>-</td>
<td>Eisenburg and Phibbs, 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phibbs and Eagon, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eagon and Phibbs, 1971</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lengeler, 1975 a,b</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td>-</td>
<td>Berkowitz, 1971</td>
</tr>
<tr>
<td></td>
<td>Klebsiella*</td>
<td>-</td>
<td>Tanaka et al., 1967</td>
</tr>
<tr>
<td></td>
<td>(Aerobacter)</td>
<td></td>
<td>Tanaka and Lin, 1967</td>
</tr>
<tr>
<td></td>
<td>Spirochaeta</td>
<td>-</td>
<td>Saier and Newman, 1976</td>
</tr>
<tr>
<td></td>
<td>Clostridium</td>
<td>+</td>
<td>Patni and Alexander, 1971</td>
</tr>
<tr>
<td></td>
<td>Streptococcus</td>
<td>+</td>
<td>Maryanski and Wittenberger, 1975</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus*</td>
<td>+</td>
<td>Friedman and Hays, 1977</td>
</tr>
</tbody>
</table>

*Mannitol-1-Phosphate dehydrogenase not reported.
If mannitol is not phosphorylated during transport, as for example, in *Pseudomonas aeruginosa* (see Table 6), a Man-1-P dehydrogenase is lacking, and the dehydrogenase substrate is unphosphorylated mannitol, which is oxidized as follows:

```
mannitol dehydrogenase
mannitol ----> fructose
NAD       NADH
```

Fructose is then phosphorylated by fructokinase (Phibbs and Eagon, 1970):

```
fructokinase
fructose ----> fructose-6-phosphate.
ATP       ADP
```

Thus, in both cases, mannitol finally enters the metabolic pathways as fructose-6-phosphate. The transport product (that is, phosphorylated or unphosphorylated mannitol) determines whether Man-1-P or mannitol dehydrogenase is present.
Mannitol phosphotransferase systems

The reactions occurring in the transport of a sugar via a PTS may be represented as follows (Dills et al., 1980):

![Diagram of PTS reactions involving EI, HPr, EII, and EIII]

(PEP = phosphoenolpyruvate; HPr = histidine-containing protein; EI, II and III = enzymes I, II and III; X~P = phosphorylated compound).

EI and HPr are soluble proteins for the PTS that function non-specifically with all the PTS sugars for that organism. The EII and EIII proteins are specific for a given sugar, and these components vary between the different PTSs. For example, the *Escherichia coli* mannitol PTS has a single membrane bound EIIImannitol (EIIImtl) but no EIII (Jacobsen et al., 1979), whereas the glucose PTS of the same organism has a protein duplex called EIIA/EIIB (Kundig and Roseman, 1971b) or EII and EIII (Kundig, 1974).

Structure and function of the *E. coli* mannitol PTS have been elucidated using three methods:
1. The isolation of strains mutated in various components of this particular PTS was carried out by Solomon and Lin (1972) - EI\textsubscript{II}mtl and Man-1-P dehydrogenase; Lengeler (1975a, 1975b) - EI\textsubscript{II}mtl and Man-1-P dehydrogenase, and Saier et al., (1976) - EI, HPr and EI\textsubscript{II}mtl.

2. Purification of general PTS proteins was achieved by Kundig and Roseman (1971a) and Anderson et al., (1971), who purified EI and HPr respectively. The mannitol specific EI\textsubscript{II}mtl was purified by Jacobsen et al., (1979). Partial purification of the Man-1-P dehydrogenase was carried out by Lee et al., (1981).

3. The genetics of the mannitol PTS was elucidated by mapping (Lengeler, 1975a, 1975b), and by cloning the mannitol (mtl) operon (Lee et al., 1981; Lee and Saier, 1983). It was found by these workers that there are 3 closely linked genes in the operon: mtl A, the structural gene for EI\textsubscript{II}mtl; mtl D, the structural gene for Man-1-P dehydrogenase, and mtl C, a cis dominant regulatory gene.

**Mannitol binding protein**

A mannitol binding protein system has been discovered in *Pseudomonas aeruginosa*. In 1970, Phibbs and Eagon reported the presence of mannitol dehydrogenase in that organism, and further studies on the kinetics of mannitol transport were carried out (Eagon and Phibbs, 1971). Mutants for mannitol utilization were isolated (Phibbs et al.,
1978), and no mannitol PTS could be detected. Eisenberg and Phibbs (1982) reported the isolation and characterization of an inducible mannitol binding protein. This protein is found in the periplasmic space between the outer and inner cell membranes. 'Permeases' required to transport mannitol from the periplasm through the inner membrane to the cytoplasm have not been described.

Distribution of phosphotransferase systems in bacteria

It will be noted in Table 6 that, with the exception of the *P. aeruginosa* binding protein system, all mannitol transport systems described so far are PTSs. Romano et al. (1970) have indicated that the possession of a PTS has a physiological advantage for organisms carrying out the fermentation of a particular sugar. The phosphorylated compound that is a product of sugar transport can be fed directly into the Embden Meyerhof pathway. ATP is conserved, as transport of PTS sugars is energized by PEP, which is an energy product of anaerobic glycolysis, performed by facultative anaerobes.

A later study of various fermentative bacteria elaborated on this hypothesis (Romano et al., 1979). It was found that homofermentative bacteria that utilize a sugar via the Embden Meyerhof pathway, possess a PTS for that particular sugar, whereas the system is absent in heterofermentative species that utilize the phosphoketolase pathway.
The frequent presence of the Entner Doudoroff pathway in obligate aerobes implies that their primary energy source is ATP, which is generated during oxidative phosphorylation. The ATP-dependent non-PTS uptake systems therefore predominate in these bacteria. With reference to mannitol, all the bacteria that ferment this sugar have a mannitol PTS, whereas the aerobic P. aeruginosa has a binding protein system as the first step in the oxidation of mannitol.

Both oxidative and fermentative pathways of mannitol utilization are found among the kelp bed bacteria. A preliminary characterization of mannitol transport and utilization was carried out using a representative from each group. Isolates of the fermentative Vibrio strain, mutated in mannitol uptake and utilization properties, were examined to further investigate the mannitol uptake system in that strain.

3.2 MATERIALS AND METHODS

Bacterial strains

A Vibrio strain, isolated from the tip of a kelp frond as described in Chapter 2, was chosen as a representative of a fermentative kelp bacterium. This strain was Gram negative, rod shaped, motile, flagellated, oxidase positive, catalase positive, non-agarolytic, and fermented glucose, mannitol and mannose.

The Pseudomonas was from the water column bacterial strain collection, whose isolation was also described in Chapter 2. These
bacteria were Gram negative motile rods with polar flagella, oxidase positive, catalase positive; they were agarolytic, and utilized mannitol, glucose and mannose oxidatively, without the production of acid end products.

Mannitol uptake and utilization

Cells were cultured in 1/10 SWB, with 0.2 - 0.5% (m/v) carbohydrate (mannitol or glycerol) added as indicated. After 16 h growth at 22°C, shaking at 150 rpm, cells were harvested and washed with SM buffer (see Appendix). The cells were resuspended at an OD600 of 0.1 - 0.5.

The uptake reaction was initiated by adding D[1 - 14C] mannitol (59 mCi/mmol) at the appropriate concentrations to aliquots of cells. The reaction was stopped at various times by filtering 50 µl subsamples through a 0.45 µm filter (Gelman). The filter was washed with 1 ml seawater and immediately dried by heating.

Incorporation was measured by precipitating macromolecules in the assay mix with 5% (final concentration) trichloracetic acid (TCA) at 0°C for at least 2 h. The samples were filtered onto Whatman GFA filters, which were washed with cold 5% TCA and dried.

Filters were placed in scintillation vials containing 5 ml of Instagel scintillation fluid. Radioactivity in each vial was determined using a Packard Tricarb 460 liquid scintillation counter.
Dehydrogenase assays

Cell extracts for dehydrogenase assays were prepared as follows: 40 ml of cell suspension grown as described were harvested and resuspended in 2 ml phosphate buffer (PB – see Appendix). The suspension was sonicated at 18 kHz in an MSE Soniprep 150 sonicator for 2 x 30s. The disrupted cells were centrifuged at 16 000 x g for 40 min, and the clear supernatant containing the soluble protein was used. The cells and extract were maintained at 0°C throughout.

Man-1-P dehydrogenase was assayed according to the method of Solomon and Lin (1972). The assay mixture contained 0.2 ml of 0.01 M Man-1-P: 0.1 ml of 0.02 M NAD; 0.1 ml of 1 M sodium carbonated buffer (pH 9.5); crude extract and PB to a final volume of 0.8 ml.

Mannitol dehydrogenase activity was determined as described in Siegel et al., (1977). The reaction mix included 50 mM Tris HCl (pH 8); 42 mM mannitol; 2 mM NAD, cell extract and PB in a final volume of 1 ml.

In both cases, the rate of reduction of NAD was assayed at 340 nm on an MSE spectrophotometer. The assays were initiated by addition of substrate, and the blanks contained no substrate.

Units of dehydrogenase activity were calculated using the following formula:

\[
U.\text{ml}^{-1} \text{ sample} = \frac{V \times \triangle E. \text{min}^{-1}}{E \times 1 \times v}
\]
U = units of dehydrogenase activity, equivalent to µmoles NAD converted to NADH per min.,

\[ V = \text{total volume of assay (ml), } l = \text{light path (cm), } v = \text{enzyme sample volume (ml),} \]

\[ \triangle E_{\text{min}^{-1}} = \text{OD}_{340}\text{.min}^{-1}, \varepsilon = \text{molar absorption coefficient} \]

which has a value of 6.22 cm².mol⁻¹ for NADH measured at 340 nm.

**Phosphorylation assay**

Cells, grown and washed as described, were incubated with 16 µM \[^{14}\text{C}]\) mannitol for 30 min. The cells were frozen, thawed and sonicated.

Samples (25 µl) were adsorbed on DEAE filter paper (Whatmans) and air dried. The filters were then washed with 2 ml H₂O on a vacuum filtration unit, dried, and the radioactivity determined by liquid scintillation counting. (This method was adapted from Jacobsen et al., 1979).

**Mutagenesis**

The *Vibrio* strain was mutagenised as follows: Cells were grown for 11 h in SWB, diluted 1 : 100 into fresh SWB and grown for a further
2 h. The culture was centrifuged and resuspended in Tris-maleic acid buffer (see Appendix) diluted 1:1 with seawater, containing 100 μg.ml⁻¹ of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The cells were incubated in the mutagenesis buffer for 15 min at 30°C, and were then washed twice with seawater to remove the NTG. The culture was resuspended in SWB, and grown for 3 h before selection was carried out.

Selection and isolation of mannitol mutants

The culture was washed, resuspended in a medium containing 1% mannitol and 0.1% casamino acids, and grown for 1 h. Ampicillin was added to a final concentration of 50 μg.ml⁻¹, and the cells were grown for a further 2.5 h. After washing, the cells were resuspended in SWB, grown for a further 3 h, and appropriate dilutions plated. Bochner's tetrazolium mannitol indicator (TZM) plates were used (Bochner and Savageau, 1977), adapted for marine bacteria (see Appendix). Fermentative colonies were white, whereas oxidative, or mannitol non-utilizing bacteria produce red colonies.

Protein determination

Protein concentration in whole cell suspensions and cell extracts was determined by the method of Lowry et al., (1951), with bovine serum albumin as the standard.
3.3 RESULTS

**Vibrio mannitol transport and incorporation**

Figure 3 shows the uptake of mannitol by *Vibrio* cells grown in different media as indicated. Mannitol was transported at virtually the same rate if the cells were grown in 1/10 SWB with or without mannitol. This is indicative of a constitutive mannitol uptake system. However, the addition of glycerol to 1/10 SWB as a growth medium caused decreased mannitol uptake. By way of contrast, cells grown in casamino acids with mannitol had almost double the rate of uptake compared with those grown without a carbon source.

Once mannitol has been transported into the cell, it may either remain in the soluble intracellular pool of substrates for metabolic processes, or be incorporated into various macromolecules such as nucleic acids, proteins, and structural components. These alternatives can be distinguished between by measuring $[^{14}C]$ mannitol incorporated into TCA precipitable material, as shown in Figure 4. The rate of transport of mannitol in cells grown in 1/10 SWB with or without mannitol was similar, however, there was a 10 fold difference in the rate of incorporation. Cells grown without mannitol incorporated 3% of the mannitol taken up, but 33% of the $[^{14}C]$ mannitol taken up by bacteria grown in the presence of the sugar was found in the TCA precipitable material within 1 min. This effect was seen to a lesser effect in cells grown in casamino acids with and without mannitol.
FIGURE 3: Uptake of mannitol by a Vibrio strain. Cells were grown in 1/10 SWB (●), 1/10 SWB + 0.2% mannitol (▲), 1/10 SWB + 0.2% glycerol (■), 0.5% casamino acids (○), and 0.5% casamino acids + 0.2% mannitol (△).

FIGURE 4: Incorporation of mannitol by a Vibrio strain, expressed as a percentage of the mannitol transported into the cells (see Figure 3). Legend as for Figure 3.
Mannitol uptake rates by cells grown in 1/10 SWB were measured over a range of mannitol concentrations. The results are shown in Figure 5, drawn as a Lineweaver-Burke double reciprocal plot. The $K_m$ for mannitol transport for this Vibrio strain was determined to be 7.69 $\mu$M. The Vibrio strain possessed a Man-1-P dehydrogenase, with a $K_m$ of 630 $\mu$M, as shown in Figure 6. No mannitol dehydrogenase was detected. The specific activities of the Man-1-P dehydrogenase in cells grown in different media is presented in Table 7. This enzyme was very active in cells grown in the presence of mannitol. If the carbon source for growth was glucose or glycerol, the specific activity was far lower. Potential phosphorylation of the $[^{14}C]$mannitol occurred at a rate of 5.95 nmol mannitol bound.min$^{-1}$.mg cell protein$^{-1}$ in the DEAE cellulose binding assay.

Vibrio mannitol mutants

Table 8 is a summary of the characteristics of the Vibrio mannitol mutants. They all formed red (non-fermentative) colonies on TZM indicator plates, with the exception of MC. This strain reverted to the wild type white (fermentative) colony type with a high frequency. Mutants MA, MD, MG and MS all lacked the ability to transport mannitol into the cell to any extent, and also did not produce Man-1-P dehydrogenase. Two strains that are potential Man-1-P dehydrogenase mutants are M3 and M6, which were able to transport mannitol into the cell and phosphorylated it, but did not exhibit dehydrogenase activity. A third type of mutant is represented by MF, MH and MI, which had functioning mannitol uptake
FIGURE 5: Double-reciprocal plot of initial rates of mannitol uptake by a Vibrio strain. Km for mannitol uptake is 7.69 µM.
FIGURE 6: Double-reciprocal plot of activity of mannitol-1-phosphate (Man-1-P) dehydrogenase of a Vibrio strain at various concentrations of Man-1-P. 1 Unit of dehydrogenase activity (U) is equivalent to 1 µmol of NAD converted, min⁻¹ (see Materials and Methods). Km for Man-1-P activity is 630 µM.
TABLE 7

Specific activities of Man-1-P dehydrogenase in all extracts of *Vibrio* grown in 1/10 SWB with the indicated carbohydrate additions. Enzyme levels are expressed as U. mg protein⁻¹, where 1 Unit is equivalent to 1 μmol of NAD converted min⁻¹ (see Materials and Methods).

<table>
<thead>
<tr>
<th>Addition to growth medium</th>
<th>Specific activity (U. mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% mannitol</td>
<td>2.03</td>
</tr>
<tr>
<td>0.2% mannitol + 0.2% glycerol</td>
<td>1.97</td>
</tr>
<tr>
<td>0.5% glycerol</td>
<td>0.33</td>
</tr>
<tr>
<td>0.5% glucose</td>
<td>0.19</td>
</tr>
</tbody>
</table>
**TABLE 8**

Mannitol transport and utilization by *Vibrio mtl-* mutants. These have been scored as follows:

- **0 - 20% of wild type (WT) activity** = 
- **20 - 70% of** = +
- **70 - 100% of** = +

ND = not determined

<table>
<thead>
<tr>
<th>Strain</th>
<th>Uptake</th>
<th>Incorporation</th>
<th>Phosphorylation</th>
<th>Man-1-P</th>
<th>Type of mutant</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-</td>
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and dehydrogenase components, but nevertheless failed to produce fermentation end products on mannitol indicator plates. These strains probably have lesions in the components of the fermentation pathway itself.

**Pseudomonas mannitol transport**

The oxidative *Pseudomonas* strain was shown to possess an inducible mannitol uptake system (Figure 7). Figure 8 is a reciprocal plot of the rate response of the transport system to different mannitol concentrations, giving a $K_m$ of 25 $\mu$M. This strain did not have Man-1-P dehydrogenase activity, but did possess a mannitol dehydrogenase, with a $K_m$ of 2.5 mM (Figure 9).

### 3.4 DISCUSSION

Heterotrophic bacteria are classified as either oxidative or fermentative, depending on the acid production which may result from sugar degradation. The strategies with which these two types of bacteria deal with the uptake and metabolism of sugars differ considerably. In the case of mannitol, the sugar is oxidized and phosphorylated, or vice versa, with the end product being fructose-6-phosphate in both situations. The latter process occurs in facultative anaerobes, where transport is mediated by a mannitol PTS whereas the former occurs in aerobes (Romano *et al.*, 1970; 1979). Two kelp bed bacterial strains were examined with respect to their mannitol transport systems, in order to test this generality.
FIGURE 7: Mannitol uptake by a Pseudomonas strain. Induced cells (●) were grown in 1/10 SWB + 0.2% mannitol, and uninduced cells (■) in 1/10 SWB.
FIGURE 8: Double-reciprocal plot of initial rates of mannitol uptake by a *Pseudomonas* strain. K_m for mannitol transport is 25 \( \mu \text{M} \).
FIGURE 9: Double-reciprocal plot of activity of mannitol dehydrogenase of a Pseudomonas strain at various mannitol concentrations. 1 Unit of dehydrogenase activity (U) is equivalent to 1 μmol of NAD converted min⁻¹ (see Materials and Methods). Km for mannitol dehydrogenase activity is 2.5 mM.
The fermentative strain, a *Vibrio*, has a constitutive, rapid mannitol uptake system, which results in mannitol entering a pathway mediated by Man-1-P dehydrogenase. Evidence in favour of the *Vibrio* mannitol uptake being carried out by a PTS is as follows:

1. The absence of mannitol dehydrogenase and presence of Man-1-P dehydrogenase presumably implies that Man-1-P is the end product of transport.

2. [¹⁴C] mannitol is rapidly converted to a negatively charged ('phosphorylated') derivative, which adsorbs to DEAE cellulose.

3. The isolation of mutants able to take up and 'phosphorylate' mannitol, but lacking Man-1-P dehydrogenase enforces the likelihood that the transport and phosphorylation processes are coupled, and occur before the dehydrogenase acts in the sequence of reactions.

It is interesting to note that one of the mannitol mutants isolated (MC) reverted rapidly. This is unusual, as the mutagen NTG usually generates relatively stable mutations (Carlton and Brown, 1981). The mutant MB, which appears to have reduced levels of all the mannitol-associated functions tested, may have multiple lesions. This is possible if the genes for these functions are grouped together, as NTG is known to induce multiple mutations in a small region of the chromosome. Two other explanations are feasible: An intermediate amount (relative to the wild type activity) of mannitol transported into the cell, due to a defective transport system, may cause a lower level of induction of the dehydrogenase.
Alternatively, this may be a polar effect, where genes further from the promoter of an operon are transcribed less frequently than those that are closer. It is also possible that the regulatory region of a group of 'mannitol genes' has been mutated.

Proof that a mannitol PTS exists in this *Vibrio* strain requires identification of sugar specific components of the transport system (EII) in cell membranes, with possible EIII membrane associated proteins, and demonstration of their absence in mannitol transport mutants. If a mannitol PTS operates, Man-1-P must accumulate intracellularly, and this can be measured by chromatographic methods after a Dowex resin procedure to obtain the sugars from cell extracts (Kundig and Roseman, 1971a).

A feature of all the mannitol PTSs identified is that they are inducible (Table 6), whereas the *Vibrio* under study here appears to have a constitutive transport system. It is possible that the *Vibrio* system is induced by trace amounts of mannitol that could be present in the low concentrations of yeast extract or peptone (0.01% and 0.05% respectively) used as a growth medium. When casamino acids with and without mannitol are used to culture the *Vibrio*, the difference in uptake rates is only 2 fold. The induction phenomena mentioned in the various genera in Table 6 may be as great as 100 fold (*Spirochaeta aurantia* - Saier and Newman, 1976), although the constitutive level of the mannitol PTS can be appreciable (*Salmonella typhimurium* - Hays, 1978).
Hays (1978) states that glucose, fructose and mannose may be considered as 'main line' PTS substrates, and bacteria usually possess constitutive PTSs for these sugars, and inducible systems for the other non 'main line' PTS sugars. He also notes that inducible and constitutive systems may be present for the same sugar in a bacterium, but that these have different affinities. Kelp bed bacteria exist in a habitat where the most predominant sugar is mannitol - it may thus be a 'main line' substrate for them.

Possession of an inducible dehydrogenase may account for the difference in incorporation of mannitol that occurs in cells grown in 1/10 SWB with and without mannitol (Figure 4). Man-1-P dehydrogenase facilitates the entry of Man-1-P into biosynthetic pathways as fructose-6-P, after which it would appear in TCA precipitable material. Uninduced cells would accumulate an intracellular pool of Man-1-P during the assay period. In the case of a buildup of intracellular Man-1-P during culturing, growth stasis would be expected, as occurs in E. coli Man-1-P dehydrogenase mutants exposed to mannitol (Solomon and Lin, 1972).

The Vibrio strain's constitutive uptake system and inducible dehydrogenase is in direct contrast to the E. coli mannitol PTS, which has coordinate regulation of induction of the EII^mtl and Man-1-P dehydrogenase. This is as a result of the close linkage of the genes for EII^mtl (mtlA), Man-1-P dehydrogenase (mtl D), and the regulatory gene (mtl C), which are situated on a single operon (Lee et al., 1981). It would be interesting to discover if the mannitol
operon from *E. coli* on the plasmids constructed by Lee *et al.*, (1981), could complement the mannitol transport and dehydrogenase *Vibrio* mutants isolated in this study.

It has been shown by various workers (Saier *et al.*, 1978; Dills *et al.*, 1980; Nelson *et al.*, 1982) that the PTS (in particular, the glucose PTS) functions not only in phosphorylation and transportation of PTS sugars, but plays a dominant role in a phenomenon called inducer exclusion. Inducer exclusion may be defined as the inhibition of certain non-PTS sugar transport systems by components of the PTS preventing entry of inducer molecules. Nelson *et al.*, (1983) have shown that in *S. typhimurium*, non phosphorylated EIII\textsubscript{glc} (glucose-specific EIII) can bind to the lactose carrier and inhibit its functioning, which represents regulation of a non PTS transport system.

A different type of regulation occurs when glycerol metabolism is inhibited by interactions of EIII\textsubscript{glc} and glycerol kinase, the first enzyme involved in glycerol metabolism, and not with the membrane bound glycerol facilitator (Postma *et al.*, 1984). The PTS can partially regulate intracellular cyclic adenosine monophosphate (cAMP) levels by interacting with adenylate cyclase, although the mechanism of action is uncertain (Nelson *et al.*, 1982).

Although the kelp bed *Vibrio* strain probably has a PTS for mannitol uptake, this does not exclude the possibility that other mannitol uptake systems may function in this bacterium under different
conditions. The oral streptococci examined by Keevil et al. (1984), all contained significant glucose PTS activity (see also Calmes, 1978). However, the PTS activity was insufficient to account for in vivo glucose uptake rates, and Keevil et al. (1984) were able to demonstrate intracellular accumulation of free glucose, driven by the proton motive force. This alternative glucose uptake mechanism could only be detected under conditions where the PTS was inactivated, as occurred in a low pH environment.

A non PTS mannitol uptake system is indicated in the kelp bed *Pseudomonas* strain, that has an inducible transport activity, and possesses a soluble mannitol dehydrogenase, but no Man-1-P dehydrogenase. The kinetic constants for transport (Km = 25 μM) and the mannitol dehydrogenase (Km = 2.5 mM) are similar to those reported for *P. aeruginosa*, which has a mannitol uptake system and a mannitol dehydrogenase with Km values of 14 μM and 5.2 mM respectively (Eagon and Phibbs, 1971, Eisenburg and Phibbs, 1982).

Eisenburg and Phibbs (1982) have purified a mannitol periplasmic binding protein from *P. aeruginosa*, but other components of the transport system have not been isolated. It is envisaged that proteins similar to the *E. coli* maltose transport proteins may exist, where an outer membrane porin and cytoplasmic membrane permease interact with the maltose binding protein to bring about the transport of that sugar. This interdependence between binding proteins and membrane permeases also occurs in some amino acid transport systems. For example, two components necessary for histidine transport in *E. coli* are a binding protein (Kustu and Ames, 1974) and a permease (Ames and Spudich, 1976).
Several attempts to demonstrate the presence of a periplasmic mannitol binding protein in the kelp bed *Pseudomonas* were unsuccessful (data not presented). The marine bacterium may have different sensitivity to osmotic conditions from *P. aeruginosa*, in which case the osmotic shock procedures used to obtain periplasmic proteins (Willis *et al.*, 1974, and Stinson *et al.*, 1976) would be ineffective. Induced kelp bed *Pseudomonas* bacteria did lose their mannitol transport activity after both types of osmotic shock, but this loss was possibly caused by damage to the cytoplasmic membrane, rather than loss of binding proteins.

Cell envelope stability of a marine *Vibrio* during various types of osmotic shock was examined by Geesey and Morita (1981), in their study of that organism's arginine binding protein. They found that complete removal of seawater salts (as used in osmotic shock procedures), results in structural alterations in the cell envelope. They also noted that the large amount of arginine-binding activity recovered in the shock fluid of cells treated with Tris-EDTA may have been due to the release of cytoplasmic constituents able to bind arginine, since 27% of the total cell protein was released under these conditions. The question of cytoplasmic proteins occurring in the shock fluid was dealt with by Eisenburg and Phibbs (1982), in their work on the *P. aeruginosa* mannitol binding protein. They demonstrated that the crude extract containing mannitol dehydrogenase (the only other mannitol induced protein in *P. aeruginosa* known to have a mannitol binding site), contained no demonstrable mannitol binding activity in the filtration assay they used to measure the presence of mannitol binding protein.
The nature of the mannitol transport system of the kelp bed
*Pseudomonas* is thus largely unknown. It has already been stated that
a mannitol PTS is unlikely, as this type of system is not common in
aerobes. The only PTS described in oxidative bacteria is that for
fructose transport, discovered by Baumann and Baumann (1975), in
*Pseudomonas doudoroffii*. This marine bacterium dissimilates
phosphorylated fructose via the Embden Meyerhof pathway, unusual in
aerobes. This is in accord with the predictions of Romano et al.,
(1979). *P. aeruginosa*, classified as an aerobic organism, can be
grown anaerobically if nitrate is provided as a respiratory electron
acceptor (Hunt and Phibbs, 1981). Under aerobic and denitrifying
anaerobic conditions, the cells have two alternative methods for
taking up glucose, neither of them involving a PTS (Hunt and Phibbs,
1983).

The 2 kelp bed isolates studied thus have very different mannitol
uptake and dissimilation mechanisms. The *Vibrio* constitutive uptake
system takes up mannitol at a faster rate (8 fold) than the induced
*Pseudomonas* system. This indicates that the *Vibrio* may be more
effective in 'mopping up' mannitol when it becomes available in the
kelp bed. However, possession of a mannitol transport system that
can be 'switched off' by the *Pseudomonas*, may give this strain an
advantage during periods of low nutrient availability, as energy
would not have to be expended on maintenance of the system (see
Chapter 4).
In general, there are more nonfermentative than fermentative bacteria found in the water column, whereas the facultative anaerobes (Vibrio strains) examined in this study were isolated from the kelp plants. Other fermentative strains, isolated independently from various parts of the kelp fronds (described in Chapter 2), showed the rapid mannitol uptake of the Vibrio strain (data not shown). They also possessed the Man-1-P characteristic of a mannitol PTS. These fermentative bacteria, being in close proximity with the kelp fronds, may be important in the utilization of leached material (including mannitol) released by the kelp plants.
SUMMARY

The starvation survival responses of various kelp bed isolates were tested. A proportion of the cells in cultures of *Vibrio*, *Pseudomonas* and *Flavobacterium* strains remained viable for long periods without exogenous energy. By comparison, a smaller proportion of *Escherichia coli* cells remained viable under the same conditions. During starvation, some of the kelp bed strains underwent fragmentation, and cell size of all the isolates tested decreased. Uptake systems, and the potential for resiping mannitol, glucose, glutamate, aspartate and alanine were maintained by the strains during starvation.

The kinetic constants for mannitol, glucose and glutamate, measured during nutrient deprivation in a *Vibrio* strain showed some changes after about five weeks starvation. Induction of the mannitol transport system in a *Pseudomonas* strain after a period of starvation was observed. Induction was found to be dependant on RNA and protein synthesis. Mannitol negative mutants were compared in co-culture with the mannitol utilizing isogenic *Vibrio* strain. The mannitol transport deficiency did not alter the specific survival rate unless mannitol was introduced into the culture flasks, when positive selection for the wild type mannitol positive strains occurred.
4.1 INTRODUCTION

Many marine bacteria are adapted to remain viable under the low nutrient conditions that exist in the ocean. Extensive work on the response to nutrient deprivation has been carried out using marine Vibrio and Pseudomonas strains (Novitsky and Morita, 1976, 1978, Tabor et al., 1981, Torrella and Morita, 1982, Amy and Morita, 1983a, 1983b, Kurath and Morita, 1983, Morita, 1984). This includes studies on changes in viability, morphology, metabolism and chemotaxis, which occur when the bacteria are subjected to starvation conditions.

Morita (1982) has coined the term 'starvation-survival', to describe the process of survival in the absence of energy yielding substrates. Amy and Morita (1983a) have pointed out that starvation survival is important in ecology, as it is a mechanism for the survival of a species, in that the genome will persist and be able to express itself when environmental conditions become favourable.

Examples of other oligotrophic habitats where bacteria may encounter starvation conditions are soil and some fresh water environments. In all these environments, bacteria have been isolated that are adapted to survive starvation. Examples of such bacteria are Arthrobacter, Bacillus and Rhizobium spp. that occur in soil (Boylen and Ensign, 1970, Nelson and Parkinson, 1978, Poindexter, 1981, Crist et al., 1984), and Caulobacter and Spirillum spp. (Matin and Veldkamp, 1978, Poindexter, 1981), which are both found in fresh and seawater.
Stevenson (1978) has proposed that dormancy is an important physiological adaption contributing to the survival of bacteria facing stress situations like starvation. Dormancy has been defined by Sussman and Halvorson (1966) as "any rest period or reversible interruption of the phenotypic development of an organism". They differentiate between constitutive and exogenous dormancy, the former being represented in bacteria by spore formation. Exogenous dormancy is defined as "a condition in which development is delayed because of unfavourable chemical or physical conditions of the environment", and this condition is addressed here.

In 1976 Novitsky and Morita reported that ANT 300, a psychrophilic marine Vibrio, decreased in size and changed in shape from a rod to coccobacillus upon starvation. Further work (Novitsky and Morita, 1977) showed that during the first week of starvation of ANT 300, there was a large increase in the number of viable cells. This period of 'fragmentation' without actual growth was followed by a decline in viable cell numbers, with 50% of the cells remaining viable for 6 - 7 weeks. A proportion of the population remained viable for more than a year, and in fact the time needed to completely eliminate a population of ANT 300 was not determined (Novitsky and Morita, 1978). The success of this bacterium in withstanding starvation must rest partly on the fact that its endogenous respiration rate dropped to less than 1% in 7 days (Novitsky and Morita, 1977), and that its ability to metabolize substrates quickly was retained during starvation (Novitsky and Morita, 1978).
In order to determine whether ANT 300 was unique in the starvation survival processes, Amy and Morita (1983a) carried out similar studies on 16 freshly isolated open ocean bacteria. They found that 3 starvation survival patterns occurred among the isolates during starvation, but in each case a constant viability was reached, and the cell size decreased. All strains tested were able to utilize the substrate glutamate after starvation.

A detailed study by Kurath and Morita (1983) showed that a marine Pseudomonas also had a successful starvation survival response. Although cultures of the strain showed a 99.9% decrease in viable cell count during the first 25 days of starvation, 10^5 viable cells per ml were maintained during a year's starvation. At 40 days starvation, the endogenous respiration of this Pseudomonas was 0.007% of the original level, similar to that for ANT 300.

Kjelleberg et al. (1983) studied the initial phases of starvation and activity of a marine Vibrio and Pseudomonas. They termed the initial period of response the 'dwarfing phase', and divided this into 2 processes: fragmentation, and continuous size reduction of the fragmented cells. By measuring O_2 uptake, and using inhibitors of proton flow, electron transport chain, and membrane-bound ATPase, the dwarfing phase was shown to be one of intense metabolic activity.

Changes in macromolecules and other intracellular compounds during starvation have been monitored in ANT 300. The DNA concentration decreased by 46% in 6 weeks starvation (Novitsky and Morita, 1977).
In a *Pseudomonas* sp., it was found that starved cells have more ATP per viable cell than non-starved cells (Kurath and Morita, 1983). The technique of protein fingerprinting was used to show that there is degradation of specific proteins in ANT 300 during starvation, and that some proteins are only synthesized during starvation (Amy and Morita, 1983b).

The importance of 'substrate capture' by starved bacteria, or by bacteria in oligotrophic waters has been stressed by Morita (1984). It is by this means that bacteria can scavenge nutrients from the environment, to be transported into the cell and used to replenish energy stores within the cell. Predominant forms of nutrients that may be encountered as DOC in the marine environment are sugars, amino acids and organic acids. As mentioned earlier, some starved marine bacteria retain the ability to utilize glutamate and glucose, and the initial step in this process is substrate capture.

ANT 300 was found to possess a high affinity transport system for glutamate, which was maintained during starvation (Glick, 1980, reported in Morita, 1984). Another amino acid transport mechanism studied in ANT 300 was that for arginine uptake. Geesey and Morita (1979) established that both a high and low affinity system for arginine transport existed in the *Vibrio*. In a later report (Geesey and Morita, 1981) an arginine binding protein was implicated in the formation of a loosely bound amino acid pool during initial binding or capture. The effects of long term starvation on the high affinity transport system were studied by Faquin and Oliver (1984), who found that a reduced but constant rate of uptake was maintained during starvation.
The challenging of starved bacteria with low concentrations of sugars or amino acids, leads to the transport of those nutrients into the cells, but does not usually result in cell division. The exact nutritional requirements necessary to bring about recovery from starvation and growth are unknown. However, the recovery response of starved ANT 300 cells on the addition of rich medium has been examined (Amy and Morita, 1983b, Amy et al., 1983b). Cell size and viable cell count increased, and the length of the lag phase during recovery was directly proportional to the length of the starvation period. On a per cell viable basis, protein, RNA and DNA increased to maximum values just before cell division, and then returned to close to the initial starved-cell value during stationary phase. Thus the dormant state brought on by starvation is reversible.

An interesting recovery phenomenon was noted by Kjelleberg et al., (1982), who showed that starved marine Vibrio cells behave differently at interfaces (air-water; solid-water), as compared with those in the aqueous phase. Nutrients added to the air-water interface produced larger cells at the surface. Regrowth and cell division at a solid-liquid interface occurred quickly, and at nutrient concentrations too low to permit growth in the aqueous phase. It was concluded that if starved cells could reach an interface, additional survival mechanisms became available to them, as interfaces constitute areas of favourable nutrient conditions.

The nearshore bacteria isolated from the kelp beds may possibly exhibit a starvation survival response similar to that of the offshore bacteria studied.
Kelp bed bacteria encounter a wide range of DOC levels, both within the kelp bed, and further from the coast, when water column bacteria are transported to offshore waters during upwelling-downwelling cycles (Koop et al., 1982a). In the kelp bed, dissolved organic carbon (DOC) levels may rise to 20 mg.l\(^{-1}\) during rough weather, and fall to 5 mg.l\(^{-1}\) within 24 hours of the onset of calm weather (Newell et al., 1980). Field et al., (1981) have reported much lower values of 0.5 - 2 mg.l\(^{-1}\) DOC measured in the kelp bed. Offshore water conditions may be described as oligotrophic (that is, having very low nutrient levels). Morita (1984) has quoted DOC concentrations for the open sea being in the range of 0.3 - 1.2 mg C.l\(^{-1}\). Particulate organic carbon (POC) is usually an order of magnitude lower. Kelp bed bacteria are presumably adapted to a 'feast-or-famine' situation as described by Koch (1971) for \textit{E. coli}, rather than the continuous 'famine' that open sea bacteria encounter.

The aim of this study was 5 fold:

1. To determine whether various nearshore kelp bed isolates possess a starvation-survival response similar to that of the offshore bacteria described.

2. To measure the uptake of various sugars and amino acids during starvation, in order to determine whether maintenance of substrate capture and transport mechanisms are an important part of the starvation survival response.
3. To examine the fate during starvation of the constitutive and inducible mannitol uptake systems of the Vibrio and Pseudomonas bacteria detailed in Chapter 3.

4. To determine whether the substrates are incorporated into macromolecules, respired, or remain in the intracellular pool after they are transported into the cell.

5. To measure the effect of possession of a carbohydrate transport system during starvation, by comparing the responses of the Vibrio mtl+ (mannitol wild type) strain, with those of a mutant unable to transport mannitol (mtl−), and the effect on recovery of the strains from starvation.

4.2 MATERIALS AND METHODS

Starvation conditions

Cells were grown for 16 h in 1/10 SWB, washed twice, and resuspended in the same volume of SM buffer. This gave an initial viable cell density of 10^7-10^9 cells ml⁻¹, depending on the strain. The cultures were starved (without shaking) at 10°C in a cooling water bath. Samples were withdrawn aseptically at appropriate times. Vibrio alginolyticus was grown in Vibrio salts medium with glucose (2.5 g.1⁻¹, w/v) added as a carbon source. E. coli B was grown in Luria broth. Starvation buffer for V. alginolyticus and E. coli were Vibrio salts and M9 salts respectively. The media and buffers are detailed in the Appendix.
Viability, total cell counts, OD and protein content

Viability was determined by plating appropriate dilutions of kelp bed bacteria and *V. alginolyticus* on SWA, and *E. coli* on Luria agar. Total cell counts were determined by AODC (see Chapter 2). OD600 was determined on an MSE spectrophotometer. Protein was measured using the Folin reagent (Lowry *et al.*, 1951).

Uptake, incorporation and respiration of substrates

Uptake and incorporation were measured as described for mannitol in Chapter 3. The amount of label lost by respiration was determined by sampling 50 µl of the assay mix at various times and measuring the total radioactivity present. Any decrease in total cpm is due to loss of 14CO2.

Specific activities of radiolabelled substrates (all from Amersham) were as follows: D[1-14C] mannitol, 59 mCi/mmol; D[1-14C] glucose, 55 mCi/mmol or 284 mCi/mmol; L[U-14C] glutamic acid, 280 mCi/mmol; L[U-14C] alanine, 165 mCi/mmol; L[U-14C] aspartic acid, 224 mCi/mmol. Final concentrations in assays were: mannitol, 11.0 µM; glucose, 11.8 or 2.9 µM; glutamic acid, 1.2 µM; alanine, 1.9 µM; aspartic acid, 1.5 µM. (Glucose of two specific activities was used: the higher specific activity sugar was used in experiments where the uptake of all 5 substrates by a number of different strains was measured, and the lower specific activity glucose was used for Km experiments with the *Vibrio* strain).
FIGURE 11: The decrease in OD_{600} of kelp bed strains during starvation. Vibrio (▲, △), Pseudomonas (■, □) and Flavobacterium (●) strains. Cells were grown in 1/10 SWB, harvested, washed and resuspended in SM salts. Aliquots were withdrawn at weekly intervals, and the OD read at 660 nm.
FIGURE 12: Protein content of starvation menstruum containing kelp bed bacterial strains. *Vibrio* (▲, △), *Pseudomonas* (■, □) and *Flavobacterium* (●) strains. Cells were grown in 1/10 SWB, harvested, washed, and resuspended in SM salts. Aliquots were removed at various times during starvation, and the protein content of these samples determined.
critical factors for storage during starvation, the effect of temperature of starvation was tested. The same initial culture of 2 strains was held in starvation buffer at 2 temperatures (Table 9). The *Pseudomonas* has a slightly lower viable cell count at the lower temperature, whereas the *Vibrio* has similar numbers of cells surviving at both temperatures. During all the experiments, bacteria were allowed to stand during starvation, and not agitated. However, before sampling from a bottle containing starved cells, the bacteria were resuspended by shaking the bottle. The frequency of sampling thus determined how often the bacteria were 'aerated', and this influenced viable cell numbers, as shown in Table 9. Cells (of both strains) that were left undisturbed showed higher numbers of viable cells after 5 weeks of starvation. Thus 2 factors were identified that may cause variability in starvation survival experiments.

Table 10 presents the results of a 3 month starvation experiment to determine whether total bacterial counts were higher than the viable counts at the end of that period. This did occur, and the plateability of the strains tested varied between 1% and 31%.

The decrease in cell size during starvation inferred from earlier results in this chapter, was shown to occur (Table 10). Starvation menstruum containing 3 month starved cells was passed through a 0.45 µm filter, and the filtrate was plated. Four of the five strains tested had formed some cells smaller than 0.45 µm, which passed through the filter and formed colonies. The menstruum of one *Pseudomonas* strain passed through a 0.22 µm filter after 3 months of starvation contained viable cells, however this did not occur when the experiment was repeated.
TABLE 9

The effect of various treatments on bacterial survival (measured as viable cell numbers) during starvation of the isolates.

<table>
<thead>
<tr>
<th>Starvation period</th>
<th>Treatment</th>
<th>Vibrio cell count</th>
<th>Pseudomonas cell count</th>
</tr>
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<tbody>
<tr>
<td>3 months</td>
<td>Storage temperature:</td>
<td></td>
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<tr>
<td></td>
<td>10°C</td>
<td>5.8 x 10^5</td>
<td>1.5 x 10^5</td>
</tr>
<tr>
<td></td>
<td>22°C</td>
<td>5.0 x 10^5</td>
<td>3.9 x 10^5</td>
</tr>
<tr>
<td>5 weeks</td>
<td>Undisturbed</td>
<td>3.8 x 10^7</td>
<td>1.0 x 10^7</td>
</tr>
<tr>
<td></td>
<td>Mixed weekly</td>
<td>1.9 x 10^7</td>
<td>5.2 x 10^6</td>
</tr>
</tbody>
</table>
# TABLE 10

Total and viable cell counts of bacteria starved for a three month period. The % plateability was calculated as follows:

\[
\text{Viable cell count} \times 100.
\]

The number of cells in the starved populations smaller than 0.45 μm was determined by filtration through a 0.45 μm filter and plating of the filtrate. (ND = Not Determined)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable cell count (cells/ml)</th>
<th>Loss in viability (P₀ / P₃)</th>
<th>AODC (cells/ml⁻¹)</th>
<th>Plateability (%) (3 months)</th>
<th>≤ 0.45 μm (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 0</td>
<td>t = 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio</td>
<td>1.4 x 10⁹</td>
<td>2.3 x 10⁶</td>
<td>6.1 x 10²</td>
<td>1.4 x 10⁸</td>
<td>2</td>
</tr>
<tr>
<td>Vibrio</td>
<td>4.0 x 10⁹</td>
<td>3.3 x 10⁷</td>
<td>1.2 x 10²</td>
<td>2.5 x 10⁸</td>
<td>13</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>3.2 x 10¹⁰</td>
<td>6.9 x 10⁶</td>
<td>4.6 x 10³</td>
<td>1.8 x 10⁸</td>
<td>4</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>4.0 x 10¹⁰</td>
<td>1.1 x 10⁷</td>
<td>3.6 x 10³</td>
<td>3.5 x 10⁷</td>
<td>31</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>8.0 x 10⁷</td>
<td>6.2 x 10⁵</td>
<td>1.3 x 10²</td>
<td>4.3 x 10⁷</td>
<td>1</td>
</tr>
<tr>
<td>V. alginolyticus</td>
<td>1.2 x 10⁹</td>
<td>1.2 x 10⁷</td>
<td>1.0 x 10²</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E. coli</td>
<td>5.5 x 10⁸</td>
<td>4.5 x 10³</td>
<td>1.2 x 10⁵</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Sampling times t=0: 0 and 3 months respectively
Plating to determine viability was carried out on SWA (high nutrient) and 1/10 SWA (low nutrient) plates. It was found that there was no difference in colony numbers on the 2 types of plates. Thus the decline in colony forming units during starvation was probably not due to the inability of starved cells to grow in the presence of the high nutrient levels in SWA plates.

The response of *V. alginolyticus* and *E. coli* to starvation is shown in Table 10. *V. alginolyticus* showed a similar loss of viability \((P_3/P_0)\) to the kelp bed isolates, whereas the *E. coli* cell numbers had dropped to well below the 'maintenance' level of the marine strains. Neither *V. alginolyticus* nor *E. coli* formed cells smaller than \(0.45 \mu m\) during this experiment.

**Maintenance of uptake systems**

Figures 13, 14 and 15 are the results of an experiment to determine whether the ability to transport and respire various substrates was maintained by *Vibrio*, *Pseudomonas* and *Flavobacterium* strains during starvation.

The *Vibrio* maintained a constant uptake activity for the sugars mannitol and glucose, and the amino acid alanine (Figure 13). Starved cells displayed an increased transport activity for the other amino acids tested, glutamate and aspartate. In general, respiration of the nutrients increased (per mg protein) during starvation. The transport and respiration have been expressed as nmoles substrate taken up or respired per mg cell protein in a 10 min or 20 hour assay.
FIGURE 13: Uptake and respiration of substrates by a Vibrio strain during starvation. Uptake of substrates (●) was measured in 10 min assays, and respiration (■) was determined after 20 h.
FIGURE 14: Uptake and respiration of sugars and amino acids by a Pseudomonas strain during starvation. Uptake of substrates (○) was measured in 80 min assays, and respiration (■) was determined after 20 h.
FIGURE 15: Uptake and respiration of sugars and amino acids by a Flavobacterium strain during starvation. Uptake of substrates (•) was measured in 80 min assays, and respiration (■) was determined after 20 h.
respectively, rather than per min or hour. This was done as the rate of uptake or respiration was not measured. Longer uptake assays (80 mins) gave slightly lower values, presumably as a result of loss of substrate as $^{14}\text{CO}_2$.

The longer assays were chosen for measuring the uptake of the 5 substrates by the Pseudomonas and Flavobacterium, as very little uptake activity was measured after 10 mins. Transport activity per mg protein increased during starvation, as did respiration. In both these strains, a greater proportion of the substrates is respired as $^{14}\text{CO}_2$, compared with the Vibrio.

**Vibrio mannitol, glucose and glutamate transport during starvation**

The kinetic constants for mannitol, glucose and glutamate transport were determined during starvation of the Vibrio strain. Michaelis Menten reciprocal plots of rates of uptake at various substrate concentrations were prepared, as described for mannitol in Chapter 3. The results are presented in Table 11.

The Km for mannitol transport after 8 weeks starvation was 1.47 µM, as compared with 7.24 µM at the start of the experiment. The lower affinity system present in unstarved cells persists for approximately 5 weeks during starvation. A similar apparent increase in substrate affinity was seen in glucose transport, although the difference between the Km values for unstarved and 5 week starved cells (6.11 and 4.33 µM respectively) was not as marked as in the case of the mannitol uptake systems.
TABLE 11

Km values for mannitol, glucose and glutamate uptake by a *Vibrio* strain during starvation. The average Km values from a number of determinations are presented.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Starvation period (weeks)</th>
<th>Km (µM)</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>0</td>
<td>7.24</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.71</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.69</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.28</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.41</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.47</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>6.11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.30</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.33</td>
<td>3</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0</td>
<td>0.83</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.50</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.83</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.25 or 0.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.23</td>
<td>2</td>
</tr>
</tbody>
</table>
Glutamate is transported into unstarved *Vibrio* cells by an uptake system with a $K_m$ of 0.83 µM. During starvation this constant decreases, and reaches 0.23 µM after 5 weeks.

**Pseudomonas mannitol transport during starvation**

The total mannitol uptake activity in the *Pseudomonas* strain was depressed 8 fold after 30 h starvation of induced cells (Figure 16). Cells grown prior to starvation in medium not containing the inducer mannitol, then starved for 6 weeks, were challenged with 11 µM [14C] mannitol (Figure 17). The mannitol transport system was induced after 5 h. This induction was not apparent if chloramphenicol (a protein synthesis inhibitor) or rifampicin (an RNA synthesis inhibitor) were present. Addition of phosphate and nitrate did not cause more rapid induction.

**Incorporation and respiration**

Figures 18 and 19 demonstrate the difference between the *Vibrio* and *Pseudomonas* strains when presented with mannitol, glucose, glutamate and alanine during dormancy (5 - 6 weeks starvation). In each strain, the response to the 2 sugars was similar, likewise with the 2 amino acids.

In the *Vibrio* strain, maximum uptake of the sugars occurred within an hour, and approximately half the sugar taken up was incorporated into macromolecules within 3 h of the start of the assay (Figure 18). The amount of sugar respired after 4 h approximately equals the quantity remaining in the cells.
FIGURE 16: Uptake of mannitol by a *Pseudomonas* strain, starved for 0 h (●) and 30 h (▲).
FIGURE 17: Induction of mannitol uptake in a Pseudomonas strain starved for 6 weeks. Additions to starved cells were as follows ('mannitol' = 11 µM [14C] mannitol): mannitol (○), mannitol + phosphate + nitrate (■), mannitol + rifampicin (▲), mannitol + chloramphenicol (×).
FIGURE 18: Uptake (●), incorporation (■) and respiration (○) of substrates by a Vibrio strain after 5 weeks of starvation.
FIGURE 19: Uptake ( ● ), incorporation ( ■ ) and respiration ( ○ ) of substrates by a Pseudomonas strain after 6 weeks of starvation.
Incorporation of the 2 amino acids into TCA precipitable material in the *Vibrio* reached a plateau after 1 h, whereas maximum uptake occurred within 30 min. Respiration of glutamate and alanine proceeded for 2 h and then stopped.

The *Pseudomonas* appeared to have an inducible glucose transport activity similar to the mannitol uptake system described in the previous chapter, and demonstrated again in Figure 19. Incorporation of mannitol and glucose was negligible for 3 h, after which time some incorporation of glucose occurred. In the case of both sugars, conversion into CO$_2$ increased dramatically as the transport system was induced.

The dominant feature of amino acid utilization by this strain is the relatively large amount of [$^{14}$C] label channelled into respiration. Uptake and respiration occurred at the greatest rate during the first hour of the assay. As the amount of [$^{14}$C] amino acid taken up stays relatively constant after 1 h, and does not decrease even though $^{14}$CO$_2$ produced increases for 2 h, some substrate must be taken up and respired immediately. Uptake rates are thus probably an underestimation.

An experiment was carried out with the *Vibrio* strain in order to determine what occurs with the substrates each at 2 concentrations. (The *Vibrio* cells had been starved for 3 weeks). Figure 20 shows that there was no difference in any of the 4 substrates tested in the proportion of substrate incorporated into TCA precipitable material, and similarly the percentage respired did not alter greatly. In the
FIGURE 20: Uptake (○) and incorporation (■) of substrates by a Vibrio strain starved for 3 weeks. Respiration (R) of the substrates after 3 h is represented by bars. Assays were performed at two concentrations of each substrate.
case of mannitol, glucose and alanine, the amount of substrate resired after 3 h approximately equalled the amount incorporated, whereas a far larger proportion of glutamate was resired than incorporated.

**Competition experiments**

The starvation survival response of one of the mannitol mutants isolated in Chapter 3 was tested, in order to determine whether lack of this particular transport activity affected survival. The viability of the WT and the mutant were virtually identical during a 5 week starvation period, as demonstrated in Figure 21.

Cells of the mutant and WT, starved separately for 2 months, were incubated in direct competition for a variety of substrates at different concentrations. The results of the experiment are presented in Figures 22 and 23. Control subsamples of starved WT and mutant cells kept unmixed, showed a 3 fold increase in cell numbers during the first day after subsampling (Figure 22). This is presumably as a result of being aerated and placed in a smaller container. The mixed population of WT and mtl- cells that had 100 µM glutamate or 100 µM mannitol added, showed a similar 3 fold increase in viable cell number. Total cell number increased almost 10 fold in the 3 mixed populations that had 1 mM glucose, 1 mM mannitol or 100 µM glutamate plus 1 mM mannitol added respectively. All major changes in cell numbers occurred within 1 d.
FIGURE 21: Viability of Vibrio mannitol wild type (WT●) and mutant (mtl− ■) strains during starvation. Viable cell numbers (P), determined by plating, are expressed as a fraction of the original population (P₀).
FIGURE 22: Viable cell numbers of co-cultures of *Vibrio* mannitol wild type (WT) and mutant (mtl-) in 'competition' for two weeks after the addition of various substrates. (WT and mtl- cells were starved separately for 2 months before the start of this experiment). Substrate additions: 100 µM mannitol (□), 1 mM mannitol ( ■ ), 100 µM glutamate ( ● ), 100 µM glutamate + 1 mM mannitol ( ○ ), 1 mM glucose ( ▲ ). Cell numbers of strains kept separately as controls: WT ( △ ), mtl- ( ▼ ).
An analysis of the proportions of WT and mtl- cells in each population is presented in Figure 23. The proportion of each cell type was determined by toothpicking colonies from SWA plates onto TZM indicator plates. The samples were not plated immediately onto TZM plates, as a variable proportion of cells were unable to form colonies on TZM after starvation.

Mtl- mutants constituted a larger proportion of the population than the WT in the subsample to which glutamate was added. The reason for this is unknown. A mixed population that was incubated with glucose (1mM) or a lower concentration of mannitol (100 µM) resulted in populations with a slightly higher proportion of WT cells. A dramatic increase in the proportion of WT cells to mutant bacteria was seen when the cells were incubated with high mannitol levels (1mM). The presence of 100 µM glutamate with this concentration of mannitol did not cause the mutant strain to increase its numbers to the same level as the WT.

After 2 weeks incubation of these mixed populations with the substrates, the WT : mutant ratios were 1.5 - 2.0 for all except the cells incubated with 1 mM mannitol. This indicates a loss of viability of the cells formed by division of the bacteria after addition of substrates. This is also recorded in the decrease in total cell numbers (Figure 22).
FIGURE 23: The proportion of *Vibrio* mannitol wild type (WT) to mutant (mtl−) cells after the addition of various substrates to co-cultures of the strains starved for 2 months (see Figure 22). Substrate additions: 100 µM mannitol (□), 1 mM mannitol (■), 100 µM glutamate (●), 100 µM glutamate + 1 mM mannitol (○), 1 mM glucose (▲).
Bacteria isolated from the kelp beds exhibited a starvation survival response similar to that described for offshore strains (Novitsky and Morita, 1976, 1978, Amy and Morita, 1983a, 1983b, Tabor et al., 1981). Three patterns of viability were observed during starvation of the nearshore Vibrio, Pseudomonas and Flavobacterium strains. The patterns were not restricted to particular species, as the 2 Pseudomonas strains tested exhibited different changes in viability. The viability patterns of 16 open ocean bacteria monitored by Amy and Morita (1983b) fell into 3 groups similar to those found in starved kelp bed bacteria.

A feature of the starvation survival response is a decrease in cell size, and the kelp bed bacteria showed this characteristic. Tabor et al., (1981) have found that a significant relationship exists between decreased cell size and increased survival of marine bacteria. This may be because the surface area : volume ratio increases, which is favourable for factors like nutrient assimilation.

An important factor determining bacterial cell size by filtration is filter type. Starved ANT 300 (Vibrio) cells were able to pass a 0.4 µm polycarbonate filter, but were retained by a 0.45 µm cellulose filter (Novitsky and Morita, 1976). Millex filters (cellulose) were used in this study, thus the numbers of 'filterable' bacteria given in Table 10 may be an underestimation.
An increase in cell numbers accompanied by a decrease in OD\textsubscript{600} as occurred in some of the kelp bed strains during starvation, is indicative of the fragmentation process described by Novitsky and Morita (1977) and Kjelleberg et al. (1983). (It is assumed that the 2 groups of workers described the same process, although the one group observed fragmentation during a period of days, rather than hours, as noted by the other group). During fragmentation, cells 'break up' into smaller 'dwarf' cells that are viable. Novitsky and Morita (1976), reported that 50% of a 3 week starved population of ANT 300 passed through a filter with a 0.45 µm pore size. This may be because fragmentation is irregular, giving rise to cells larger and smaller than 0.45 µm, or because all the cells passing through the filter may not be able to form colonies on the medium used to determine cell number. A feature of the starvation survival and fragmentation process is that 'non viable' cells (cells that do not form colonies) do not lyse, as indicated by the AODC levels remaining high when viable cell counts decrease.

Fragmentation of cells during starvation differs from minicell formation as occurs in \textit{E. coli}, as the latter process results in anucleated cells, whereas miniaturized starved cells have nuclei. Non starved marine bacteria such as ANT 300 may have from 1.4 to 4.0 nuclear bodies per cell (Novitsky and Morita, 1977). The complete nuclei which comprise the nuclear bodies are distributed amongst the cells during fragmentation, thus DNA synthesis is not required. This has been verified by the use of nalidixic acid, which did not prevent an increase in viable cell number when added to cultures of ANT 300 at the start of starvation (Novitsky and Morita, 1977). A similar
experiment was carried out on a kelp bed *Vibrio* strain, and starved cells treated with nalidixic acid showed the same increase in cell number as untreated cells (data not shown).

Humphrey *et al.* (1983) are of the opinion that dwarfing (fragmentation) is a process mediated by constitutive enzymes. The protein synthesis inhibitor, chloramphenicol, did not affect the size decrease of cells during starvation. However, dwarfing was reversibly inhibited by low temperature (0°C) and low pH (5.3).

During starvation survival, various factors may influence the percentage survival of the original population. Novitsky and Morita (1978) showed that as the density of various populations of ANT 300 decreased, there was a magnified initial increase in viable cell numbers and increased longevity. This 'population effect' was not tested with the kelp bed bacteria. However, it was shown that these bacteria were affected by the degree of 'aeration' during starvation. A similar effect has been reported by Robb *et al.* (1980), and Robb *et al.* (1982), who were working on *V. alginolyticus* (Achromobacter), which is possibly of marine origin. Stationary phase cultures differed physiologically and morphologically, depending on the aeration conditions. In comparison with non-aerated standing cultures, vigorously aerated cultures showed a decrease in viability, as well as increased protein synthesis levels, but decreased RNA synthesis, membrane transport and intracellular ATP levels.
The possibility that the increase in cell numbers demonstrated in some kelp bed bacterial strains is due to cryptic growth (growth on materials released from dead cells), cannot be discounted. This is unlikely however, as it has been calculated that it is necessary for several cells to die to permit the doubling of each remaining cell (Druilhet and Sobek, 1976). In addition, attempts by Kurath and Morita (1983) to show that a starved marine *Pseudomonas* utilized nutrients leached from nonviable cells in the culture were unsuccessful.

Having established that a high proportion of the bacterial cells remained viable under starvation conditions with no exogenous energy source present, the extent to which active transport systems for substrates were maintained was examined. Using *Vibrio*, *Pseudomonas* and *Flavobacterium* strains, it was found that, in general, the bacteria were able to take up and respire the 2 sugars and 3 amino acids tested, although the responses of the strains showed different utilization patterns.

Amy and Morita (1983a), and Kurath and Morita (1983) found that various marine isolates all maintained the ability to take up and respire glucose and glutamate. Amy and Morita (1983a) also reported cellular incorporation of these 2 compounds. They point out that the ability to produce CO\(_2\) from the substrates is a good indication that the metabolic systems necessary to produce energy in the starved cells remain intact. It is interesting to note that the kelp bed bacteria can utilize mannitol as well as glucose when starved, although mannitol is not an 'essential' nutrient as particular amino acids may be, nor is it a 'mainline sugar' according to Hays (1978).
Comparisons of rates of uptake and respiration during starvation of different bacterial strains are difficult to make. This is from lack of knowledge about which cells in a population are responsible for the activity, as all the cells in a starving culture are not physiologically the same. Kurath and Morita (1983) showed that glucose and glutamate uptake decreased per ml of culture during starvation, but increased when calculated per number of respiring cells. Faquin and Oliver (1984) have calculated the arginine uptake per AODC numbers during starvation of ANT 300. Kurath and Morita (1983) state that the actively respiring subpopulation, rather than the viable or total cell numbers is the most appropriate denominator for interpretation of observed activities. The actively respiring proportion of the kelp bed bacteria was not determined during starvation, thus this could not be used in the present study.

A measurement in substrate transport studies that is independent of cell numbers is the affinity constant or Km. The mannitol uptake system (Km = 7.6 µM) of the Vibrio strain, described in Chapter 3, was maintained for approximately 5 weeks. At this time the Km decreases. This may be indicative of a higher affinity system dominating the mannitol uptake process as starvation of the cells progresses. Alternatively the existing uptake system may be modified resulting in this change. Changes in the Km values for glucose and glutamate also occurred during starvation of the Vibrio. It would presumably be beneficial for starving bacteria to have high affinity uptake systems ready to utilize any low concentrations of substrates. There is no evidence to show that a different uptake system becomes functional when the Km for transport of a substrate changes. Faquin
and Oliver (1984) have suggested that multiple substrate uptake systems may also function during starvation of bacteria, where one uptake system may serve to transport a number of substrates.

A different approach has been taken by Nissen et al. (reported in Azam and Ammerman, 1984), working on glucose transport in a marine bacterial isolate. They measured uptake kinetics over a wide range of substrates (in unstarved cells), and found four kinetic phases (the highest with a millimolar Km). These facts were interpreted to be indicative of a single multiphasic transport system that may undergo (allosteric) transitions at defined concentrations, with changes in affinity, rather than of multiple uptake systems. Similar multiphasic kinetics for glucose uptake in natural marine bacterial assemblages were found by Azam and Hodson (1981), with Km ranges of $10^{-9} - 10^{-4}$ M.

There are a few reports of affinity constants for amino acid and sugar transport in aquatic bacteria. Km values ranging from 0.15 to 0.09 µM were characteristic of glutamate transport for ANT 300 during a starvation period of 20 days (Glick, 1980, reported in Morita, 1984). Hayasaka and Morita (1978) measured a Km of 4.8 µM for galactose transport in a psychrophilic marine Vibrio. Arginine transport in ANT 300 was characterized by Geesey and Morita (1979). The organism possessed both a high and low affinity system, with Km values of 0.017 µM and 4.5 µM respectively. The high affinity system was maintained during starvation (Faquin and Oliver, 1984). An Aeromonas strain, isolated from an oligotrophic area of Lake Biwa by Ishida et al. (1982), and grown in either high or low nutrient
concentrations, had either 2 or 3 distinct Km values for glutamate assimilation. These were 0.28 µM and 0.15 mM for high nutrient cells, and 0.074 µM, 8.3 µM and 0.17 mM for low nutrient cells, and possibly represent high and low affinity transport systems. A Spirillum and Pseudomonas species isolated in a chemostat by lactate limitation from pond water had Km values for lactate transport of 5.8 and 20 µM respectively. At low concentrations of lactate, the more efficient scavenging capacity of the Spirillum (manifested by its lower Km) contributed to that organism outcompeting the Pseudomonas (Matin and Veldkamp, 1978).

The non oligotrophic E. coli has Km values for glutamate, glucose and mannitol transport of 23 µM, 5-10 and 0.1 µM, and 0.37 µM respectively (reported in Tempest and Neijssel, 1978). On the basis of results obtained from experiments on the same organism, Koch and Wang (1982) have proposed that diffusion of nutrient molecules through the outer membrane of Gram negative bacteria could restrict growth at low nutrient concentrations. Similar factors may affect transport processes in starving bacteria.

When considering the data about substrate transport, 3 things become apparent.

(1) Most transport systems have Km values in the micromolar range, with occasional low affinity systems being present that function at millimolar substrate levels. This is true for oligotrophic and copiotrophic bacteria (for a summary of further Km values, see Tempest and Neijssel, 1978).
(2) The affinity constants for amino acids are usually lower than those of sugars, and the uptake rates higher. This may reflect that amino acids are present at lower concentrations than carbohydrates in aquatic waters. Some bacteria have a requirement for particular, essential amino acids, whereas carbohydrates, as energy sources, are usually interchangeable.

(3) The kinetics of substrate transport may be dependent on the nutrient status of the cells. Cells grown prior to starvation in high nutrients differ from those grown at low nutrient concentrations, and starved and nonstarved cells may have different Km values for the same transport function.

Bacteria are found in the kelp beds that differ in their maintenance of mannitol transport systems during starvation. A *Vibrio* strain maintained its mannitol transport system during nutrient deprivation, whereas a *Pseudomonas* with an inducible system had only a basal level of uptake. These types of transport system may be advantageous for different reasons during starvation. The presence of a constitutive transport system in starved bacteria allows the cells to make use of any substrates that become available without delay. However, energy of maintenance is presumably required to keep the system functional. By way of contrast, any system that is present at basal levels only when the cells are not induced requires little maintenance energy. Endogenous energy has to be expended during induction of the system, and there is a lag phase between the time of appearance of the inducer, and full functioning of the induced transport system.
Experiments using inhibitors of transcription and translation showed that de novo RNA and protein synthesis was required for induction of the kelp bed *Pseudomonas* mannitol transport system. No additional exogenous energy source was necessary, and transport activity in cells starved for 5 weeks could be induced by a low concentration of mannitol (11 μM). Addition of phosphates and nitrates did not increase the rate of induction, but the effect of amino acids on induction was not tested. Hayasaka and Morita (1978) reported that an amino acid, in addition to the inducer, was required for induction of the galactose transport system in a marine bacterium.

Amy et al. (1983a) found that during starvation of ANT 300, RNA levels increased to 2.5 times its minimum level. These nucleic acids may be either part of the transcription machinery ready to make proteins such as those for inducible transport proteins, or may be storage products that can be catabolically used as energy sources. The former alternative is unlikely however, because although RNA levels rise during starvation, the lag period needed to relieve starvation increases instead of decreases (Amy et al., 1983a). Starved kelp bed *Pseudomonas* cells appear to lack RNA transcripts of the mannitol transport proteins, as rifampicin inhibited induction of the uptake system.

Once the substrates have been transported into the cells, they may be respired, used to replenish endogenous energy reserves, or utilized for macromolecular synthesis. Amino acids and sugars taken up by starved kelp bed bacteria were channeled into all 3 pathways. The same proportion of substrate was found in each compartment, when 2
concentrations of the nutrients were tested, which indicates that no compartment requires an absolute amount of the substrate, or becomes saturated at the substrate levels employed.

ANT 300 respired up to 60% of the arginine that was transported into the cells after 35 days of starvation (Faquin and Oliver, 1984). The same organism starved for 3.5 months responded to glutamate and glucose in different ways: Incorporation of the former was much lower than the amount respired, whereas the converse was true of glucose (Amy and Morita, 1983a). The Pseudomonas studied by Kurath and Morita (1983), respired approximately 30% and 70% of the glucose and glutamate taken up. A similar result was obtained using a kelp bed Vibrio strain (Figure 20). An Aeromonas strain from Lake Biwa was found to respire 60% of glutamate taken up at substrate levels less than $10^{-6} \text{M}$, and 30 - 50% if glutamate levels were greater than $10^{-6} \text{M}$ (Ishida et al., 1982). Kjelleberg et al. (1982) reported that 5 day starved Vibrio cells increased their $O_2$ consumption rates when offered glucose or casamino acids. The transport systems and metabolic pathways were apparently saturated by 25 mg.l$^{-1}$ glucose (140 $\mu$M), whereas the amino acid pathway was not fully saturated by the same concentration (mg.l$^{-1}$). Varying levels of $CO_2$ production and cellular incorporation of glutamate were measured in the open ocean bacteria isolated by Amy and Morita (1983a) and starved for 8 - 9 months.

The processes involved in 'shut down' of cellular functions during starvation must be finely controlled, and likewise, the reverse processes needed for recovery from starvation must be regulated. In
experiments involving a kelp bed *Vibrio* mannitol transport mutant (*mtl*−), and the wild type (WT) strain, it was found that the addition of 1 mM mannitol to starved cells of both phenotypes caused a dramatic increase in cell numbers of the WT. Two weeks after the addition of the carbohydrate, cell numbers of the WT decreased, possibly as a result of unbalanced growth of cells formed by cell division at the nutrient increase, causing loss of viability of these cells. The decrease is probably also due to restarvation of the cells. The phenomenon of substrate accelerated death has been described by Calcott and Postgate (1972) for *Klebsiella*. The addition of glycerol to glycerol-starved cells caused accelerated death of the cells. They expressed the view that the phenomenon is related to intracellular repression-derepression processes, as it could be relieved by cAMP. Their experiments were carried out after only 14 hour starvation of the cells.

Addition of 100 µM mannitol to starving WT and *mtl*− *Vibrio* cells caused a small increase in cell numbers, similar to that in control experiments, where no additions were made. It is not known why the latter occurred. Addition of high concentrations (1 mM) of glucose caused both the WT and *mtl*− mutant to increase in numbers. Thus it appears that comparatively high concentrations (such as 1 mM) can stimulate cell division of starved cells, whereas lower concentrations (100 µM) do not cause this marked effect. Amino acids such as glutamate at concentrations of 100 µM can probably not reverse the effects of starvation as far as cell division is concerned. Kjelleberg *et al.* (1982) found that 5 mg.1−1 of tryptone and yeast extract were able to cause 7 day starved *Vibrio* cells to divide and become motile, although this concentration of nutrients did not support growth of the strain in batch culture.
The fact that the *Vibrio* mtl- mutant lacking a functional mannitol transport system showed a similar viability pattern to the WT strain may be an indication that the energy burden for maintenance of a particular uptake system is low. However the mutant cells may have to maintain defective transport proteins that are nonfunctional.

A question that has occupied many workers is that of what supplies the energy needed for functioning of a cell during starvation survival. This is the concept of 'energy of maintenance' – energy consumed for purposes other than the production of new cell material (Pirt, 1965). Various types of molecules may be degraded for maintenance energy: Storage carbohydrates, nucleic acids, lipids, and intracellular pool components.

Nelson and Parkinson (1978) showed that levels of endogenous substrates such as carbohydrates and protein decreased during starvation of 3 isolates from Arctic soil. However, Jones and Rhodes-Roberts (1981) concluded that no peculiar physiological property such as the ability to survive at the expense of intracellular carbohydrate storage products (for example, poly-β-hydroxybutyrate) fully accounted for the starvation resistance of some marine bacteria.

Amy and Morita (1983a) have examined the changes in protein and nucleic acid levels during starvation of ANT 300. They found that protein and DNA decreased to a constant level, but that RNA increased with time. They did not determine which form of RNA was stored (ribosomal, transfer, or messenger), but suggest that the nitrogen limitation experienced by the cells triggers the synthesis and storage of RNA as a nitrogen reserve. Proteins are known to be
degraded during starvation, but it is not known whether specific representatives of this class of molecule are catabolized. Work on ANT 300 by Oliver and Stringer (1984) showed that the total lipid phosphate levels decreased by 65% during starvation, indicating utilization of these molecules. More specifically, a selective increase in palmitoleate at the expense of myristate was detected in membrane lipids, which would affect membrane fluidity, and this in turn may affect nutrient transport systems. Novitsky and Morita (1978) reported that 30% of cellular carbon is contained in low molecular weight compounds in ANT 300. This intracellular pool was completely eliminated during starvation, and the organism's capsule was also degraded and utilized.

Thus it appears that most cellular components may be degraded for use as energy and nitrogen sources during starvation and thus energy storage is not the limitation on starvation survival. It is probable that bacteria with successful starvation survival responses have the ability to lower their endogenous metabolic rates, and suppress endogenous respiration capabilities. From their studies, Matin and Veldkamp (1978) consider that the more active and/or efficient respiratory chain of a Spirillum sp. may give it an advantage over a Pseudomonas strain.

The active transport systems that are maintained during starvation need to be powered by some form of energy, such as ATP, proton motive force or PEP. ATP levels per cell have been reported to increase during starvation in ANT 300 and a Pseudomonas strain (Amy et al., 1983a, Kurath and Morita, 1983), although Oliver and Stringer (1984)
state that ATP levels per cell decreased in ANT 300. Faquin and Oliver (1984) found that a proton gradient across the membrane rather than ATP was required for transport of arginine during starvation of ANT 300. The dependance of arginine transport on respiration rather than on ATP hydrolysis had been suggested by Geesey and Morita (1979), who showed that succinate and D-lactate (oxidized via respiratory pathways) are better stimulants of arginine uptake than glucose (which generates ATP via substrate level phosphorylation).

As mannitol transport appears to be effected by a PEP-dependent PTS in the kelp bed Vibrio, it would be interesting to monitor PEP levels during starvation, in this organism as well as others, as the most common sugar transport systems in facultative anaerobes may be PTSs.

A comparison of the response to starvation by the kelp bed bacteria and E. coli showed that the latter is considerably less successful in terms of survival than the former. This may be an indication of adaptation at the genetic level by marine bacteria to waters which are often oligotrophic. It is interesting to note that the Bacillus strain studied by Nelson and Parkinson (1978) which presumably carried genetic information for spore formation, did not form spores during starvation. The details of some starvation studies carried out on nonmarine strains, and bacteria from nutrient rich environments are presented below.

Reeve et al. (1984a, 1984b) have shown that both the degradation and synthesis of proteins are necessary for starvation survival by E. coli (which has a less efficient response than the marine bacteria described). The cells in an E. coli culture starved for glucose lost
50% of their initial viability in 6 days. The 50% viability level was reached after 4 days by a mutant lacking a single peptidase activity, and half of the cells of a strain mutated in 5 peptidase activities were non viable after only 2 days (Reeve et al., 1984a). Similar results were obtained for *S. typhimurium*, indicating that protein degradation plays a role in the survival of both these species. The need for protein synthesis in *E. coli* during starvation was demonstrated by Reeve et al. (1984b), who found a more rapid loss in viability in cultures to which chloramphenicol was added, than in those without the inhibitor.

*Salmonella enteriditis* was shown to have a half life survival time of approximately 5 days, and utilized RNA, protein and acid-alcohol-soluble material as endogenous reserves during starvation (Druilhet and Sobek, 1984). *Klebsiella pneumoniae* and *Enterobacter cloacae*, which were classed as survivors in a dilute aquatic environment by Sjogren and Gibson (1981), had far higher levels of ribonuclease than 'non survivors' such as *E. coli*. This indicates that RNA degradation may be an important factor in determining starvation survival.

Sinclair and Alexander (1984) have attempted to correlate the ability of some strains of bacteria to resist starvation in buffer, and their survival in natural habitats such as lake water and sewage. In a comparison of random isolates from nutrient poor and rich habitats, the percentage of survival of 17 of 19 bacteria isolated from lake water was found to be greater than of all 11 isolates from human skin and mouth. They suggest that starvation susceptible bacteria will not persist in environments that are nutrient poor, or in which they fail to compete for organic nutrients.
The final point to be discussed is whether the small cells that result from starvation of marine bacterial cultures bear any relation to the very small bacteria that are encountered in aquatic waters. It is likely that the 'microbacterial' population consists of 2 types of cells:

(1) Starved cells of various copiotrophic strains, and

(2) True oligotrophic bacteria (Tabor et al., 1981, Torrella and Morita, 1981, MacDonell and Hood, 1982).

A simple definition of oligotrophs described them as bacteria able to grow at carbon (C) levels of 0 - 10 mg.l\(^{-1}\) and below. An example of an oligotroph is a marine bacterium isolated by Carlucci and Shimp (1974), capable of growing (multiplying) in unsupplemented seawater. Ishida and Kadota (1981) have described the growth patterns of 18 isolates of 'obligate oligotrophs' that were able to grow in unsupplemented lake water, but could not grow in rich medium (5 g.l\(^{-1}\) nutrients).

However, it may not be that easy to distinguish between copiotrophs and oligotrophs. Martin and MacLeod (1984) have examined the nutritional requirements of 4 marine bacteria, 2 designated as oligotrophic, and the other 2 as eutrophic or copiotrophic, because they grew on media containing 10 mg.l\(^{-1}\) C (as peptone), or higher, respectively. They found that with appropriate carbon and energy sources, the difference between the oligotrophs and copiotrophs disappeared, and all could grow at 10 mg.l\(^{-1}\) C. In a comparison of
2 marine bacteria (an *Acinetobacter* and *Vibrio* strain) isolated by extinction dilution, Baxter and Sieburth (1984) noted that diverse metabolic and ultrastructural responses occurred when the isolates were grown at a variety of glucose concentrations. They propose that most marine bacteria are eurytrophic (rather than oligotrophic or copiotrophic), and thus are able to utilize a very wide range of dissolved organic nutrients.

Amy and Morita (1983b) have pointed out that some microbacteria may be formed by lack of specific nutrients such as vitamins and essential amino acids, since they are found in nearshore environments (like the kelp beds) where organic carbon is more abundant. Rather than distinguish between oligotrophs and copiotrophs on the basis of the ability to grow at various nutrient levels, Kjelleberg et al. (1982) describe copiotrophs as those bacteria that survive nutrient deprived environments by forming small inactive cells, and oligotrophs as those adapted to grow at low nutrient levels. Baross and Morita (1978) define the difference (actually between psychrophiles - a different type of stress) in terms of metabolism: Copiotrophs would be considered to have an efficient secondary metabolism functioning in conditions where they cannot grow, whereas oligotrophs must have a primary metabolism able to function at very low nutrient levels.

This oligotroph-copiotroph dilemma remains largely unresolved, but it can be assumed that bacteria starved in laboratory experiments may be similar in some ways to the forms in which they are found in the marine environment. Studies of starvation survival responses by possible copiotrophs such as the kelp bed bacteria, may give insight into the dynamic changes that occur in populations as ambient nutrient levels fluctuate in marine waters.
CHAPTER 5

DEGRADATIVE ENZYMES OF THE KELP BED BACTERIA

SUMMARY

Kelp bed bacterial isolates from the water column and kelp fronds were tested for the ability to degrade various compounds. Plate assays showed that the strains were able to hydrolyze proteins (casein and gelatine), lipids (Tween 80), starch, cellulose (carboxymethylcellulose), and chitin. The kelp components laminarin and alginate were degraded by some strains. Inducible laminarinase and alginase activity was found among the Pseudomonas, Vibrio, and Acinetobacter strains. Generally, these two enzymes had maximum activity at 30°C. The motile kelp bed strains were chemotactic towards nutrients such as amino acids, as assayed by the swarm plate method. Thymidine and uracil uptake by the kelp bed strains was measured. The degradative enzymes may be important in the kelp bed ecosystem, where a large fraction of the primary production is recycled via a detrital pathway.
5.1 INTRODUCTION

A wide variety of organic nutrients are available to bacteria in the marine environment. The nutrients may exist either as dissolved substrates (DOM) or detrital particles (POM), and these can be of macrophyte or phytoplankton origin.

Kelp derived substrates have been identified as proteins, fats, sugars and polyols, alginates and laminarins (Newell et al., 1980), and agar in red seaweeds. Phytoplankton cells contain all of these components except alginates and agar, and have cell walls composed of cellulose. Another complex polymer found in the marine environment is chitin, which forms the exoskeleton of crustacea such as shellfish. The chemical compositions of the complex polysaccharides are summarized in Table 12.

### TABLE 12

Characteristics of some polysaccharides found in the marine environment.

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Monomers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminarin $\beta 1 - 3$</td>
<td>glucose, mannitol</td>
<td>Chesters and Bull, 1963</td>
</tr>
<tr>
<td>Alginate $\beta 1 - 4$</td>
<td>$\beta$-D mannanuronic acid</td>
<td>Preiss and Ashwell, 1962</td>
</tr>
<tr>
<td>Starch $\alpha 1 - 4$, $\alpha 1 - 6$</td>
<td>glucose</td>
<td>Shallenberger et al., 1974</td>
</tr>
<tr>
<td>Agar $\beta 1 - 4$</td>
<td>D + L galactose</td>
<td>Hodgson and Chater, 1981</td>
</tr>
<tr>
<td>Cellulose $\beta 1 - 4$</td>
<td>glucose</td>
<td>Shallenberger et al., 1974</td>
</tr>
<tr>
<td>Chitin $\beta 1 - 4$</td>
<td>N-acetyl-glucosamine</td>
<td>Reid and Ogrydziak, 1981</td>
</tr>
</tbody>
</table>
Many bacteria have been isolated that can utilize these complex substrates, and thus produce enzymes which hydrolyse them. Chesters and Bull (1963) described the distribution of laminarinase among microorganisms such as bacteria, yeasts and fungi. When studying the bacteria associated with kelp fronds, Laycock (1974) found many strains able to hydrolyze laminarin and alginate. Quatrano and Caldwell (1978) isolated a marine bacterium capable of growth on a wide variety of macrophyte polysaccharides such as alginate, laminarin, agar, cellulose and starch. Alginases or alginate lyases of bacterial origin have been studied in detail (Preiss and Ashwell, 1962, Stevens and Levin, 1977, Doubet and Quatrano, 1982, 1984). An agarase has been characterized from Pseudomonas atlantica (Groleau and Yaphe, 1977). Carroad and Tom (1978) isolated a number of chitinolytic (chitinoclastic) bacteria from various sources of chitin, and Reid and Ogrydziak (1981) have characterized a chitinase-overproducing Serratia mutant. Much work has been done on bacterial cellulases that decompose plant matter (for example, Robb et al., 1979).

Other degradative enzymes from marine bacteria have been identified. A detailed study of the proteases of a marine Pseudomonas was made by Makino et al. (1983). Bacteria possessing lipases and enzymes for starch hydrolysis have been reported in studies on marine bacterial communities (Kaneko et al., 1979, Martin and Bianchi, 1980, Fukami et al., 1981).

Bacteria in the kelp beds on the West Coast encounter all of the abovementioned compounds, which are available due to fragmentation of
kelp plants and growth of phytoplankton blooms. The kelp derived substrates have been shown to serve as nutrients for the bacteria in flask experiments (Linley et al., 1981, Lucas et al., 1981, Stuart et al., 1981). These workers demonstrated increases in bacterial numbers and biomass that occurred as the levels of macrophyte mucilage and particulate matter incubated with the bacteria decreased. However, bacterial strains responsible for the differential utilization of various components were not identified. This chapter describes some of the hydrolytic activities of bacteria isolated from the kelp bed water column and surface of the kelp fronds. The strains were also tested for the ability to take up thymidine, as this is used as an indicator of heterotrophic activity. Transport of uracil by some strains was measured to test whether this could be used instead of thymidine uptake.

5.2 MATERIALS AND METHODS

Bacterial strains

The isolation and maintenance of bacterial strain collections from the kelp bed water column and from kelp plants has been described in Chapter 2. An enrichment for bacteria on the surface of kelp plants able to utilize released mucilage was carried out. Kelp fronds were sampled as described in Chapter 2, the pieces of kelp were incubated in sterile seawater for 24 h at 22°C, and the resultant population of bacteria were plated on SWA. Thirty randomly chosen colonies were restreaked until pure cultures were obtained, and the strains were maintained as described for the other collections.
Plate assays

Protease, gelatinase, lipase, chitinase, starch hydrolysis, agarase, cellulase and alginate enzymes produced by the strains were identified using plate assays. Fermentation properties of strains from the kelp enrichment culture were determined. Details of the composition of the media and solutions are given in the Appendix. Some of the assay plates contained a basal layer of agar (BA), overlaid with a layer containing the test substrate. Plates were inoculated by toothpick, from colonies on SWA plates.

<table>
<thead>
<tr>
<th>Assay plate</th>
<th>Overlay</th>
<th>Incubation</th>
<th>Flooding of plate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk (protease)</td>
<td>-</td>
<td>1 - 2 d</td>
<td>-</td>
<td>Kaneko et al., 1979</td>
</tr>
<tr>
<td>Gelatine (gelatinase)</td>
<td>-</td>
<td>16 h</td>
<td>mercuric chloride</td>
<td>Cowan and Steel, 1970</td>
</tr>
<tr>
<td>Tween 80 (lipase)</td>
<td>-</td>
<td>3 d</td>
<td>-</td>
<td>Holding and Collee, 1971</td>
</tr>
<tr>
<td>Chitin (chitinase)</td>
<td>+</td>
<td>3 + d</td>
<td>-</td>
<td>Reichenbach and Dworkin, 1981</td>
</tr>
<tr>
<td>Starch (starch hydrolysis)</td>
<td>-</td>
<td>16 h</td>
<td>Lugol's iodine</td>
<td>Cowan and Steel, 1970</td>
</tr>
<tr>
<td>Agar (agarase)</td>
<td>-</td>
<td>2 d</td>
<td>Gran's iodine</td>
<td>Hodgson and Chater, 1981</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>-</td>
<td>1 d</td>
<td>Congo red, dilute hydrochloric acid</td>
<td>Teather and Wood, 1982</td>
</tr>
<tr>
<td>(cellulase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alginate (alginate)</td>
<td>+</td>
<td>2 d</td>
<td>acetone</td>
<td>Laycock, 1974</td>
</tr>
<tr>
<td>Tetrazolium + glucose or mannitol (fermentation)</td>
<td>-</td>
<td>2 d</td>
<td>-</td>
<td>Bochner and Savageau, 1977</td>
</tr>
</tbody>
</table>
Positive reactions were visualized as zones of clearing in the following plates (after flooding and rinsing them with water where necessary):

- skim milk, gelatine, chitin, starch, agar and alginate. Lipase activity in Tween 80 plates was seen as zones of opacity, composed of calcium soaps, around the colonies. The presence of 'pits' or 'depressions' in the agar plates was additional evidence for agarase activity. Strains positive for cellulase activity made violet blue zones in a paler blue background. Fermentation of a particular sugar caused white colonies on the tetrazolium indicator plates, and oxidative reactions produced red colonies.

**Laminarinase**

This assay is based on the Nelson Somogyi method of determining reducing sugars (Nelson, 1952).

1. **Preparation of crude extract:**
   Cells were grown in 10 ml of 1/10 SWB (see Appendix) supplemented with 0.1% laminarin, for 16 h at 22°C with aeration. After harvesting by centrifugation, the bacteria were resuspended in 1 ml phosphate buffer (PB – see Appendix). The cells were disrupted by sonication as described in Chapter 3. The extract was not clarified.

2. **Substrate**
   Laminarin (0.1% w/v) in PB.
3. **Assay mix**

Enzyme (0.3 ml crude extract) + 1.7 ml substrate. This was incubated at 22°C unless otherwise stated, and subsamples (0.5 ml) were taken at various times.

4. **Assay**

Somogyi reagent (0.5 ml) was added to 0.5 ml assay mix, boiled for 10 min and cooled. Nelson reagent (1 ml) and 3 ml water were added, the samples were mixed thoroughly, and then left at 22°C for 20 min for the colour to develop. The samples were read at 660 nm on the spectrophotometer.

5. **Calibration**

A glucose calibration curve was prepared by assaying dilutions of a glucose solution (400 µg.ml⁻¹). This included glucose concentrations in the range of 5 - 200 µg.

**Alginase**

This activity was determined colorimetrically using the thiobarbituric acid (TBA) method of Jacober *et al.* (1980). TBA reacts with the aldehyde derivatives of guluronic and mannuronic acids.

1. **Preparation of crude extract**

This was exactly the same as that described for laminarinase, except that the cells were grown in alginate medium (see Appendix) containing SW3 and alginate.
2. **Substrate**

Sodium alginate (0.4% w/v) in PB.

3. **Assay mix**

0.25 ml enzyme (crude extract) + 0.75 ml substrate. This was incubated at 22°C unless otherwise stated, and aliquots (0.2 ml) removed at appropriate times.

4. **Assay**

Freshly prepared periodate solution (0.25 ml) was added to 0.2 ml assay mix, and left to incubate at 22°C for 20 min. NaAsO$_4$ (0.5 ml) was used to stop the periodate oxidation, and 2 ml TBA was added after 2 min. The samples were heated to 100°C for 10 min, cooled and read at 550 nm on the spectrophotometer.

5. **Calibration**

Malondialdehyde was used to draw up a calibration curve, according to the method of Jacober et al. (1980), as modified by Seiderer (1983). Dilutions of malondialdehyde (a liquid) were assayed, to give a standard curve in the range of 0.003 - 2 μl malondialdehyde.

**Thymidine and uracil uptake**

The procedure was exactly the same as that described in Chapter 3 for mannitol uptake. Cells were grown in 1/10 SWB. Details of the radiolabelled substrates were as follows: [2 - $^{14}$C] thymidine, 55 mCi:mmol$^{-1}$, final concentration in assays, 79nM, and [5,6 - $^{3}$H] uracil, 58 Ci:mmol, final concentration, 6.6 nM.
Protein determinations

These were carried out using the Folin reagent (Lowry et al., 1951), with bovine serum albumin as a standard.

Chemotaxis: Swarm plate assays

Two kinds of soft agar swarm plates containing attractants were made (see Appendix): Tryptone Soy (TS) and minimal plates containing amino acids and sugars. Bacteria were grown for 10 h in SWB, washed twice with sterile seawater, and concentrated 10 fold. Aliquots (15 µl) of the cell suspension were pipetted into the agar. TS and minimal plates were viewed for chemotaxis 'rings' after approximately 5 and 10 h respectively.

5.3 RESULTS

Plate assays

Bacteria isolated from the kelp bed water column and from kelp fronds had a wide variety of degradative enzymes, as determined by plate assays (Table 13). Pseudomonas strains with various combinations of enzymes for hydrolyzing proteins, gelatine, chitin, Tween 80, starch and CMC were present in the water column strain collection. The Acinetobacter strain (nonfermentative, nonmotile rod) isolated from the water column had a wider spectrum of activity than the representative of this species isolated from a kelp frond. Six of the seven Vibrio strains from kelp plants could degrade all the
TABLE 13

Summary of the degradative enzymes of water column and kelp frond bacterial isolates, as determined by plate assays. Classification of the strains was described in Chapter 2. The number of strains possessing the indicated combination of enzymes is presented in the last column. (Plates: Milk = skim milk, Gel = gelatine, Chit = chitin, Fat = Tween 80, St = starch, Cell = CMC).

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Classification</th>
<th>Plates</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water column</td>
<td>Pseudomonas</td>
<td>Milk</td>
<td>Gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(N = 13)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Acinetobacter</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kelp frond</td>
<td>Pseudomonas</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(N = 9)</td>
<td>Acinetobacter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Vibrio</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
substrates tested except cellulose. Protease and gelatinase activity were exhibited by a *Flavobacterium* from the water column, but this strain had none of the other enzymes tested. Results of alginase activity obtained by the plate assay are not presented in this table, as all of the isolates were also screened using the TBA assay, and these results are given in Table 15.

**Enrichment culture for bacteria on kelp fronds**

Incubation of kelp frond samples in sterile seawater were carried out. Four bottles contained $5.0 \times 10^1$, $1.0 \times 10^1$, $2.0 \times 10^1$ and $1.0 \times 10^1$ bacteria per ml at the start of the experiment. After 24 h incubation, $2.0 \times 10^4$, $2.6 \times 10^3$, $6.0 \times 10^2$ and $1.0 \times 10^1$ bacteria per ml respectively were counted in the bottles. Thirty randomly chosen colonies from plating of one sample were isolated for enzyme characterization by the plate assays. Twenty seven of the thirty strains were fermentative, and seventeen of these possessed all of the enzyme activities tested except cellulase (Table 14). Cellulase activity was not found in any of the fermentative isolates, and was present in only one of the oxidative strains. Alginase activity was absent in only five of the thirty strains. All of the strains could hydrolyze skim milk and gelatine.

**Laminarinase activity**

The ability to produce reducing sugars during degradation of laminarin was widespread amongst the bacteria in the water column strain collection (Table 15). Laminarinase activity of five of the
TABLE 14

Summary of the enzyme activities (determined by plate assays) of bacterial isolates from a kelp enrichment culture. The number of strains possessing the indicated combination of enzymes is presented in the last column. (Plates: Milk = skim milk, Gel = gelatine, Chit = chitin, Fat = Tween 80, St = Starch, Cell = CMC, Alg = alginate.

<table>
<thead>
<tr>
<th>Fermentative/</th>
<th>Plates</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidative</td>
<td>Milk</td>
<td>Gel</td>
</tr>
<tr>
<td>Fermentative</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(N = 27)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Oxidative</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(N = 3)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
TABLE 15

Laminarinase and alginase activities of kelp bed bacterial strains. Laminarinase activity is expressed as mg glucose equivalents. mg protein\(^{-1}\) released in a 4 h assay. Alginase activity is expressed as µl malondialdehyde released. mg protein\(^{-1}\) in a 5 h assay. (ND = not determined).

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Strain</th>
<th>Laminarinase (mg gluc/mg prot/4 h)</th>
<th>Alginase (µl mal./mg prot/5 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water column</td>
<td>Pseudomonas P1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(N = 13)</td>
<td>P2</td>
<td>0.88</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>3.33</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>0.32</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>0.92</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>1.33</td>
<td>6.40</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>1.44</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>3.70</td>
<td>3.71</td>
</tr>
<tr>
<td></td>
<td>P9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P10</td>
<td>2.08</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>P11</td>
<td>0.55</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Acinetobacter</td>
<td>3.45</td>
<td>4.96</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Kelp frond</td>
<td>Pseudomonas</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(N = 9)</td>
<td>Acinetobacter</td>
<td>2.98</td>
<td>11.17</td>
</tr>
<tr>
<td></td>
<td>Vibrio V1</td>
<td>-</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>V2</td>
<td>-</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>V3</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>V4</td>
<td>-</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td>V5</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>V6</td>
<td>-</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>V7</td>
<td>-</td>
<td>0.31</td>
</tr>
</tbody>
</table>
eleven *Pseudomonas* strains released more than 1 mg glucose equivalents per mg protein (crude cell extract) in the 4 h assay period, and only two of the water column strains showed no activity against laminarin. The *Acinetobacter* strains from the water column and kelp plants had active laminarinases. None of the other isolates from the kelp plants produced laminarinas.

Laminarinase activity of two strains was studied in more detail. The *Acinetobacter* isolated from a kelp frond, and *Pseudomonas* P8 both had inducible laminarinase activities. Uninduced cells of P8 grown in a medium containing no laminarin had no detectable laminarinase, and the *Acinetobacter* had a low basal level of activity (0.23 and 2.98 mg glucose mg protein$^{-1}$ per 4 hr for uninduced and induced cells respectively). The results of an experiment to measure laminarinase activity by the two strains in the 4 hr assay period are presented in Figure 24. Release of glucose equivalents was linear for 3 hr after a slight lag period with the assay conditions as described.

Activity of the laminarinases of both strains at various temperatures was determined (Figure 25). The temperature at which maximum activity was measured differed in the two strains: 22°C and 30°C for the *Acinetobacter* and P8 respectively. The laminarinase of the former was more active at 4°C than at 37°C, whereas the opposite was true of the *Pseudomonas* enzyme.

**Alginase activity**

The alginase (alginate lyase) activity of the water column and kelp frond strain collections is presented in Table 15. Five of the ten
FIGURE 24: Laminarinase activity in cell extracts of two kelp bed bacterial isolates, *Acinetobacter* (●) and *Pseudomonas* P8 (■), expressed as mg glucose equivalents released per mg protein in crude cell extracts.

FIGURE 25: Laminarinase activity at various temperatures in cell extracts of *Acinetobacter* (●) and *Pseudomonas* P8 (■).
water column *Pseudomonas* strains had alginase activities of greater than 1 (expressed as µl malondialdehyde produced per mg protein (crude cell extract) in 5 hr). The highest 'specific alginase activity' amongst the bacteria was shown by the kelp frond *Acinetobacter*. All of the *Vibrio* strains from kelp fronds were able to degrade alginate.

*Pseudomonas* P8 and *Vibrio* V4 produced alginase provided the growth medium contained alginate. The kelp frond *Acinetobacter* grown in 1/10 SWB possessed some alginase activity in the 4 hr assay (2.55 µl malondialdehyde. mg protein −1).

Figure 26 shows the alginase activity of three strains during a 4 hr period. The responses in activity of the enzymes to various temperatures are presented in Figure 27. The enzymes from all three strains have maximum and minimum activity at 30°C and 4°C respectively.

**Chemotaxis**

The chemotactic responses of two water column *Pseudomonas* strains (P8 and P11), two kelp frond *Vibrio* strains (V3 and V8), and a water column *Flavobacterium* were tested. The four motile strains (*Pseudomonas* and *Vibrio*) were all chemotactic towards the mixture of amino acids in tryptone. This was visualized as a number of rings in a TS chemotaxis plate (Figure 28). Each ring consists of bacteria responding to a particular attractant, for example, serine, oxygen and aspartate (Adler, 1976). The nonmotile *Flavobacterium*, included as a control, produced no rings.
FIGURE 26: Alginase activity in cell extracts of three kelp bed bacterial isolates, *Acinetobacter* (●), *Pseudomonas P8* (■), and *Vibrio V4* (▲), expressed as µl of malondialdehyde released per mg protein in the crude cell extracts.

FIGURE 27: Alginase activity at various temperatures in cell extracts of *Acinetobacter* (●), *Pseudomonas P8* (■), and *Vibrio V4* (▲).
FIGURE 28: Plate assay for chemotaxis. Rings of bacteria attracted to various substrates in tryptone can be seen round the inoculum of Pseudomonas (A), whereas there are no rings around the nonmotile Flavobacterium (B).
The response of the motile strains to individual attractants was tested in minimal plates. All four isolates were chemotactic towards alanine, glutamate, aspartate, valine and serine. Glucose and mannitol elicited a positive reaction from V3, V7 and P11.

**Thymidine and uracil uptake**

Thymidine uptake by eighteen water column and kelp frond bacteria is summarized in Table 16. $[^{14}C]$ thymidine with a relatively low specific activity (52.5 mCi/mmol) was used in the assays. Bacteria able to take up more than 200 pmoles thymidine per mg protein in a 14 min assay were considered as positive for thymidine uptake. $[^{3}H]$ uracil with a higher specific activity (58 Ci/mmol) was used, thus these assays were carried out at a low substrate concentration (6.6 nM).

The results indicate that eleven and thirteen of these bacterial strains were able to take up thymidine and uracil respectively. Four *Vibrio* strains transported uracil but not thymidine, whereas another *Vibrio* strain took up thymidine but not uracil, as was also the case with the *Flavobacterium* tested. Both substrates were transported by nine strains, and neither by three isolates.

5.4 **DISCUSSION**

Previous chapters of this thesis have shown that bacteria isolated from the kelp bed environment can take up and utilize simple sugars (glucose and mannitol) and amino acids (glutamate, aspartate and
TABLE 16

Thymidine and uracil uptake by kelp bed bacterial isolates. Thymidine concentration in assays was 79 nM, and strains taking up > 200 pmol.mg protein\(^{-1}\) in a 14 min assay were considered positive (+). Uracil concentration in assays was 6.6 nM, and strains taking up > 10 pmol.mg protein\(^{-1}\) in a 14 min assay were considered positive (+).

<table>
<thead>
<tr>
<th>Source of bacteria</th>
<th>Classification</th>
<th>Thymidine uptake</th>
<th>Uracil uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water column (N = 11)</td>
<td>Pseudomonas</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acinetobacter</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Kelp frond (N = 7)</td>
<td>Vibrio</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>V7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
alanine) that are available in marine waters. The results presented in this chapter suggest that the bacteria have a spectrum of enzymatic activities enabling them to utilize the more chemically complex nutrients that occur.

Compounds such as polysaccharides are too large to be transported into the bacterial cell, and thus have to be broken down extracellularly prior to uptake. Plate assays are useful in the identification of exoenzymes, as 'producer' bacteria are localized as colonies, but the enzymes can diffuse through the agar and degrade the substrate, at the same time remaining at a higher concentration than would occur in liquid culture. Protein hydrolysis tested with skim milk and gelatine plates showed that kelp bed strains producing enzymes for one substrate usually degraded the other. 'Lipolytic' activity was determined with Tween 80, which is a thermostable soluble ester of oleic acid. Cellulose activity was found predominantly amongst the free living water column bacteria, whereas starch was hydrolyzed by bacteria from both strain collections. The polymer chitin was degraded by many strains, notably of the genus *Vibrio*.

The enrichment experiment indicated that the numbers of a predominantly fermentative population of colony forming bacteria increased when 'fragmentation' of kelp (during sampling) took place. This process was dependant on the bacteria associated with the pieces of kelp, as identically treated sample bottles developed different numbers and types of bacteria. A population of nonfermentative, non
motile strains similar in colony morphology to *Acinetobacter* isolated from one sample bottle, survived only one plating. The scarcity of *Acinetobacter* isolates in strain collections may be because they are recalcitrant to plating.

Seventeen of the thirty random isolates were fermentative, and had protease, lipase, alginase and chitinase activity. These are similar to the characteristics of *Vibrio* strains as determined by Ducklow and Mitchell (1979). *Vibrio* strains were commonly isolated from the decomposing tips of *Laminaria* by Laycock (1974), although at certain times of the year *Pseudomonas* strains predominated. In their work on bacteria associated with phytoplankton, Martin and Bianchi (1980) found that vibrio-like organisms were most common when phytoplankton blooms decayed. Another environment where *Vibrio* species were found to be important in decomposition processes is the coral reef, where these bacteria are adapted to utilizing the mucus layer of living corals (Ducklow and Mitchell, 1979). All these lines of evidence suggest that *Vibrio* strains may be associated with the decay processes of plant and animal material. The ability to degrade chitin is widespread in this genus (R. Colwell, pers. comm.).

Many bacterial strains isolated from the kelp beds were shown to hydrolyze laminarin and alginate. Laminarin may occur in plant material either as a cell wall constituent or as a reserve material (Chesters and Bull, 1963). Laycock (1974) found that laminarin hydrolyzing bacteria, associated with Laminaria fronds were restricted to a psychrophilic population. The *Acinetobacter* isolated in this study had a laminarinase that was more active at 4°C than at 37°C (Figure 25). It is not clear whether this is due to inhibition or inactivation of the enzyme at the higher temperature.
The organisms hydrolysing alginate isolated by Laycock (1974) were mesophilic, and were considered to be an integral component of the spoilage flora. Detailed studies of the alginase activity of marine bacteria have been carried out by Stevens and Levin (1977), and Doubet and Quatrano (1982, 1984). Alginate is a glycuronan polymer of guluronic and mannuronic acid. Doubet and Quatrano (1982, 1984) have demonstrated that a guluronic acid specific lyase was recoverable from the growth medium, whereas a mannuronic acid specific lyase was retained within the bacteria. These lyases may cleave the polymeric alginate molecules in an exo or endo manner (that is, the chains may be attacked near the end or within the chains). The implications of these findings are that enzymatic activities may be the result of the action of two or more enzymes, and that these may be extracellular or cellbound. In this study, all of the laminarinase and alginase activities presented in Table 15 were cell associated (in the crude cell extract). Culture fluids of the strains were tested for activity, and some were weakly positive, indicating extracellular enzymes.

Marine bacteria thus have the genetic potential to produce enzymes that degrade complex organic compounds under laboratory conditions. However, production of these enzymes in natural waters is likely to be a slow process, with many regulatory controls. *Vibrio alginolyticus*, commonly found in the marine environment, has been shown to produce extracellular proteases in response to an inducer (collagenase). This response is sensitive to levels of various nitrogen sources (Hare et al., 1983, Long et al., 1981). An effective method for measuring exoenzymatic activities in natural
waters has been developed by Hoppe (1983), using fluorogenic methylumbelliferyl (MUF) substrates. By this technique, protease activity was found in all of the marine water samples tested, and glucosidase and glucosaminidase in some. The production of exoenzymes in an aquatic system appears at first to be 'wasteful' in energetic terms, due to the dilution factors involved. However, many of these enzymes are inducible (for example, the laminarinases and alginases of the kelp bacteria), and may thus only be produced if the substrate is present at concentrations that stimulate synthesis of the enzymes. Secondly, Azam and Ammerman (1984) have proposed the existence of microzones rich in DOM around decaying or autolyzing detrital particles and substantiated this with experimental data. These microzones may cause clustering (not attachment) of bacteria around the particles, and thus create a non-random distribution of free living bacterial populations. The release of exoenzymes, breakdown of the polymers, and assimilation of the products may all occur within the microzones. This proposed rather loose association of bacteria and POM, rather than the formation of aggregates of bacteria attached to particles would explain why many workers have reported a greater proportion of free living bacteria compared with numbers of attached bacteria (for example, Palumbo et al., 1984).

An important consideration in the nutrient enriched microzone concept, is how bacteria find and remain localized in the zone. Azam and Ammerman (1984) propose that the properties of motility and chemotaxis enable bacteria to respond to nutrient gradients around particulate matter. They examined natural assemblages of marine bacteria and found that a significant fraction were motile. Bell and
Mitchell (1972), and Chet and Mitchell (1979) have reported chemotaxis of marine bacteria towards potential nutrients. In this study, kelp bed bacterial isolates were shown to be chemotactic towards sugars and amino acids. Preliminary results indicate that chemotaxis towards laminarin and alginate may occur. In the plate assay used, bacteria deplete the local supply of the attractant, and then follow the attractant gradient they themselves have produced.

The last experimental section of this chapter dealt with the uptake of thymidine and uracil by the kelp bed bacteria. Thymidine incorporation has been used as a measure of heterotrophic bacterial production in many aquatic systems (for example, Fuhrman and Azam, 1982, Bell et al., 1983). This method assumes that all of the active bacteria in the system will transport and incorporate thymidine. However, results presented in this chapter show that some isolated kelp bed bacteria, (which would presumably be included in the 'active' fraction) do not take up the DNA precursor. Thus this method of determining production may be subject to the same disadvantage of other radiolabelled substrates, namely, that appropriate transport systems for the substrate may not be present in the cell. In E. coli and S. typhimurium, thymidine has been shown to be transported by a direct uptake system involving thymidine kinase (review by Pandey, 1984).

Uracil uptake by marine bacteria has been reported (Baross et al., 1974). In E. coli and S. typhimurium, transport of the base may be mediated by a group translocation system (Pandey, 1984). The uptake of uracil (as a possible alternative to thymidine for production
measurements) by the kelp bed bacteria was determined. Not all of
the strains transported uracil however, so it is probably not a
suitable alternative to thymidine. Pyrimidines such as uracil may be
degraded to urea and ammonia (Lehninger, 1975), and thus not solely
used for nucleic acid synthesis.

There is another consideration to be taken into account when using
thymidine incorporation to measure production. 'Production' by
bacteria is considered to be synonymous with biosynthesis. However,
bacteria may carry out biosynthesis, and increase in size, without
increasing in numbers, in which case DNA synthesis may not accompany
production. Experiments using nucleic acid precursors to estimate
production must thus be interpreted with caution.

The results presented in this chapter show that marine bacteria
possess a wide spectrum of degradative enzymes. The occurrence of
chitinase amongst the Vibrio strains, and the frequent association of
these bacteria with degrading plant and animal material, suggest that
Vibrio might be adapted to coastal habitats. Their ability to grow
under aerobic or anaerobic conditions makes them suited to promote
breakdown of degrading material, possibly under fermentative
anaerobic conditions, both in the water column and in stranded
material on the beaches. The Pseudomonas strains and other aerobic
bacteria may be important in aerobic degradation of water column
detritus in both coastal and oceanic waters.
BACTERIOLYSIS IN THE BIVALVE CHROMYMILUS MERIDIONALIS

SUMMARY

Bacteria were isolated from the crystalline style and gut of a filter feeder in the kelp beds, the mussel, Choromytilus meridionalis. Some of the style bacteria produced a lysozyme-like factor (LLF) that lysed marine bacterial cells. The LLF of one isolate was examined with respect to its spectrum of activity against various bacteria. It was inactive against several terrestrial bacterial strains, but active against the majority of marine bacteria. The spectrum of hydrolytic enzymes of the gut and style bacteria was determined, and their ecological significance is discussed.
6.1 INTRODUCTION

Bacteriolytic enzymes such as lysozyme are widely distributed in nature and have often been assigned a host defense function (for example, Hardy et al., 1976). Lysozyme-like activity has been found in a number of bivalves (McHenery and Birckbeck, 1979, 1982; McHenery et al., 1979). These workers found that the lytic activity was associated primarily with the digestive system in a range of invertebrates, and therefore proposed that it had a function in bivalve nutrition.

The necessity for bivalve utilization of bacteria as a food resource may be estimated by examining the carbon : nitrogen (C : N) ratios of bacteria and macrophyte-derived detritus. Bacteria have a low C : N ratio, with estimates ranging from 3.5 (Seiderer et al., 1984) to 4.0 (Fukami et al., 1981), whereas macrophyte detritus has the much higher value of 17.2 (Koop et al., 1982), and this means that bacteria are a better nitrogen source than the detritus. This may be important for filter feeders in the kelp beds such as Choromytilus meridionalis, which are able to meet their carbon requirements from a detrital diet but may become nitrogen limited when filtering detritus only (Seiderer et al., 1982).

Seiderer et al., (1982) showed that C. meridionalis possessed the necessary carbohydrases to digest detrital plant material. Further
work aimed to discover whether the mussel produced any enzymes able to digest bacteria, and thus make this nitrogen rich resource available to the animal.

The crystalline style of bivalves such as *C. meridionalis* is a structure which contains an assemblage of digestive enzymes attached to a glycoprotein structure (Jacober *et al.*, 1980). This was the source of the carbohydrases and lysozyme described by Seiderer *et al.* (1982), and McHenry *et al.*, (1979) respectively. Seiderer *et al.* (1984) found that the style of *C. meridionalis* contained a bacteriolytic enzyme, capable of lysing free living, water column kelp bed bacteria. During this study, it was noted that the style extract occasionally contained bacteria capable of causing bacteriolysis in the plate assay used for lytic activity. This resulted in the subsequent isolation of bacterial strain collections from the style and gut of *C. meridionalis*.

In this chapter, some aspects of the bacteriolytic activity of one of the style bacteria are examined. The spectrum of degradative enzymes of a number of the style and gut bacteria is presented, and the significance of the bacteria and bacterial enzymes is discussed.

6.2 **MATERIALS AND METHODS**

Isolation and culture of bacterial strains

The isolation of style and gut bacteria was carried out by D. Muir.
1. **Style bacteria:** Mussels (*C. meridionalis*) were collected at Bloubergstrand, and transferred to the laboratory, where the crystalline styles were removed, rinsed and homogenised in sterile seawater. The homogenate was plated on seawater agar (SWA) plates (see Appendix).

2. **Gut bacteria:** The guts of mussels were removed aseptically, and dilutions of the contents were cultured on SWA plates, after treatment with trypsin (1% w/v) for 30 min at 30°C. Strains were also isolated from homogenised gut tissue.

3. **Water column and kelp frond bacteria:** These were isolated as described in Chapter 2.

All plates were incubated at 22°C. Strains were stored on SWA slants.

**Preparation of bacterial lytic factor**

'Producer' style bacteria were grown for 16 h in seawater broth (SWB) or in casamino acids (0.5% w/v made up in seawater). Cells were separated from the culture medium by centrifugation, and aliquots of the supernatant fluid were assayed for activity. The cells were resuspended in sterile seawater, and sonicated (2 x 30s at 16 kHz) on ice (see Chapter 3). The cell debris was separated from the soluble cytoplasmic contents by centrifugation, and both fractions were assayed for activity.
Preparation and incubation of plates

Bacteriolytic activity was assayed using agarose plates containing heat killed 'target' bacteria, and activity was visualized as zones of clearing in the opaque bacterial background. Marine 'target' bacteria were cultured for 16 h in SWB with aeration at 22°C, and harvested by centrifugation. The cells were resuspended in seawater, and the suspension was heated to 65°C for 10 - 15 min to kill the bacteria. The pasteurized cell suspension was added to a warm (approximately 65°C) agarose solution (0.8% w/v, made up in seawater unless otherwise stated) to give a final OD_{600} reading of 0.1 - 0.5. Wells were cut in the agarose plates to receive the enzyme preparation (15 µl per well).

Other 'target' strains included *Vibrio alginolyticus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Micrococcus luteus*. These were cultured in the following media: *V. alginolyticus* - SWB, *P. aeruginosa*, *E. coli* - Luria broth (LB - see Appendix). A commercially available, freeze dried preparation of *M. luteus* (Sigma) was used.

Plate assays for other degradative enzymes of style and gut bacteria

These were performed as described in Chapter 5.

Laminarinase and alginase assays

The methods for these assays were described in Chapter 5.
6.3 RESULTS

**Bacteriolytic factor of bacterial origin**

Bacteria isolated from the style of *C. meridionalis* were able to cause lysis of 'target' bacteria in an agarose assay plate. Twenty-six strains were screened by toothpicking them onto an assay plate, and thirteen created zones of lysis. Similarly, seven of twelve isolates from the mussel gut were positive in producing a bacteriolytic factor (see Table 19).

Figure 29 shows an assay plate with wells containing culture medium (supernatant fluid - SNF) of style bacteria. Zones of lysis are seen around three wells.

**Lytic activity against various target bacteria**

The lytic agent produced by one style bacterial isolate (S6) was studied with regard to activity against a range of target bacteria. The sensitivities of inshore and gut bacterial isolates to the lytic agent are presented in Table 17. In these experiments, 'inshore bacteria' refers to the water column and kelp frond strain collections described in Chapter 2. Some strains with similar characteristics (for example, whole cell protein polyacrylamide gel electrophoresis profiles - determined by D. Muir), were grouped together, and assay plates were made of these pooled 'target' strains, as well as of single strains. The results show that, in general, the inshore strains contained a higher proportion of isolates that were more sensitive to the lytic agent than the gut
FIGURE 29: Plate assay for lysozyme like factor. Culture fluids of bacterial isolates from the style of Choromytilus meridionalis were placed in wells in a plate containing heat killed target bacteria suspended in seawater agarose. Zones of bacteriolysis can be seen around three of the wells.
TABLE 17

A summary of the marine target bacteria sensitive and resistant to the lytic factor produced by a style bacterial isolate.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Target bacteria</th>
<th>Inshore</th>
<th>Gut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td></td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td><em>Vibrio</em></td>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td></td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total number of strains</td>
<td></td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>92%</td>
</tr>
</tbody>
</table>
bacteria. Variation in sensitivity to the agent occurred within the species (for example, two inshore \textit{Vibrio} strains were resistant to lysis, whereas five were sensitive).

Table 18 shows the activity spectrum of the lytic agent against a number of nonmarine bacteria. No zones of lysis were seen in plates made of \textit{M. luteus} and \textit{E. coli} target bacteria, but \textit{P. aeruginosa} and \textit{V. alginolyticus} were found to be sensitive to lysis. Larger zones of lysis were formed in plates made up with phosphate buffer (PB - see Appendix) than in seawater assay plates.

Hens egg lysozyme (100 µg.ml⁻¹) was tested for activity against the marine inshore bacteria, and no zones of lysis were observed.

\textbf{Characteristics of the lytic factor}

The lytic factor was tested for heat sensitivity by heating SNF to 100°C for 10 min, and then assaying it against inshore target bacteria. No zones of lysis were seen after this treatment.

Cell debris and the soluble cytoplasmic fraction of sonicated producer cells were assayed for lytic activity, and both fractions caused zones of lysis in assay plates. The cytoplasmic fraction produced somewhat smaller zones than those formed by the cell wall fraction and the SNF.

The effect of growth medium on production of the lytic factor was tested. Cells produced lytic factor if they were grown in either SWB or casamino acids.
TABLE 18

Activity of lytic factor against a range of target bacteria. Lysis assay plates were made using the buffers as indicated.

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>Zones of lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Micrococcus luteus</strong> in</td>
<td></td>
</tr>
<tr>
<td>a) phosphate buffer (PB)</td>
<td>-</td>
</tr>
<tr>
<td>b) acetate buffer</td>
<td>-</td>
</tr>
<tr>
<td>c) Tris EDTA buffer</td>
<td>-</td>
</tr>
<tr>
<td>d) seawater</td>
<td>-</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> in</td>
<td></td>
</tr>
<tr>
<td>a) PB</td>
<td>-</td>
</tr>
<tr>
<td>b) seawater</td>
<td>-</td>
</tr>
<tr>
<td><strong>Vibrio alginolyticus</strong> in</td>
<td></td>
</tr>
<tr>
<td>a) PB</td>
<td>++</td>
</tr>
<tr>
<td>b) seawater</td>
<td>+</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong> in</td>
<td></td>
</tr>
<tr>
<td>a) PB</td>
<td>++</td>
</tr>
<tr>
<td>b) seawater</td>
<td>+</td>
</tr>
<tr>
<td><strong>Inshore bacteria</strong></td>
<td>++</td>
</tr>
<tr>
<td><strong>Gut bacteria</strong></td>
<td>+</td>
</tr>
</tbody>
</table>

++ Large zones of clearing
+ Zones of clearing
- No clearing
An experiment was performed to determine at which stage of the bacterial growth cycle the lytic factor was produced. It was found that activity was present in the SNF of cultures in stationary phase (after 13 h growth).

Concentration of the lytic factor

Several attempts were made to concentrate the lytic factor.

(1) The SNF was lyophilized.

(2) Active SNF was dialized against
   (a) 1M ammonium acetate buffer, pH 7.0 and
   (b) triethanolamine buffer (0.05 M),
   and after dialysis the samples were lyophilized.

(3) Ammonium sulphate precipitation (with a 5M saturated ammonium sulphate solution) of the SNF was carried out.

Methods 1 and 3 inactivated the lytic factor completely. Dialyzed and freeze dried SNF (2), produced zones of lysis on assay plates equal in size to those from unconcentrated SNF.

Degradative enzymes of gut and style bacteria

A summary of the degradative enzymes (identified by plate assays) of representative strains of the gut and style bacterial isolates is presented in Table 19. A notable feature of the style bacterial
A summary of the degradative enzymes of the gut and style bacterial isolates, as determined in plate assays. (Plates: Milk = skim milk, Gel = gelatine, Chit = chitin, Fat = Tween 80, St = starch, Cell = CMC).

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>Classification</th>
<th>Fermentative/oxidative</th>
<th>Bacteriolytic</th>
<th>Plates</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut (N = 12)</td>
<td>Vibrio</td>
<td>Fermentative</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>-</td>
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<td>-</td>
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<td>+</td>
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<td>-</td>
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<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Proteus</td>
<td>Fermentative</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>Oxidative</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>Oxidative</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Style (N = 10)</td>
<td>Not determined</td>
<td>Fermentative</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
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<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Oxidative</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
collection is the relative scarcity of degradative enzymes, with protease and bacteriolytic activities being most common. By contrast, the gut bacterial isolates were able to hydrolyze all of the substrates except carboxymethylcellulose.

The Nelson-Somogyi and thiobarbituric acid assays were used to determine laminarinase and alginase activities respectively in the gut and style bacterial isolates (Table 20). Only two strains produced reducing sugars in the laminarinase assay. Alginase activity was common in the Vibrio strains isolated from the mussel gut, whereas only one style isolate could degrade alginate.

6.4 DISCUSSION

Bacteria isolated from the crystalline style and gut of the mussel C. meridionalis produced a bacteriolytic factor against some marine bacteria. Some characteristics of the lytic agent produced by one style isolate were determined, but the nature of the factor remains uncertain. It is unlikely that bacteriolysis was caused by a true lysozyme (N-acetylmuramyl hydrolase) capable of degrading cell walls, as bacteria such as M. luteus and E. coli were not lysed by it, even in the presence of the chelating agent, EDTA.

The lytic factor may be a protein, as it was inactivated by heat. It is possible that lysis of sensitive target bacteria was caused by one or more extracellular proteases produced by the style isolate. Production of the lytic factor during stationary phase of the bacterial cell cycle may be similar to the secretion of extracellular
TABLE 20

Laminarinase and alginate activities of gut and style bacterial isolates. Laminarinase activity is expressed as mg glucose equivalents released per mg protein (crude cell extract) in a 4 h assay. Alginate activity is expressed as µl malondialdehyde released per mg protein (crude cell extract) in a 5 h assay. (* = No alginate as determined in plate assay).

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>Classification</th>
<th>Laminarinase</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut (N = 12)</td>
<td>Vibrio</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>G6</td>
<td>-</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>G7</td>
<td>-</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td>G8</td>
<td>1.11</td>
<td>5.37</td>
</tr>
<tr>
<td></td>
<td>G9</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>G12</td>
<td>-</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>G13</td>
<td>-</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>G15</td>
<td>-</td>
<td>5.63</td>
</tr>
<tr>
<td></td>
<td>G16</td>
<td>-</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas</td>
<td>G4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium</td>
<td>G18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Proteus</td>
<td>G1</td>
<td>-</td>
</tr>
<tr>
<td>Style (N = 7)</td>
<td>Fermentative</td>
<td>S1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>-</td>
<td>-*</td>
</tr>
<tr>
<td></td>
<td>S6</td>
<td>0.54</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>S15</td>
<td>-</td>
<td>-*</td>
</tr>
<tr>
<td></td>
<td>S20</td>
<td>-</td>
<td>-*</td>
</tr>
<tr>
<td></td>
<td>Oxidative</td>
<td>S2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>S18</td>
<td>-</td>
<td>-*</td>
</tr>
</tbody>
</table>
proteases as reported for *V. alginolyticus* (Hare et al., 1983). However, two of the bacteriolytic style isolates had no proteolytic activity against casein and gelatine (Table 19).

It has been suggested (L. Seiderer, pers. comm.) that bacteriolysis may be caused by a Bdellovibrio-like organism. The bdellovibrions are a group of bacteria that attack host (target) bacteria, and penetrate through the cell wall into the periplasmic space, where they multiply, and subsequently cause lysis of the infested individual (Stolp, 1981). Marine *Bdellovibrio* strains have been isolated (Miyamota and Kuroda, 1975, Varon and Shilo, 1976). Varon (1981) has shown that various marine host strains have different levels of sensitivity to the 'parasite'.

These facts would explain why the lytic factor was not active against *M. luteus* (*Bdellovibrio* strains are by definition restricted to Gram negative host strains), and explain the diverse range of bacterial strains sensitive to its lytic activity. Gram stains of some of the bacteriolytic style isolates showed the bacteria to be very small (*Bdellovibrio* cells may be as small as 0.25 x 1 μm - Shilo, 1966). A feature of the style bacteria is that some produce proteases, and this is similar to *Bdellovibrio* isolates, which produce no carbohydrases (Stolp, 1981). The major difficulty with this interpretation of the facts is that no *Bdellovibrio* strains have been isolated that can form colonies on agar plates (as the style bacteria can), although some host independant strains that can grow in liquid medium without host cells have been described (Starr and Seidler, 1971).
Attempts to establish whether the bacteriolytic activity present in crystalline style extracts of *C. meridionalis* was of bacterial origin (and thus similar to the lytic factor produced by style and gut isolates) were inconclusive. However, bacteriolytic activity produced in the style of *Mytilus edulis* appears to be of bacterial origin (L. Seiderer, pers. comm.). Features that the style and bacterial lytic factor share are:

(1) heat sensitivity,
(2) no activity against *M. luteus*,
(3) activity against a higher percentage of strains amongst the inshore compared with the gut bacteria.

Attempts to concentrate and purify the style lytic agent have also been unsuccessful (L. Seiderer, pers. comm.).

Bacteria isolated from the gut and style of the mussel may be part of a resident microflora that is maintained in the digestive tract of the bivalve. A number of the gut isolates were facultative anaerobes (*Vibrio* strains), and tolerance of anaerobic conditions would be advantageous in an environment that may become partially anaerobic when the mussel, an intertidal animal, is not actively filtering. If the bacteriolytic factor plays a role in the digestive processes of the mussel, a resident bacterial population would have to be resistant to the lytic activity. This does appear to be the case, as the gut bacteria were more resistant to lysis than the inshore bacteria. The more sensitive inshore bacteria may serve as an important source of nitrogen, due to the release of bacterial cell components after bacteriolysis (Seiderer et al., 1984).
It has been mentioned that the carbon requirements of the mussel *C. meridionalis* can be met by utilization of kelp detrital material (Seiderer *et al.*, 1982). This is mediated by an array of enzymes, including amylase, cellulase, laminarinase and alginase, which are localized in the crystalline style. It is unlikely that the style bacteria are at all responsible for some production of these enzymes, as they possess few degradative enzymes (Table 19 and 20). For example, the style has strong laminarinase activity, whereas only one style bacterial isolate caused any breakdown of laminarin. However, release of extracellular enzymes shown to be produced by gut bacteria (Table 19 and 20), may be important in the digestion of detrital material by the mussel. The relationship between the mussel and the gut bacteria may be symbiotic, with the bacteria benefitting by existing in a microenvironment with a reliable source of nutrients due to the filtering action of the mussel, and the mussel having an extra source of degradative enzymes, produced by the gut bacteria.

A remarkable phenomenon was noted by Seiderer *et al.*, (1984), who found that the bacteriolytic agent in the style of *C. meridionalis* was subject to considerable adaptive changes in activity, correlated with water temperature. Water temperatures less than 10°C (that is, upwelled water, depleted in POM), were associated with induction of the bacteriolytic agent. The interpretation of these results was that bacteriolysis, stimulated by the low water temperature, or associated environmental parameter, permitted the mussel to utilize free living bacteria present in the upwelled water, in compensation for the low phytoplankton availability. It is not clear whether changes in bacteriolysis by the style lytic factor was due to varying concentrations or changes in the specific activity of the factor.
Seiderer (1983) also showed that production of the protease by the crystalline style mentioned earlier was coregulated with the induction of bacteriolysis. If the lytic agent produced by the style bacteria plays any role in bacteriolysis by the style, these lytic factors may be coregulated. Thus the bacterial lytic factor may be responsive to environmental changes such as temperature. Regulation of other bacterial degradative enzymes may also be mediated by ambient water temperatures.

In conclusion, it may be said that, although the molecular nature of the lytic factors remains unclear, bacteriolysis probably plays an important role in mussel-bacterial trophic interactions.
CONCLUSION

In all habitats, bacteria are considered the primary agents of remineralization. Morita (1982) has called microbes the principal catalysts in the ocean. This thesis has examined some physiological properties of heterotrophic bacteria isolated from kelp bed habitats, to gain some insight into the role of bacteria in the recycling of organic carbon and nitrogen.

The first step in the utilization of any compound is transport of the substrate across the membrane into the cell. Membrane uptake systems for mannitol of kelp bed Pseudomonas and Vibrio strains were examined. Transport of the sugar into the cells, and its subsequent oxidation for metabolic purposes, although they were different in the two strains, were similar to these processes in P. aeruginosa and E. coli respectively. This suggests that the mechanisms of membrane transport in marine bacteria may be the same as those described for nonmarine strains.

Another purpose of this study was to determine whether the transport systems for two sugars and three amino acids remained functional when the bacteria underwent nutrient deprivation, as may occur in the natural environment. It was found that, in general, the uptake systems were maintained in kelp bed strains kept for extended periods of time in the absence of exogenous energy sources. Thus some bacteria may be able to utilize substrates in the labile fraction of the DOM pool immediately after they become available. There are many reports of bacteria in natural
waters that take up sugars and amino acids (for example, Williams et al., 1976, Wright, 1978, Ferguson and Sunda, 1984). It is possible that the low levels of sugars and amino acids measured in some marine ecosystems are due in part to rapid turnover of the substrates by bacteria.

A kelp bed *Pseudomonas* strain had a mannitol transport system that could be induced after weeks of starvation. The induction process required RNA and protein synthesis. A similar result has been reported for amino acid uptake in natural bacterial populations by Kirchman and Hodson (1984), who suggest that in aquatic environments amino acids are taken up by active transport, which is coupled to protein synthesis. This may vary from strain to strain, as some of the kelp bed isolates respired amino acids.

Little is known of the factors affecting substrate transport, and substrate preferences of natural bacterial assemblages, and this should be a fruitful area for future research. Kirchman and Hodson (1984) have shown that amino acid uptake by aquatic bacterial populations is inhibited by peptides. It is likely that availability of nitrogen sources may affect the transport of a carbon source, and vice versa. Using pure cultures, two factors affecting substrate transport have been determined:

(1) attachment (Bright and Fletcher, 1983), and
(2) hydrostatic pressure (Baross et al., 1974).

Barotolerance may be an important feature in determining transport activity of bacteria in the kelp bed system, where upwelled water containing bacteria from great depths reaches the surface.
The relationships between uptake of substrates, respiration and biosynthesis, cell growth and cell division need to be examined. Some estimates of bacterial production depend on the assumptions that gross uptake of amino acids and sugars is measured in the standard radiolabelled substrate – filtration method, and that assimilation (biosynthesis) can be calculated using the following formula:

\[
\text{Assimilation} = \frac{\text{Uptake}}{\text{Respiration}}
\]

(see for example, Joint and Pomroy, 1982). Experiments with starved kelp bed isolates showed that both these assumptions may not necessarily be valid. Uptake of a radiolabelled substrate measured by filtration of the bacteria is likely to be an estimate of net rather than gross uptake, due to respiration of the transported substrate. Bacteria such as starved kelp bed isolates do not assimilate all of the unrespired substrate, and the sugars or amino acids may remain in an intracellular pool until all of the components necessary for biosynthesis are present. This has been shown for natural marine bacterial populations by Palumbo et al., (1983).

In contrast to the constitutive membrane transport proteins that many of the kelp bed bacteria possess, enzymes involved in breakdown of complex polymers such as laminarin and alginate are inducible. Bacterial extracellular enzymes such as chitinase and cellulase may be responsible for the breakdown of the more refractory substrates throughout the ocean. The existence of these enzymes in natural marine waters remains to be demonstrated, and the levels of substrates and mechanisms needed to induce production of the proteins are unknown.
Genetic techniques may be useful in determining the molecular processes governing the survival of bacterial strains in the aquatic environment. For example, a mannitol uptake negative mutant, otherwise isogenic with its parental *Vibrio* strain, was used to show that possession of a functional transport system for the sugar gave the wild type strain no survival advantage or disadvantage compared with the mutant unless mannitol became available, in which case a higher proportion of the wild type cells survived.

This study has shown that in the kelp beds there are fluctuations in the colony forming fraction of the total bacterial numbers, and that variations occur in bacterial populations found in adjacent habitats. The efficiency of uptake systems specific for various substrates of different bacterial strains may play a role in determining which strains are dominant in assemblages under different nutrient regimes, and in various habitats. Other physiological properties that may be important in these selection processes are:

1. possession of appropriate extracellular degradative enzymes,
2. chemotaxis, and
3. a fermentative or oxidative metabolism.

An understanding of the interactions between bacteria and organisms on the next trophic level is important, if accurate carbon and nitrogen flow patterns are to be drawn up. A bacteriolytic agent, of possible bacterial origin, present in the digestive system of the mussel *C. meridionalis*, suggests that bacteria may be an important food resource for this filter feeder.
Finally, a major problem remaining in assessing marine bacterial activity, is the low percentage of strains that can actually be isolated and studied. Every effort must be made to develop methods to examine the 'non colony-forming' bacteria, which may play a vital role in remineralization in marine ecosystems.


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Utilization of bacteria as nitrogen resource by kelp-bed mussel

significance of style enzymes from two marine mussels (Choromytilus

activity in the crystalline style of the surf clam (Spissula solidissima).
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Agar plates
(For agarase activity)

1/10 SWB
Agar

To visualize zones of activity, flood the plate with Gran's iodine:
0.05 M iodine in 0.12 M KI.

Alginase assay reagents

1. Periodate (make up fresh every time).
   0.025N K1O4 in 0.125 N H2SO4

2. NaAsO2
   2% NaAsO2 in 0.5N HCl

3. Thiobarbituric acid (TBA) pH 2.3
   0.3% TBA in distilled H2O
   (Heating is necessary to dissolve TBA).

Alginate medium

Dissolve sodium alginate (2% w/v) in phosphate buffer (PB), by stirring
the buffer vigorously with a magnetic stirrer bar, and sprinkling the
alginate on the vortex. Dilute this solution with 1/10 SWB in the ratio 1
alginate : 3 1/10 SWB. Raise the pH to 7.0 using 1N NaOH. There is
slight precipitation of the medium during autoclaving.
Alginate plates

1. $\text{K}_2\text{HPO}_4$ 0.3 g
   Distilled $\text{H}_2\text{O}$ 1 ℓ
2. Sodium alginate 25 g
   NaCl 30 g

Make up the potassium phosphate solution (1), and stir vigorously using a magnetic stirrer bar. Sprinkle the dry sodium salts (2) onto the vortex. When the solution is complete, raise the pH to 7.0 with 1N NaOH. Add 12 g agar and autoclave. This is poured as a top layer on basal layer agar (BA).

To visualize zones of activity, flood the plate with acetone.

Basal layer agar (BA)

Peptone 1 g
Yeast extract 1 g
Agar 15 g
Seawater 1 ℓ

Carboxymethylcellulose (CMC) plates

(For cellulose activity)

Dissolve 10 g CMC in 100 ml distilled water (heating may be necessary). Mix this solution with 900 ml of basal agar (BA), and autoclave.
To visualize zones of activity, flood the plate with an aqueous solution of Congo red (0.1% w/v) for 15 min, wash plate with distilled H$_2$O, and then flood with 0.1 N HCl.

**Chitin plates**

Precipitated (swollen) chitin  
(see chitin precipitation procedure)  
30 ml  
Agar  
15 g  
Seawater  
1 l

After autoclaving, this is poured as a top layer on basal layer agar (BA).

**Chitin precipitation procedure**

Dissolve 40 g chitin (powdered crab shells, Sigma) in 400 ml cold concentrated HCl (1 h). Precipitate the chitin by pouring the solution into 2 l of distilled H$_2$O (5 - 10°C), while stirring vigorously. Collect the precipitate by filtration (Whatman's No. 1 filter paper), and dialyze overnight against running tap water. Adjust the pH to 7.0 with 1 N KOH, and autoclave. Do not let the suspension dry out.

**Gelatine plates**

Gelatine  
10 g  
Basal agar (BA)  
1 l
To visualize zones of activity, flood the plate with an acid mercuric chloride solution, which is made up by adding mercuric chloride (12 g) to 80 ml distilled H$_2$O, then acidifying with 16 ml concentrated HCl.

**Laminarinase assay reagents**

(Nelson Somogyi assay for reducing sugars)

1. **Somogyi reagent.**

   A : Na$_2$CO$_3$  
   NaHCO$_3$  
   Potassium sodium tartrate  
   Na$_2$SO$_4$  
   Distilled H$_2$O  

   Heat until boiling, seal in a brown bottle.

   B : CuSO$_4$.5H$_2$O  
   Na$_2$SO$_4$  
   Distilled H$_2$O

   Mix A and B in a ratio of 4 : 1 before use.

2. **Nelson reagent (poisonous)**

   Make up a solution of (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O (25 g) in distilled water (450 ml). Add 21 ml H$_2$SO$_4$, and 3 g Na$_2$HA$_8$O$_4$.7H$_2$O dissolved in distilled water (25 ml). Keep at 37°C for 24 h and seal in a brown bottle.
Luria broth (LB)

- Tryptone: 10 g
- Yeast extract: 5 g
- NaCl: 10 g
- Distilled H₂O: 1 l

Luria agar (LA)

- Luria broth (LB): 1 l
- Agar: 15 g

M9 salts

- Na₂HPO₄: 6 g
- KH₂PO₄: 3 g
- NaCl: 0.5 g
- NH₄Cl: 1 g
- MgSO₄·7H₂O: 0.246 g
- CaCl₂·2H₂O: 14.7 mg
- Distilled H₂O: 1 l

Minimal chemotaxis plates

- Agar: 3 g
- Seawater: 1 g
Autoclave medium, and distribute 20 ml aliquots into plates. Add 20 ul of a stock solution of the attractant (100 mM for amino acids, 10 mM for sugars), and allow agar to 'solidify' for at least 2 h before inoculating.

Nitrosoguanidine (NTG)

Make up in 10% ethanol and 90% distilled water.

Oxidase reagent

1% tetramethyl-p-phenylenediamine (aqueous solution), protect from light.

Phosphate buffer (PB) pH 7.0

This contains 150 mM NaCl.

\[
\begin{align*}
\text{NaCl} & : 8.78 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 2.10 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 1.09 \text{ g} \\
\text{Distilled } \text{H}_2\text{O} & : 1 \ell
\end{align*}
\]

Seawater agar (SWA)

Seawater broth (SWB) : 1 \ell

Agar : 15 g
Seawater broth (SWB)

Peptone \( \frac{1}{10} \) g
Yeast extract \( \frac{1}{10} \) g
Seawater (Whatman's No. 1 filtered) \( \frac{1}{10} \) l

1/10 SWB

Seawater broth 1000 ml
Seawater 900 ml

Skim milk plates
(For protease activity)

1. Skim milk powder 10 g
   Distilled H\(_2\)O 100 ml

2. Basal agar (BA) 900 ml

Autoclave 1 and 2 separately, mix together before pouring plates.

SM buffer

NaCl 21.6 g
KCl 0.66 g
MgSO\(_4\).7H\(_2\)O 6.3 g
MgCl\(_2\).6H\(_2\)O 4.7 g
Tris 1.2 g
Distilled H\(_2\)O 1 g pH 7.8
Starch plates

Potato starch 5 g
Basal agar (BA) 1 ℓ

To visualize zones of activity, flood the plate with Lugol's iodine: 10 g iodine + 20 g potassium iodide in 1 ℓ distilled H₂O.

Tetrazolium indicator plates (Bochner)

Peptone 2 g
Carbohydrate 2 g
(mannitol or glucose)

Tetrazolium 0.025 g
Agar 15 g
Seawater 1 ℓ

Tris maleic acid buffer

NaCl 46.8 g
Tris 24.2 g
CaCl₂·2H₂O 0.58 g
Maleic acid 20.0 g
Distilled H₂O 1 ℓ pH 6.8

Dilute 1 : 1 with seawater for marine bacteria.
Tryptone soy (TS) chemotaxis plates

Tryptone soy broth 3.5 g
Agar 3.0 g
Seawater 1 ℓ

Autoclave medium, pour plates, and leave agar to 'set' for at least 1 h before innoculating.

Tween 80 plates
(For lipase activity)

CaCl$_2$.2H$_2$O 0.1 g
Tween 80 10 ml
Basal agar (BA) 1 ℓ

Vibrio MM salts

1. Salts

K$_2$HPO$_4$ 10.6 g
KH$_2$PO$_4$ 4.56 g
Sodium citrate 0.48 g
(NH$_4$)$_2$SO$_4$ 1.0 g
MgSO$_4$.7H$_2$O 0.1 g
Distilled H$_2$O 100 ml

2. Saline

NaCl 23.4 g
Distilled H$_2$O 900 ml

Autoclave 1 and 2 separately, and mix before use.
APPENDIX B

Chemical Suppliers

BDH Chemicals Ltd., Poole, England
Boehringer Mannheim, West Germany
Difco, Michigan, USA
Merck, Darmstadt, Federal Republic of Germany
Oxoid, Herfordshire, England
Sigma, Missouri, USA

Acridine orange
Agar
Agarose
Alginate, sodium salt
Amino acids:
  alanine, aspartate, glutamate, serine
  valine
Antibiotics:
  ampicillin, chloramphenicol, rifampicin
Bovine serum albumin, fraction V
Carboxymethylcellulose (CMC)
Casamino acids
Chitin
Gelatine
Glucose
Glutaraldehyde
Immersion oil
Laminarin

Maleic acid
Malondialdehyde
Mannitol
Mannitol-1-Phosphate
NAD
NTG
Peptone
Photoflo
Radioactive substrates:
  mannitol, glucose
  alanine, aspartate, glutamate
  thymidine, uracil
Scintillation fluid:
  Instagel
  Scintillator 299
Skim milk powder
Starch (potato)
Tetrazolium
Tryptone
Tryptone soy broth
Tween 80
Yeast extract

Merck
Merck
BRL, Federal Rep. of Germany
Kelco/AIL, Ayrshire, England
Sigma
BDH
Boeringer Mannheim
Seravac, S.A.
BDH
Difco
Sigma
Sigma
Merck
NT Laboratory Suppliers, S.A.
TAAB Laboratory Equipment, England
Nikon
Gift of E. Percival, Univ. London
Sigma
Koch-Light Laboratory, England
Sigma
Merck
Merck
Sigma
Boeringer Mannheim
Sigma
Difco
Kodak
Amersham, United Kingdom

Packard United Technologies,
USA, Switzerland,
Netherlands
Oxoid
British Drug Houses Ltd., London
Merck
Merck
Oxoid
BDH
Merck
APPENDIX C

AODC  acridine orange direct counts
 cfu  colony forming units
 C : N ratio  carbon : nitrogen ratio
 cpm  counts per minute
 DC  direct count
 DOC  dissolved organic carbon
 DOM  dissolved organic matter
 EDTA  ethylenediaminetetra-acetic acid
 HN  high nutrient (agar plates)
 Km  Michaelis Menten constant
 LN  low nutrient (agar plates)
 Man-1-P  mannitol-1-phosphate
 MM  minimal medium
 mtl-  mannitol uptake/utilization mutant
 NAD  nicotinamide adenine dinucleotide
 NTG  N-methyl-N-nitro-N-nitrosoguanidine
 OD  optical density
 PB  phosphate buffer
 PEP  phosphoenolpyruvate
 POC  particulate organic carbon
 POM  particulate organic matter
 PTS  phosphotransferase system
 SNF  supernatant fluid
 SWA  seawater agar
 SWB  seawater broth
 TBA  thiobarbituric acid
 TCA  trichloracetic acid
 TS  tryptone soy (chemotaxis agar)
 TZN  tetrazolium mannitol (agar plates)
 UV  ultraviolet
 VC  viable count
 WT  wild type