Regulation of Beta-Glucosidase in Marine Bacteria

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Regulation of Beta-Glucosidase in Marine Bacteria

A Dissertation in partial fulfillment of the
Doctor of Philosophy in Ocean Sciences
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by
Dennis Stetter

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DOCTOR OF PHILOSOPHY DISSERTATION

OF

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Approved
Dissertation Committee

Major Professor

NOVA SOUTHEASTERN UNIVERSITY

1996
I wish to dedicate this dissertation to

Bill and Ruth Walker

whose friendship made this possible.
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3. References
The following is a study of the regulation of production of a catabolic enzyme, beta-glucosidase, by isolated strains of marine bacteria. Catabolic enzymes transform organic matter to monosaccharides which are utilized as an energy source for growth by bacteria. The bacterial strains were isolated from the Gulf Stream off the coast of Florida, as well as from particulate matter collected from waters adjacent to the Florida coast.

The first section describes the preparation of a liquid medium using sterile saltwater supplemented with inorganic nutrients and a carbohydrate component. This medium allowed growth of marine bacteria under carbohydrate-limiting conditions. A solid agar version of the media was also prepared, which allowed isolation of individual colonies of marine bacteria under carbohydrate-limiting conditions.

The second section describes analyses of the regulation of beta-glucosidase production by five isolated bacterial strains using methylumbelliferyl-glucopyranoside (MUF-glu) as a model substrate. The beta-glucosidase hydrolysis of MUF-glu to glucose and a highly fluorescing product, methylumbelliferon (MUF), allowed a measurement of enzyme activity in laboratory cultures.

The experiments showed that four of the five bacterial strains isolated could regulate production of beta-glucosidase. When cellobiose, in particular, was the only carbohy-
drate present, the four strains showing regulatory ability produced elevated levels of enzyme activity. This elevated enzyme activity was not observed when glucose was provided as the only carbohydrate source. The fifth strain showed only low-level enzyme activity in the presence of cellobiose or glucose. This is the first evidence of the regulation of beta-glucosidase activity in particular strains of marine bacteria.

Authenticity of beta-glucosidase activity was confirmed with known inhibitors of beta-glucosidase, gluconic acid, and glucose. The enzyme activities of all the isolated strains, measured by hydrolysis of MUF-glu to fluorescent MUF, showed sensitivity to both enzyme inhibitors. The sensitivity was observed as lower MUF production compared to control assay samples with no inhibitor added.

The first isolated bacterial strain, from Gulf Stream waters, also showed an ability to repress the production of beta-glucosidase in the presence of glucose. This strain was tested with cyclic AMP, known to neutralize glucose repression of beta-galactosidase in E. coli. Cyclic AMP, however, did not neutralize the effect of glucose on repressing beta-glucosidase activity in the isolated marine bacterium.
1. PREPARATION OF CARBOHYDRATE-LIMITING MEDIA FOR IN-VITRO
STUDIES OF CARBOHYDRATE METABOLISM IN MARINE BACTERIA

ABSTRACT

An adaptation of Guillard's phytoplankton culture medium (Guillard, 1963) was prepared for the cultivation of marine bacteria in vitro. Gulf Stream seawater samples were membrane-filtered (0.45 micron pore size), and the filtrates were used as inoculum in the adapted medium. Growth curves of cultures growing at several different glucose concentrations were determined. The adapted liquid medium provided a carbohydrate-limiting environment in which the marine bacterial response to various concentrations of different carbohydrates could be analysed.

A carbohydrate-limiting saltwater agar medium was also prepared for this study. The medium was composed of dialyzed agar with supplemental inorganic nutrients and 50 mg/l cellobiose dissolved in saltwater. The solid medium was used to grow colonies of individual strains of marine bacteria with the ability to utilize cellobiose as a carbohydrate energy source. Bacteria capable of hydrolyzing cellobiose to glucose would possess a beta-glucosidase enzyme.
1.1 Introduction

Studies of bacteria have shown that enzymes which direct metabolic pathways may be induced or repressed depending on significant metabolites in their immediate environment (Paigen and Williams, 1970). The standard reference model for induction and repression of enzyme production is the group of genes which catabolize lactose in *Escherchia coli* (Jacob and Monod, 1961). In this model (Figures 1, 2, and 3) (Tortora et al. 1995) a segment of DNA contains a group of genes known as the lactose operon. The operon consists of a regulatory gene (I) which produces a repressor protein, a control region which has a promoter site (P) where RNA polymerase attaches for transcription and an operator site where the repressor protein attaches to block transcription, and three structural genes (Z, Y, and A) which are needed in lactose metabolism.

Figure 1.

In the absence of lactose (Figure 2), the product of the I gene (the repressor protein) attaches to the operator
and blocks RNA polymerase from transcribing the structural genes (Z, Y, and A).

**Figure 2.**

When lactose is in the environment (Figure 3), a derivative of lactose (allolactose) binds to the repressor protein, preventing the attachment of the repressor protein to the operator. This opens the operator, and the induction process begins as RNA polymerase attaches to the promoter site and transcribes the structural genes. The Z gene produces the enzyme beta-galactosidase, which hydrolyses lactose into glucose and galactose.

**Figure 3.**
The repression of enzyme production is also demonstrated by the \textit{E. coli} lactose operon. In the presence of glucose, the operon cannot be transcribed. Glucose causes a reduction in intracellular concentrations of the regulatory molecule, cyclic AMP. RNA polymerase requires cyclic AMP in order to attach to the promoter. The absence of cyclic AMP results in RNA polymerase not being able to attach to the promoter, thus preventing transcription (Pastan and Adhya, 1976).

The hydrolysis of polysaccharides is an acknowledged rate-limiting step in the transformation of organic matter to utilizable monosaccharides by bacteria (Billen, 1982). With few exceptions, bacteria cannot transport polymers and thus depend on the degradation of large macromolecules to small oligomers or monomers (Degens, 1975). However, in spite of the significance of polymer hydrolysis, relatively little is known about its characteristics, dynamics, or controls in marine ecosystems (Meyer-Reil, 1981). The production and regulation of enzymes which direct carbohydrate metabolism in other types of bacteria, such as \textit{Escherichia coli}, is better understood (Pardee \textit{et al.} 1959, Paigen \textit{et al.} 1970). Some studies have examined inputs of particulate organic compounds and subsequent changes in marine microbial activities or metabolic end products (Molongoski, 1980). Others have examined the rates of transformation of added radiolabeled polymers, such as lignocelluloses (Benner, 1984). However, there have been relatively few detailed
studies of the patterns and rates of polymer hydrolysis or of the enzymes that are involved in marine ecosystems.

Polymers are catabolized by a series of reactions to provide a group of essential metabolites which are used as the basic building blocks for the synthesis of the monomers, coenzymes, and fundamental structural units of the cell (Doelle, 1969). Since these catabolic reactions are oxidative in character they also give rise to the production of ATP and NAD(P)H.

Enzymes catalyzing the glycolytic pathway and the tricarboxylic acid cycle constitute the basal intermediary metabolism. They are concerned both with the production of energy and reducing equivalents and with the synthesis of the essential cell building blocks (Kelly, 1971). Such enzymes and pathways which can function in both catabolism and anabolism are known as amphibolic and are usually present irrespective of the environment conditions. They are referred to as constitutive because they are produced continually by the cell.

In contrast to the constitutive enzymes, are the enzymes leading into catabolic pathways which are concerned with the specific utilization of particular carbon sources. Since many heterotrophic micro-organisms are capable of using over a hundred different carbon sources (Doelle, 1969) and since many specific enzymes may be involved in each of the pathways, a cell has the potential to produce a very large number of catabolic enzymes. In enteric bacteria, such as E. coli, these enzymes are only formed when they are
actually required, i.e., they are induced when the specific carbon source is present in the environment, the example already mentioned being beta-galactosidase produced in the presence of lactose.

Another example of an inducible enzyme is beta-gluco-sidase which can hydrolyze celllobiose (O-beta-D-glucopyranosyl-1,4-beta-D-glucopyranoside) and chemically related glucosides (Grabnitz, 1988). Together with endo- and exo-glucanases, beta-glucosidase plays an essential role in the biological saccharification of cellulose (Shewale et al. 1982). Many species of micro-organisms produce beta-glucosidases and different forms of the enzyme have been isolated. These different enzyme forms have different mechanisms of action. Hydrolysis of celllobiose may occur by cleavage of the glucosidic bond to yield two molecules of glucose. This reaction is catalyzed by the enzyme cellobiase (beta-D-glucoside glucohydrolase) which is present in enteric bacteria and yeasts. A second mechanism consists of phosphorolysis of celllobiose into one molecule of glucose and one molecule of glucose-1-phosphate. This reaction is catalyzed by celllobiose-phosphorylase, an enzyme which has been described in enteric bacteria (Alexander et al. 1968) and fungi (Heale et al. 1971). There is a third mechanism of phospholysis which yields glucose and glucose-6-phosphate (Palmer et al. 1971).

The metabolic activity of microheterotrophs is also important from another point of view: small organisms such as bacteria have short generation times and one of the
highest metabolic rates per unit of biomass. Most organic matter (75-99%) in aquatic environments exists as dissolved organic matter (DOM), and the rest is living (biomass) or dead (detritus) particulate fractions (Sharp, 1973). All DOM sources are particulate. During the conversion of living biomass to nonliving detritus, autolytic enzymes from the organisms themselves initiate the solubilization of certain cell compounds. In successive decomposition steps the exoenzymatic capacity of bacteria and fungi is certainly involved. Through microbial exoenzymatic activity, particulate organic matter (POM) is transformed stepwise into DOM (Halemjko, 1986). Other mechanisms of DOM production include phytoplankton exudation (Chrost and Faust, 1983), excretion by microzooplankton and macrozooplankton (Webb and Johannes, 1967), and herbivorous zooplankton (Lampert, 1978). Only a small fraction (less than 5%) of DOM, however, can be directly taken up by bacteria. A major fraction of DOM consists of compounds with high molecular weight such as proteins, polysaccharides, nucleic acids, and humic materials which are not directly available to aquatic bacteria (Ammerman et al. 1984).

The idea that bacteria form an important trophic link between DOM and higher trophic levels has been presented in the concept of the microbial loop (Azam et al. 1983). A major step in the concept of the microbial loop is the conversion of POM, often characterized as "marine snow" (Alldredge and Cohen, 1987), and DOM into bacterial biomass. The marine snow macroaggregates in surface waters are hydro-
lysed to DOM; they reform at mid-depth, then sink, dissolve, and reform again on their descent to the ocean floor, providing nourishment to bacteria with each cycle of hydrolysis to DOM. The bacteria, subsequently, serve as an additional food source to zooplankton at the base of the marine food chain (Alldredge and Silver, 1988).

The penetration of organic molecules across a bacterial cell wall is an active process occurring with the intervention of specific enzymes called permeases. Only small molecules (monomers and small polymers) can therefore be taken up. Through the production of exoenzymes and after subsequent hydrolysis of compounds to monomers or oligomers, DOM can be ultimately absorbed by bacteria (Rogers, 1961). The products of exoenzymatic decomposition serve as nutrients for heterotrophic bacteria as well as an input of substrates to the pool of low-molecular-weight DOM which is rapidly incorporated and mineralized by bacteria.

Polysaccharides are common structural and storage polymers in both aquatic and terrestrial organisms and represent the major form of photosynthetically fixed carbon in the biosphere. In aquatic ecosystems carbohydrates may contribute 10-20% and more than 25% to the DOM and POM respectively (Munster, 1984). Dissolved carbohydrates are also major sources of carbon and energy for aquatic bacteria (Hagstrom et al. 1984). Only some of these compounds are ready for assimilation by bacteria, while others must undergo further enzymatic hydrolysis before use. Therefore, the hydrolysis of polymeric carbohydrates is generally accepted
as the first and rate-limiting step in their mineralization by heterotrophs (King, 1986). Many of these transformations can only be mediated by heterotrophic bacteria since the enzyme systems required are not found in higher organisms (Morita, 1982). In spite of the significance of carbohydrate hydrolysis, however, relatively little is known about the regulation of these enzyme systems, whether or not they must be induced, and if so, their response time to substrate inducers.

Recent studies (Karner and Herndl, 1992; Rath and Herndl, 1994) have focused on measurement of extracellular enzymatic activity by both free-living and marine-snow-associated bacteria in the pelagic environment of the northern Adriatic Sea. Although bacterial density and production were similar, hydrolytic activity (beta-glucosidase for example) was significantly higher in marine-snow-associated bacteria, in terms of both absolute and per-cell rates. Because concentrations of dissolved total and monomeric carbohydrates and free amino acids in marine snow were very close to those in the ambient water, Karner and Herndl suggested that the differences in extracellular enzyme activity between free-living and marine-snow-associated bacteria did not simply reflect repression of catabolic enzyme expression as reported in other studies (Chrost, 1989). One hypothesis was that substrate induction of enzyme activity was responsible for the observed higher hydrolase activity in marine snow bacteria. Another hypothesis was that there were distinct bacterial species associated with
marine snow producing constitutive, unregulated higher levels of exoenzymes.

The observed differences in hydrolase activity per cell in ambient water and in marine snow may be caused by a different percentage of bacteria actively expressing a distinct enzyme and/or by different bacterial species compositions between the ambient water and marine snow. Wрангстадт et al. (1989, 1990) found enhanced extracellular enzymatic activity during the early phase of starvation in marine bacteria and speculated that free-living bacteria become more active when they attach to particulate organic matter.

The elevated extracellular hydrolase activity in bacteria associated with marine snow does not simply reflect that the attached bacteria have a larger surface, since the attached and free-living bacteria did not differ significantly in size (Herndl, 1988). Recent findings indicate that the carbon demand of attached bacteria cannot account for the apparent disappearance of particles with depth as estimated from sediment trap deployments (Cho and Azam, 1988). In order to explain this "particle decomposition paradox", Karl et al., 1988, have hypothesized that attached bacteria exhibit "hyperproduction" of enzymes and the resulting organic matter (DOM) becomes available to the free-living bacterial community.

Another reason for the observed differences in specific activity of hydrolase enzymes between marine snow and free-living bacteria might have been the control of enzymatic
activity by substrate and/or end products. Unlike Chrost (1989), who detected repression of glucosidase activity by glucose, Karner and Herndl (1992) were not able to detect such a regulation mechanism, i.e., there was no inverse ratio between the concentration of glucose and the activity of beta-glucosidase in their analyses. Whether there was substrate induction affecting marine-snow-associated bacteria or whether there were distinct bacterial species capable of high enzyme activity associated with marine snow remained unresolved.

1.2 The Purpose of This Study

This study sought to determine if individual strains of marine bacteria could regulate their production of beta-glucosidase depending upon their carbohydrate environment. This would be in contrast to the hypothesis that different bacterial species produce different levels of beta-glucosidase, each in a constitutive, i.e., unregulated, manner.

It is possible to grow aquatic bacteria in continuous cultures with filtered seawater for several generations (Bjornsen, 1986). Supplementation of the filtered seawater with inorganic nutrients and 50 mg/liter of glucose could raise the level of growth in continuous culture (Morel and Ahn, 1990) sufficiently to provide an enrichment culture for the subsequent selection on solid agar medium of bacterial strains with the capacity for beta-glucosidase production.
A seawater agar medium on which to isolate colonies of marine bacteria was developed. It was carbohydrate-limiting in order to select bacterial strains with the ability to utilize cellobiose as the only carbohydrate energy source. The bacterial strains which metabolized cellobiose possessed beta-glucosidase, which hydrolyzes cellobiose to utilizable glucose. The individual isolated strains were subsequently tested in carbohydrate-limiting liquid medium for their growth with different carbohydrate substrates to test the effect of the substrates on the regulation of beta-glucosidase production. A quantitative measure of enzyme activity, using a fluorogenic enzyme assay, was used to determine whether or not an individual strain could regulate the production of the enzyme.

1.3 Methods and Materials

1.3.1 Liquid Medium Preparation

Starting in the fall of 1991, seawater samples were collected at Port Everglades, Florida, east of Nova Southeastern University Oceanographic Center. The coastal seawater had a salinity of approximately 34 parts per thousand measured by an a YSI Model 33 conductivity-temperature meter. The seawater pH was approximately 8.1 measured by an Allied (Fisher) portable pH meter. Two liter samples of the coastal seawater were passed through a Whatman GF/F fiber-
glass filter apparatus utilizing vacuum pressure to exclude eucaryotic cells and large particulate matter. The filtered seawater was decanted into 500ml polypropylene storage bottles and sterilized by autoclaving at 121°C (15 pounds per square inch) 15 minutes. The bottles were loosely capped during autoclaving to allow steam contact and to prevent bottle expansion. This filtered, sterilized seawater was used as the base of the liquid medium to be prepared for the culture of marine bacteria in vitro.

In order to utilize carbohydrate as a growth-limiting constituent, a defined inorganic salts supplement was prepared to supply ample nitrate, phosphate, and other inorganic growth factors. There are few published defined inorganic media for the growth of marine microbes in vitro (Rheinheimer, 1986). One exception is Guillard's F medium for the growth of marine centric diatoms (Guillard, 1963). Guillard's formula is shown in Table 1.

A modified version of Guillard's F formula was developed to grow marine bacteria in vitro in this study. Bacteria have the ability to synthesize vitamins and do not require silicon, therefore, the formula was simplified by dropping these components. The concentrations of the remaining supplemental nutrients were divided by four, because procaryotic bacteria require lower concentrations of inorganic nutrients than eucaryotic diatoms, and were tested as to whether or not these reduced concentrations were sufficient for bacterial growth in vitro. The adapted formula was referred to as F/4 medium and is given in Table 2.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>Sea Water</td>
<td>1 liter</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>150 mg (1.765 mM)</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>10 mg (72.5 μM)</td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
<td>30 mg (10 μM)</td>
</tr>
<tr>
<td>Ferric sequestrene (1)</td>
<td>10 mg (1.3 mg Fe)</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0196 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.044 mg</td>
</tr>
<tr>
<td>CoCl₂·4H₂O</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.360 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.0126 mg</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>1.0 ug</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>1.0 ug</td>
</tr>
</tbody>
</table>

(1) Ferric sequestrene is the sodium iron salt of ethylene diamine tetra acetic acid, 13% iron.
A stock solution of the F/4 medium ingredients was prepared at 10X the desired concentration of the medium and sterilized by autoclaving. A stock solution was diluted 10-fold when added to sterile filtered seawater to prepare the medium.

In initial studies the growth of marine bacteria in F/4 medium was measured spectrophotometrically which required turbid levels for detection. Marine bacteria do not grow to turbid levels at the natural nutrient levels of the ocean. In the laboratory, therefore, the F/4 medium was supplemented with known levels of carbohydrates to generate turbid cultures. This is referred to as a minimal medium because it contains only the inorganic nutrients plus a defined carbohydrate required by bacteria for growth, as well as the constituents of seawater.
To determine the effectiveness of this medium, glucose was added at a concentration of 50mg per liter (1.7mM carbon) which has been used in other studies of marine bacterial growth in vitro (Goldman et al. 1991). 50mg glucose per liter is relatively high compared to natural dissolved organic carbon concentrations in the ocean of about 1mg per liter (Holm-Hansen et al. 1966). A stock solution of glucose was prepared at a concentration 100X of the desired 50mg/l and sterilized by autoclaving. The stock solution was diluted 100-fold when added to the minimal medium.

1.3.2 Collection of Marine Bacteria from Ocean Waters

The initial bacteria collected for this study were from seawater samples taken inside the Gulf Stream off the coast of Port Everglades, Florida, utilizing a Niskin sampler at a depth of 90 feet. The samples were transferred to sterile 500ml polypropylene bottles and stored at 5°C.

Marine bacterial cells are at the low end of the spectrum of procaryotic cell size. Marine cocci can be as small as 0.5 micron in diameter or less, and marine bacilli can be 0.5 micron or less in cross-section and 1.0 micron in length (Bergery's Manual, 1986). Millipore Type HA filters have mean pore diameters of approximately 0.5 micron and can capture enteric bacteria, which are as large as 1 micron in cross-section and 8 microns in length. These filters will, however, allow some of the smaller marine bacteria to pass through.
In order to obtain an exclusively bacterial inoculum for growth curve experiments in F/4 medium, 20 ml samples of Gulf Stream water were vacuum filtered through a sterile Millipore filter apparatus. The filtrates were decanted into sterile test tubes and stored at 5°C.

1.3.3 The First Growth Experiment in F/4 Medium

The first experiment to test the ability of the F/4 medium to support marine bacterial growth was done in a set of six 18ml test tubes filled with 10ml of F/4 medium supplemented with 50mg/l glucose. Each culture was initiated by inoculating with 0.1 ml of the same Gulf Stream filtrate. The six cultures were placed on an inclined rotating tube rack allowing a slow turnover of media in the tubes (60 rotations/hour). The experiment was conducted at room temperature (22°C).

The test tube cultures showed no visible turbidity during the first 48 hours of the experiment. On the third day, slight turbidity could be seen and measured. Culture growth was measured by turbidity readings taken at 450nm on a Milton Roy Spectronic 20D spectrophotometer. From that point onward the turbidity of each test tube culture was measured every 6 hours until the culture passed through stationary phase and began to decline. The experiment was stopped on the fifth day, and one test tube culture was preserved at 5°C as a more concentrated inoculum for future experiments.
Each sample culture tube was viewed microscopically at each sampling interval. Gram-stain slides were also prepared. All slides were viewed at 1000X on an Olympus Model CHT microscope with bright-field illumination.

1.3.4 Growth Experiments at Varying Glucose Concentrations

A second set of experiments tested a range of glucose concentrations while maintaining the concentrations of all other components of the F/4 medium (Table 2). Five sets of six test tubes, each containing 10 ml of F/4 medium were prepared. Each set of test tubes contained a different concentration of glucose: 50mg/l, 25mg/l, 10mg/l, 5mg/l, and 1mg/l. All tubes were inoculated with 0.1ml of the stock culture of Gulf Stream bacteria preserved from the first experiment and incubated on an inclined wheel rotating at one revolution per minute at room temperature (22°C).

The duration of these growth experiments was limited to four days. Bacterial cultures growing over longer periods of time might be affected by organic by-products of metabolism causing changes in growth patterns not associated with the particular substrate of interest.

The five sets of culture tubes were visually inspected for the first signs of turbidity over the four day period. When turbidity appeared in each set, levels were quantified by reading their absorbances at 450nm periodically until the turbidity reached a maximum and began to decline. The absor-
bance readings were plotted over time for each glucose concentration. Regression slopes were computed from the set of readings for each glucose concentration to compare rates of growth. The test of the significance of difference between two slopes (Edwards, 1973) was used to compare growth rates at successively higher glucose concentrations.

1.3.5 Preparation of the Solid Agar Medium

In order to preserve the carbon-limiting characteristic of the solid version of the F/4 liquid medium, commercial agar was dialyzed to remove impurities (Sieburth, 1979). Powdered Difco-brand agar was added to boiling seawater, previously filtered and autoclaved, at twice the normal 15 gm per liter concentration. When the agar powder had melted in the boiling seawater, the mixture was poured in wide trays to a depth of about 4 mm. After the rehydrated agar had gelled at room temperature, the agar sheets were cut into 2 cm squares. Deionized water was then poured over the agar squares to a depth of 2 cm, and the trays were placed in a refrigerator (5°C) for 48 hours. Every 48 hours over a period of eight days, the deionized water in the trays was poured off, and fresh deionized water was added. This allowed diffusion of free glucose, soluble polysaccharides and dissolved inorganic materials from the agar. There was a noticeable lightening of color in the agar squares as a result of the dialysis.

The dialyzed agar squares were then used to prepare a
solid salt-water medium by mixing dialyzed agar squares with a two-fold concentrate of F/4 saltwater medium of equal volume to agar in a glass beaker. The mixture was autoclaved at 121°C for 15 minutes. After autoclaving, the sterile melted medium was placed in a water bath at 50°C. A desired carbohydrate supplement was added and the mixture was aseptically poured into sterile Petri dishes at 15 ml per plate to solidify at room temperature. This was then called F/4 dialyzed agar medium (F4Dagar).

In the first set of experiments, F4Dagar medium was prepared in two batches, one with and one without 50 mg/l supplemented glucose. Six plates from each batch were inoculated with 0.1 ml of 0.45 micron Millipore-filtered Gulf Stream seawater spread over the agar surface with a sterile glass rod. The plates were kept at room temperature (22°C) and observed for growth at 24 hour intervals.

In a second set of experiments, dialyzed agar squares, prepared with deionized water, rather than seawater, were added to deionized water supplemented with two-fold F/4 mineral ingredients as described above and glucose at 50 mg/l. Six plates were aseptically filled with 15 ml of this sterile medium and inoculated in the same manner as the first set of experiments.

1.4 Results

1.4.1 Microbial Growth in Liquid Medium

Figure 4 shows the results of the first growth experiment. Growth in F/4 medium took nearly 72 hours to produce
turbidity which caused an increase in absorbance readings on the spectrophotometer. The microscopic slides indicated that only bacteria one micrometer or less in length and one-half micrometer in width were present in the test tube cultures, generally Gram-negative bacilli and cocci of mixed morphologies.

The results of the second set of growth experiments are presented in Figures 5 and 6. Regression lines of the exponential growth phase from each growth curve are included. The slope values of the growth curve regression lines compared to the glucose concentrations of the culture medium are shown in Figure 7 and Table 3.

Cultures containing 50 mg/l glucose level (Figure 5) showed faint turbidity after only 24 hours and increased thereafter exponentially until they exhausted the glucose and went into stationary phase at about 48 hours.

The sets of cultures containing 25 mg/l, 10 mg/l, and 5 mg/l glucose (Figure 6) each developed turbidity more slowly, with declining glucose concentration over the four day period. The 5 mg/l glucose culture had only faint turbidity on the fourth day. The cultures supplemented with 1 mg/l glucose did not produce visible turbidity during the four day period. Microscope wet slides indicated, however, the presence of live minute bacteria similar in size and morphology to the inoculum present in the culture in scarce quantity.
Figure 4. The microbial growth in the Gulf Stream Millipore filtrate culture in F/4 medium supplemented with 50 mg/l glucose measured by turbidity readings taken at 450nm on a Milton Roy Spectronic 20D spectrophotometer. Two sample readings were taken at each time period; the mean values plus and minus the standard deviations are plotted.

Filtrate Growth Curve at 50mg Glucose/L
Absorbance: 450nm
Figure 5. Microbial growth in F/4 medium with 50 mg/l and 25 mg/l glucose supplements.

Growth Curves at Various Glucose Conc.
Absorbance: 450nm

Figure 6. Microbial growth in F/4 medium with 10 mg/l, 5 mg/l and 1 mg/l supplements.

Growth Curves at Various Glucose Conc.
Absorbance: 450nm
Figure 7. Slope values (± the standard error) of the growth curve regression lines compared to the glucose concentrations of the culture media of microbial cultures (Table 3).

GROWTH CURVE REGRESSION SLOPES AT VARIOUS GLUCOSE CONC.

Table 3. Glucose concentrations and slope values of regression lines of growth curves presented in Figures 5 and 6.

<table>
<thead>
<tr>
<th>Glu. conc.:</th>
<th>R:</th>
<th>N:</th>
<th>Slope Value:</th>
<th>Std. Err.:</th>
<th>t:</th>
<th>df:</th>
<th>p:</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/l</td>
<td>0.99</td>
<td>3</td>
<td>0.003509</td>
<td>0.000179</td>
<td>2.24</td>
<td>4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>25 mg/l</td>
<td>0.98</td>
<td>5</td>
<td>0.002514</td>
<td>0.000238</td>
<td>2.76</td>
<td>8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10 mg/l</td>
<td>0.96</td>
<td>7</td>
<td>0.001552</td>
<td>0.000180</td>
<td>4.06</td>
<td>12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5 mg/l</td>
<td>0.98</td>
<td>8</td>
<td>0.000765</td>
<td>0.000052</td>
<td>9.80</td>
<td>14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1 mg/l</td>
<td>0.66</td>
<td>8</td>
<td>0.000036</td>
<td>0.000015</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The differences between the increasing slope values are significant, re. computed P values <0.0001 (Edwards, 1973).
1.4.2 Microbial Growth on Solid Agar Medium

The growth of marine bacteria on the two sets of experimental F4Dagar medium, the dialyzed agar mixture containing saltwater and inorganic salts, with or without glucose supplement, was observed over a five day period. During the first 72 hours there was no visible growth on either set of agar plates, i.e., with or without glucose supplement. After 72 hours, pin-point colonies, approximately 50 per plate, started to appear only on the first set of saltwater F4Dagar plates supplemented with glucose. The colonies were viewed under a dissecting microscope (40X) in order to study the diverse colony morphology. After five days the colonies reached maximum size generally not exceeding one millimeter in diameter.

There were no observable colonies under 40X magnification after 5 days on the second set of saltwater F4Dagar without glucose supplement.

There was no growth after 5 days on the third set of plates in the experiment containing a dialyzed agar mixture of deionized water instead of seawater, inorganic salts, and glucose supplement (Table 4).

Table 4.

<table>
<thead>
<tr>
<th>Type of Medium</th>
<th>After 5 Days:</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4Dagar with 50 mg/l glucose:</td>
<td>50 colonies/plate</td>
</tr>
<tr>
<td>F4Dagar without glucose added:</td>
<td>No colonies/plate</td>
</tr>
<tr>
<td>F4D freshwater agar + 50 mg/l glucose:</td>
<td>No colonies/plate</td>
</tr>
</tbody>
</table>
1.5 Discussion

The increasing slope values of the five growth curve regressions corresponding to the five increasing concentrations of glucose (Table 3) show that the lower glucose concentrations are growth rate limiting. Figure 7 shows that a glucose concentration of 50 mg/l produces a growth curve slope value which levels-off asymptotically compared to slope values at lower glucose concentrations. This indicates a rate-saturating concentration of carbohydrate where other factors become growth limiting. Therefore, this particular adaptation of Guillard's medium was carbohydrate-limiting within the range of 1 mg/l to 50 mg/l glucose supplement. In F/4 medium supplemented with 50 mg/l glucose the molarities of carbon, nitrogen, and phosphate are 1667 : 441 : 18.1 respectively indicating molar ratios of 92 : 24 : 1. These values show relatively high concentrations of nitrogen and phosphate versus carbon when compared to the average molar concentrations of these substances in ocean waters, 106 : 16 : 1 (Redfield, 1934). The carbon/nitrogen ratio (C/N) in F/4 medium is 3.8 compared to the Redfield C/N ratio of 6.6 which indicates that nitrogen was supplied in excess and that the carbohydrate component in F/4 medium is rate-limiting for bacterial growth.

The set of culture tubes containing a 5 mg/l glucose supplement produced low turbidity after 48 hours (Figure 6). This indicated that a bacterial culture grown in F/4 medium with a initial glucose concentration between 1 mg/l and 5
mg/l could be inoculated and grown for approximately 16 hours below turbid levels. This non-turbid in vitro growth would be similar to non-turbid marine environment bacterial concentrations (Hobbie et al. 1977). Pulses of other types of carbohydrates could be added thereafter to study their effect on growth of the culture.

The procedures used in these experiments were derived from techniques used in the study of enteric bacteria in vitro. Enteric bacteria are grown in defined freshwater inorganic media to study carbohydrate utilization (Cappucino and Sherman, 1987).

Table 4 shows that the solid media (F4Dagar) could be used for the isolation of individual strains of marine bacteria under carbohydrate-limiting conditions. There was growth only on the F4Dagar plates containing seawater and 50 mg/l glucose. The supplemental glucose was the controlling factor required by the bacteria to grow to colony size. In future experiments, individual colonies of bacterial species with abilities to grow on particular carbohydrates could be isolated by adding those particular carbohydrates as supplements. These individual colonies could be different species of marine bacteria, each with distinct abilities to utilize the particular carbohydrate supplements.

No growth on F4Dagar medium containing seawater without glucose supplement indicated that the dialysis procedure removed extraneous carbohydrate from the agar, and the bacteria could not produce visible colonies without a carbohydrate supplement. Therefore, the dialyzed agar medium was
suitable for growth experiments where the type and concentration of carbohydrates must be controlled. No growth results on the medium made with deionized water indicated that the cultivated bacteria were obligate seawater strains unable to grow on freshwater medium despite the presence of glucose.
2. A FLUOROMETRIC STUDY OF BETA-GLUCOSIDASE REGULATION IN MARINE BACTERIA

ABSTRACT

Bacterial strains were isolated from the Gulf Stream and from marine snow-like macroaggregates in Florida coastal waters. These strains were analyzed for their ability to regulate the production of beta-glucosidase under carbohydrate concentrations approaching those of the natural marine environment. Cell concentrations in batch cultures at approximately $1 \times 10^6$ cells/ml were counted by a modified hemocytometer procedure and confirmed by epifluorescence microscopy. Assays of beta-glucosidase production by the selected strains at carbohydrate concentrations of 2.5 mg/l were performed using methyumbelliferyl-glucopyranoside (MUF-glu) as a model substrate. Enzyme activities indicated that several strains could regulate the production of beta-glucosidase related to the carbohydrate available in the medium. One isolated strain produced constitutively low levels of beta-glucosidase irrespective of which carbohydrate was in the medium. The enzyme activities of all the isolated strains were sensitive to chemical inhibitors of the beta-glucosidase enzyme. The one strain showing repressibility of enzyme activity by glucose did not respond to cyclic AMP in an attempt to neutralize the glucose repression, unlike the ability of cyclic AMP to overcome the repression of beta-galactosidase in the bacterium *Escherichia coli*. 
2.1 Introduction

2.1.1 Characteristics of Beta-Glucosidase

Recent studies based upon anion-exchange and size exclusion chromatography report similar molecular characteristics in beta-glucosidase isoenzymes isolated from mixed microbial assemblages in marine snow and in surrounding ambient waters in the Adriatic Sea (Rath and Herndl, 1994). Significant difference has been reported in quantity of enzyme activity in these microbial assemblages, which appears to be higher inside marine snow than in ambient waters (Karner and Herndl, 1992).

This study reports on four strains of marine bacteria which show the ability to induce relatively high levels of beta-glucosidase activity when provided with cellobiose as a sole carbohydrate source. This suggests a common ability of cellulose-utilizing marine bacteria to regulate their production of beta-glucosidase depending upon their immediate carbohydrate environment.

Applications of several substrate analogs have proven useful in characterizing various catabolic enzymes. The analogs provide colorimetric or fluorogenic products upon hydrolysis, not available in natural substrates, which allows quantitative measure of enzyme activity. The substrate analog methylumbelliferylglucopyranoside (MUF-glu), can be hydrolyzed by the enzyme beta-glucosidase, to one molecule of glucose and one molecule of methylumbelliferone.
which is fluorogenic in ultraviolet light and can be measured quantitatively with a fluorometer (Mead et al. 1955). Hoppe (1983) and Somerville (1984) have used substrate analogs to determine the temporal and spatial distribution of several enzyme systems and their relationships with bacterial biomass and productivity. The hydrolysis of these glycoside analogs have been used to determine the kinetic properties of various enzyme systems and their responses to key environmental variables, e.g., temperature, salinity, pH; King, 1986. King reported on several characteristics of beta-glucosidase from marine sediments. The pH and salinity optima tended to reflect the in situ marine environment values for these parameters with maximum activities at pH 8.5 and 30 ppt salinity.

The apparent evolutionary adaptation of the genes which produce beta-glucosidase to ambient salinity is particularly intriguing because sodium at seawater salinities inhibits a number of enzymes (Hochachka and Somero, 1984). The addition of sodium has been reported to inhibit a variety of soil exoenzymes (Frankenburger and Bingham, 1982). It is possible that exoenzymes localized on the outer surfaces of marine bacteria differ significantly in composition relative to that in intracellular systems as an adaptation to high ambient sodium concentrations.

The beta-glucosidase system also has been shown to exhibit a positive response to added calcium and magnesium. The response to magnesium was greater than that to calcium, which may result from a requirement for a divalent ion, with
magnesium more effective than calcium. This relative response to magnesium and calcium has also been reported in studies of beta-D-galactosidase (Cesca et al., 1984).

In contrast to the response to pH and salinity, the beta-glucosidases exhibit no obvious adaptation to ambient temperature because the optimum, 40°C, was considerably higher than in situ temperatures (King, 1986). It could be that the observed optimum maximizes hydrolysis during the summer when the marine ecosystem is most productive and that optimization is less critical during colder periods. However, there are no data indicating such optimization in temperate systems in general.

Beta-glucosidase has the ability to hydrolyze the disaccharide cellobiose. Cellobiose (Figure 8) is composed of two glucose molecules, bonded by a beta-linkage between the two molecules.

![Figure 8.](image)

(+) Cellobiose (β-anomer)
4-O-(β-D-Glucopyranosyl)-D-glucopyranose

During preliminary experiments isolated bacterial strains were grown to turbid culture levels and tested for their production of beta-glucosidase spectrophotometrically.
Using the procedure of Gonzales-Candelas (1989), a synthetic substrate, p-nitrophenyl-glucopyranoside (PNPG) was hydrolyzed by beta-glucosidase to produce a colorimetric product, p-nitrophenol. One isolated strain showed elevated beta-glucosidase activity when provided with cellobiose as a carbohydrate supplement but no increase in enzyme activity in the presence of other carbohydrates such as maltose, lactose, or starch. Beta-glucosidase hydrolyzes the beta-linkage of cellobiose producing two individual glucose molecules available to the cell for its metabolism. The working hypothesis of this dissertation is that, if the production of beta-glucosidase were controlled by a cell, a substrate such as cellobiose might be effective in inducing the production of the enzyme under carbohydrate-limiting conditions.

2.1.2 Regulation of Enzyme Production in Bacteria

The purpose of this study was to determine if marine bacteria can regulate the production of the enzyme beta-glucosidase depending upon carbohydrate availability in the immediate environment. An enzyme assay procedure was devised which was free of substances which might interfere with the accurate measurement of the quantity of enzyme produced by the marine bacteria in the presence of either cellobiose or glucose. The designs of the experiments were based on knowledge, cited below, of enzyme production, regulation, and sensitivity to substances in the immediate environment.
from research on the genes and enzymes of enteric bacteria.

Bacterial genes, through the processes of transcription and translation, direct the synthesis of proteins, many of which serve as enzymes used for cellular metabolism. The genetic and metabolic machinery of a bacterial cell are integrated and interdependent. Because protein synthesis requires a tremendous expenditure of energy (ATP), the regulation of protein synthesis is important to the cell's energy economy. It has long been known that the cell conserves energy by making only the proteins needed at a particular time (Pardee et al. 1959).

Induction and repression mechanisms regulate the transcription of messenger RNAs (mRNAs) and consequently the synthesis of enzymes from the mRNAs. These genetic control mechanisms regulate the formation and amounts of enzymes in the bacterial cell, not the behavior of the enzymes once they are produced.

Induction is the process that turns on the transcription of a gene or genes. A substance that acts to induce transcription of a gene is called an inducer, and enzymes that are synthesized in the presence of inducers are inducible enzymes. The genes required for lactose metabolism in \textit{E. coli} are a well-known example of an inducible system (Jacob and Monod, 1961). One of these genes produces the enzyme beta-galactosidase, which splits the substrate lactose into two simple sugars, glucose and galactose. If \textit{E. coli} is placed in a medium in which no lactose is present, the bacterium synthesizes almost no beta-galactosidase.
However, when lactose is added to the medium, the bacterial cells produce a large quantity of the enzyme. Lactose is converted in the cell to the related compound allolactose which binds to a repressor protein, interfering with the repressor protein's normal function to bind to and repress the genes required for lactose metabolism. This inactivation of the repressor protein allows RNA polymerase to transcribe the gene for beta-galactosidase. The presence of lactose, therefore, indirectly induces the cells to synthesize more enzyme. This response, which is under genetic control, is termed enzyme induction.

In this report cellobiose and glucose were tested for their effect on production of beta-glucosidase in marine bacterial strains. The observed results were discussed in comparison with the known model of induction of beta-galactosidase by lactose in *E. coli*.

The regulatory mechanism that turns off gene expression and decreases the synthesis of enzymes is called repression. An example of catabolic repression is the effect which glucose has on the production of beta-galactosidase in *E. coli*. Glucose controls the intracellular level of the molecule: cyclic AMP (Paigen and Williams, 1970). The cell prefers glucose to lactose as a carbon source because glucose is more directly metabolized as a carbon and energy source. When glucose is not available, the cell responds by producing cyclic AMP (cAMP). The increased level of cAMP in the cell triggers a series of events that result in a low-level continuous, i.e., constitutive, production of beta-
galactosidase and allows induction of high levels of beta-galactosidase in the presence of lactose, as discussed above. In this study the isolated marine bacteria were grown in the presence of glucose to observe the glucose effect on beta-glucosidase production, and the observed results are discussed in comparison with the known model of glucose effect on beta-galactosidase production in E. coli.

The study of induction and repression of enzyme production in bacteria is separate from the study of the behavior of the enzymes once they are produced. The behavior of enzymes can be inhibited by substances in their environment (Doelle, 1969). Competitive inhibitors fill the active site of an enzyme and compete with the normal substrate for the active site. The competitive inhibitor is able to do this because its shape and chemical structure are similar to those of the normal substrate. Noncompetitive inhibitors do not compete with the substrate for the enzyme's active site, but interact with another part of the enzyme. In this process, allosteric inhibition, an enzyme's efficiency is reduced because of a change in shape caused by the binding of an inhibitor at a site other than the substrate's binding site.

Glucose and gluconic acid are known competitive inhibitors of beta-glucosidase activity. In this study, these two inhibitors were used to test the authenticity of the enzyme activity observed in the assays.
2.2 Materials and Methods

2.2.1 Isolation of Cellobiose-Utilizing Strains

Marine bacteria for this study were isolated from two marine environments: seawater samples from a depth of 30 meters in the Gulf Stream and samples from Florida coastal waters containing marine snow-like macroaggregates at a depth of 10 meters. 20 ml samples of the coastal water were transferred to a sterile test tube and vortexed to separate bacterial cells from particulate organic matter. The vortexed sample was vacuum filtered through a sterile 0.45 micron Millipore filter using the procedure explained in section 1.2.2 of this study in order to separate procaryotic bacterial cells from eucaryotic cells and macroscopic particulate organic matter. The seawater sample from the Gulf Stream and the filtrate from the coastal waters sample were used as sources of inoculum for in vitro continuous cultures designed to isolate marine bacteria with the ability to metabolize cellobiose as a carbohydrate source.

A chemostat was used to maintain a continuous culture of marine bacteria in F/4 medium with cellobiose as the only carbohydrate substrate. The chemostat consisted of a 500ml separatory funnel which delivered sterile F/4 medium by gravity to a 250ml side-arm distillation flask which was the culture vessel. The flow rate was controlled by a stopcock and a medical intravenous drop monitor. The culture vessel was continuously mixed by a magnetic stir bar, and overflowed through the sidearm at its neck.
Starter bacterial cultures were prepared in the chemostat culture flask. A starter culture from the Gulf Stream seawater sample consisted of 250 ml of the seawater which had been passed through a sterile 0.45 micron Millipore filter. This filtered seawater sample was supplemented with F/4 medium and a final concentration of 50 mg/l glucose to promote exponential growth. A starter culture from the Florida coastal waters sample consisted of the 20 ml Millipore filtered inoculum described above, supplemented with F/4 liquid medium (Table 2) to a final volume of 250 ml plus a final concentration of 50 mg/l glucose. The starter cultures were batch incubated at room temperature for 72 hours until visible turbidity was observed. After 72 hours, continuous culture was initiated, using F/4 medium with a final concentration of 50 mg/l cellobiose. This medium was supplied at a drop rate which allowed for complete turnover of the 250 ml cultures every 24 hours. The continuous cultures were maintained for five days.

At the end of five days, 0.1 ml samples of the cultures were streaked out on F4Dagar plates supplemented with 50 mg/l cellobiose in order to isolate colonies of the marine bacterial strains. The plate cultures were incubated at 22°C.

Minute colonies, visible with the aid of a dissecting microscope (40X magnification), were observed after five days incubation. The colonies never reached a size which could be visually identified without this magnification.
The procedure described above was performed twice with seawater samples from the Gulf Stream. In the first set of experiments one colony was selected for study which appeared as a small, white colony under 40X. In the second set of experiments a second colony, more minute and with a darker hue of white than the first isolated colony, was selected.

The two isolated colonies were each aseptically transferred from the agar using a straight inoculating wire into sterile test tubes containing 10 ml of F/4 medium supplemented with 50 mg/l glucose (one colony per tube) and incubated at 22°C in a shaker bath for five days.

The two five-day cultures of isolated colonies were then re-streaked on plates of F4Dagar with 50 mg/l cellobiose in order to check for contamination. The second generation of colonies were once again isolated, and a sample from each plate was taken to be grown in cellobiose-supplemented F/4 liquid medium, as before, for further testing.

The cultures which grew after five days were considered to be pure cultures of individual strains of marine bacteria capable of metabolizing cellobiose as a carbon-energy source. Therefore, these two test tube cultures (Strain 1 and Strain 2) were preserved at 5°C, and a cellobiose-supplemented F4Dagar plate was inoculated with a lawn of each sample. After five days of growth at 22°C, the two culture plates were also preserved at 5°C.

Three colonies differing in morphology and color characteristics were isolated from a continuous culture of
the 20 ml sample filtrate from Florida coastal waters. The three colonies were sampled, and a pure culture from each of the three isolated colonies was prepared using the procedure mentioned above. These three isolated strains from Florida coastal waters (Strains 3, 4 and 5) were also preserved in F/4 liquid culture and on F4Dagar supplemented with 50 mg/l cellobiose at 5°C.

2.2.2 Characterization of the Selected Strains

Wet mount and Gram-stain microscopic slides were prepared for each isolated culture and observed at 1000X with an Olympus CHT microscope.

To verify that the isolated strains were truly marine (having an obligate seawater requirement) the dialyzed agar medium was prepared as above, except that deionized water was used in place of seawater. The five strains were each inoculated onto three F4D freshwater agar plates, supplemented with 50 mg/l cellobiose: one plate with an inoculum streak, one plate spread with 0.05 ml inoculum, and one plate spread with 0.1 ml inoculum. Three F4Dagar plates, containing saltwater and 50 mg/l cellobiose, were inoculated with each strain in the same way.

2.2.3 Controlled Growth of the Selected Strains

Each isolated bacterial strain was grown in a starting culture of F/4 liquid medium with a glucose supplement at
2.5 mg/l. A 930ml culture inoculated with 0.1 ml stock culture of each bacteria and incubated at 22°C for 15 hours produced a cell population in the range of $1 \times 10^6$ cells/ml. The cell counts were determined by placing culture samples on a hemocytometer, beneath a plastic coverslip, and viewing the sample on an Olympus CHT microscope at 1000X magnification. The number of cells in ten of the smallest squares on the hemocytometer grid was counted and multiplied by a factor of $4 \times 10^5$. The accuracy of the hemocytometer procedure was double-checked by filtering 2ml samples of Strain 1 culture onto black nuclope filters and performing epifluorescent cell counts according to the procedure of Hobb, et al. 1977.

2.2.4 Carbohydrate Pulse Experiments

Thirty ml of each 930ml starting culture was taken to determine baseline beta-glucosidase activity and the remainder was subdivided into three 300ml subcultures to begin carbohydrate pulse experiments to study the ability of the each strain to produce beta-glucosidase under different carbohydrate substrate conditions.

The first 300ml subculture received no supplemental carbohydrate pulse and functioned as an original (control) culture. The second 300ml subculture received a 2.5mg/l pulse of glucose, and the third 300ml subculture received a 2.5 mg/l pulse of cellobiose. Cell counts were done for each of the two assay samples taken at hourly intervals for the duration of the experiment.
2.2.5 Fluorometric Assay of Beta-Glucosidase

Methylumbelliferyl-glucoside (MUF-glu), which produces fluorogenic methylumbelliferone (MUF), when hydrolyzed by beta-glucosidase (Mead, et al. 1955), was utilized in this assay procedure. Muf-glu was obtained from Sigma Chemicals.

Two 15 ml samples were pipetted from each 300ml subculture hourly. These were centrifuged at 4000 RPM for 30 minutes to isolate a cell pellet from each sample. The two cell pellets were each resuspended in 4ml of pH 7.0 phosphate buffer. In order to release the beta-glucosidase enclosed within the bacterial cell walls, 0.1 ml of toluene was added to each assay sample (Cappuccino and Sherman, 1987), which was then stirred on a vortex mixer for 10 seconds. Finally, 2ml of $1.0 \times 10^{-6}$ molar MUF-glu was added to each 4ml sample, bringing the final concentration of MUF-glu to $3.3 \times 10^{-7}$ molar. The samples were placed in a shaker bath at 28 °C for 24 hours after which their MUF fluorescence was measured on a Turner Model 110 fluorometer. The MUF fluorescence was measured at a wavelength of 460 nm with an excitation wavelength of 365nm.

A standard curve relating MUF concentration and fluorescence was prepared from known concentrations of MUF-glu incubated with extracts of cultures grown with 50mg/l cellobiose. The beta-glucosidase levels in these turbid cultures were sufficient to completely hydrolyze the MUF-glu. The fluorescence of the liberated MUF in each sample was measured, and standard curves were produced (Figures 9 and 10).
Figure 9. A standard curve relating MUF concentration and fluorescence on a Turner Model 110 fluorometer using a 1X excitation shutter. A regression slope was computed from two readings at each of six MUF concentrations.

**MUF STANDARD CURVE**

1X Excitation Shutter Setting

<table>
<thead>
<tr>
<th>Moles of MUF (Times 10E-7)</th>
<th>Fluorometer Reading (Mean ± Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 X 10^-9</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>1.5 X 10^-8</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>3.0 X 10^-8</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>5.0 X 10^-8</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>8.0 X 10^-8</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>1.0 X 10^-7</td>
<td>82 ± 3</td>
</tr>
</tbody>
</table>

Slope Value: 8.02 X 10^8  Std Err: 5.5 X 10^7  R^2: 0.98  N: 12

Fluorometer Reading = (Conc. of MUF * 8.02 X 10^8) + (-1.83481)
Figure 10. A standard curve relating MUF concentration and fluorescence on a Turner Model 110 fluorometer using a 3X excitation shutter. A regression slope was computed from two readings at each of four MUF concentrations.

**MUF STANDARD CURVE**

3X Excitation Shutter Setting

<table>
<thead>
<tr>
<th>Moles of MUF (Times 10E-8)</th>
<th>Fluorometer Readings (Mean ± Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 X 10^{-9}</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>1.5 X 10^{-8}</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>3.0 X 10^{-8}</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>5.0 X 10^{-8}</td>
<td>87 ± 3</td>
</tr>
</tbody>
</table>

Slope Value:1.73 X 10^9  Std Err:2.14 X 10^8  R^2:0.97  N: 8

Fluorometer Readings=(Conc. of MUF * 1.73 X 10^9) + 6.277372
2.2.6 Calculation of Beta-Glucosidase Activity

Graphs of the growth curves and enzyme assays were used to compare the relative number of cells and amount of MUF-hydrolysis present at any particular hourly sample. The enzyme activity was determined by dividing the concentration of MUF produced in a subculture sample by the number of cells in that subculture sample.

Enzyme activities of two samples from each subculture taken at hourly intervals were plotted, and computed regression lines were added to represent the rate of change in enzyme activity for a particular subculture during a pulse experiment. The slope values of glucose and cellobiose subcultures were compared to the control subculture using the test of significance of difference between two slopes (Edwards, 1973).

2.2.7 Carbohydrate Analysis of the Culture Medium

Strain 1, isolated from the Gulf Stream, was used to study relative uptake of glucose and cellobiose during a set of pulse experiments consisting of a glucose supplemented subculture, a cellobiose supplemented subculture and a mixed-pulse (glucose + cellobiose) subculture. Glucose and/or cellobiose concentrations remaining in the liquid media of the three subcultures were analyzed by the 3-methyl-2-benzothiazolinol hydrozone hydrochloride (MBTH) method both with and without pre-hydrolysis to hydrolyze glucosidic
bonds (Johnson and Sieburth, 1981; Burney and Sieburth, 1981).

The procedure allowed for the measurement of total carbohydrate concentrations in the 2.5 mg/l range which was suitable for the carbohydrate concentrations of subcultures. Two separate determinations were done (one hydrolyzed and one unhydrolyzed) on each supernatant of a subculture sample after centrifugation to remove cells, using the carbohydrate hydrolysis method of Burney and Sieburth (1981). Six replicate samples were analyzed for the hydrolyzed determination and three replicate samples were analyzed for the unhydrolyzed determination. The mean and standard deviation for each set of determinations of hydrolyzed and unhydrolyzed samples was computed and plotted. By comparing the hydrolyzed and unhydrolyzed results it was possible to estimate the amount of cellobiose and glucose in a mixed carbohydrate pulse consisting of 2.5 mg/l of each carbohydrate. An increase in the ratio of cellobiose to glucose would indicate preferential uptake of glucose by Strain 1 during the experiment. Preferential uptake of glucose would be indicative of an ability by the marine bacterial strain to repress its production of beta-glucosidase, needed to metabolize cellobiose, until the glucose was diminished.

The formalized differentiation between glucose and cellobiose in the mixed pulse was calculated by the following method. The additions of 2.5 mg/l glucose and 2.5 mg/l cellobiose are theoretically 13.9 micromoles of glucose and 7.3 micromoles of cellobiose. The glucose reading of the
hydrolyzed pulse would be 13.9 micromoles (glucose component) + 2 X 7.3 micromoles (cellobiose component) totalling 28.5 micromoles. The reading of the carbohydrate concentration of the unhydrolyzed pulse would be 21.2 micromoles (13.9 micromoles + 7.3 micromoles) because one molecule of unhydrolyzed cellobiose theoretically reacts like one molecule of glucose in the MBTH test.

The actual readings using the initial mixed pulse supernatant as a standard were 28.1 micromoles for the hydrolyzed sample (very close to the theoretical 28.5 micromoles) and 19.8 micromoles for the unhydrolyzed sample (0.93 of the theoretical 21.2 micromoles). Since it is assumed glucose reacts quantitatively, the reaction of cellobiose in the unhydrolyzed analyses must have been 81 percent efficient, i.e., the cellobiose contribution (19.8 micromoles - 13.9 micromoles)/7.3 micromoles = 0.81.

Therefore, using the micromolar values of the actual reading; the following set of simultaneous equations was derived:

\[
\begin{align*}
\text{Hydrolyzed} &= \text{glucose} + 2 \text{ cellobiose} \\
\text{Unhydrolyzed} &= \text{glucose} + 0.81 \text{ cellobiose} \\
\text{Hydrolyzed} - \text{Unhydrolyzed} &= 1.19 \text{ cellobiose} \\
\text{Cellobiose} &= (\text{Hydrolyzed} - \text{Unhydrolyzed}) / 1.19
\end{align*}
\]

Substituting the derived value for cellobiose (equation 4) in equations 1 and 2:

\[
\text{Glucose} = 1.68 \text{ Unhydrolyzed} - 0.68 \text{Hydrolyzed}
\]
Carbohydrate analyses were also performed on the supernatants of the 2.5 mg/l glucose pulse subculture and the 2.5 mg/l cellobiose pulse subculture to confirm the accuracy of the procedure to determine cellobiose and glucose concentrations in the mixed pulse subculture.

2.2.8 Computation of Induction and Repression Rates

The rate of beta-glucosidase induction and repression in Strain 1 were determined by linear regression analysis. In order to analyze the rates at which induction and repression occurred, a series of experiments was performed utilizing cellobiose and glucose pulses over a 2.5 to 50mg/l range. Strain 1 was grown overnight in F/4 liquid medium supplemented with 2.5 mg glucose/liter. Starting cultures were divided into subcultures and carbohydrate pulses were added at the following concentrations: 2.5mg/l, 5mg/l, 15mg/l, 25mg/l, and 50mg/l. Hourly samples were taken for each carbohydrate pulse up to seven hours following administration of the pulse. Cell counts (hemocytometer) and enzyme assays (MUF-glu hydrolysis) were determined for each subsample, and per-cell enzyme activities were calculated. Enzyme induction or repression rates were determined from time series data by linear regression analysis. The rates from the regression slopes were then plotted versus the initial carbohydrate pulses to determine if the rate of induction or repression of beta-glucosidase production was affected by the pulse concentrations.
2.2.9 Test For Gluconic Acid Sensitivity

Gluconic acid (D-glucono-lactone) is a known competitive inhibitor of beta-glucocidase (Boschker and Cappenberg, 1994). To test if beta-glucosidase enzyme activities were truly being measured, both gluconic acid and glucose were added separately to the assay reaction mixtures, to test whether or not changes in MUF production would occur in their presence. Instead of resuspending the centrifuged cell pellets in 4ml of phosphate buffer, as in the previous enzyme activity tests, they were resuspended in 2ml of buffer followed by the addition of 0.1 ml toluene to lyse cells and liberate beta-glucosidase. Then 2ml of 7.5 X 10^{-3} M gluconic acid, (Sigma Chemical Co.), or 2ml of 7.5 X 10^{-3} M glucose were added to the reaction mixture before the addition of 2 ml 1.0 X 10^{-6} molar MUF-glu. The complete reaction mixture contained either 2.5 X 10^{-3} molar gluconic acid or 2.5 X 10^{-3} molar glucose competing with 3.3 X 10^{-7} molar MUF-glu as substrates for beta-glucosidase hydrolysis.

2.2.10 Cyclic AMP Effect on Beta-Glucosidase Production

Uptake of adenosine 3',5'-phosphate (cAMP) by marine bacteria is found in a minority of cells in mixed bacterial assemblages (Ammerman and Azam, 1987). The addition of cAMP to cultures of E. coli will overcome the effect of glucose in repressing the low-level constitutive production of beta-
galactosidase (Pastan and Adhya, 1976). Three concentrations of cAMP (1.0 X 10^{-4} molar, 5.0 X 10^{-4} molar and 2.5 X 10^{-3} molar) were separately added to three cultures of Strain 1 given 2.5 mg/l glucose as a sole carbohydrate source to test if any of the above concentrations of cAMP would overcome the effect of glucose in repressing beta-glucosidase activity observed in initial tests. Cyclic AMP was obtained from Sigma Chemical Co.

2.3 Results

2.3.1 Characteristics of the Isolated Bacterial Colonies

Microscopic examination of Strain 1 on a wet mount slide showed motile bacilli, approximately 1.0 micron in length. Their cross-section width did not appear to exceed 0.5 micron when visually measured with an ocular micrometer which is consistent with the strain's ability to pass through a 0.45 micron millipore filter. Examination of Strain 2 showed non-motile diplococci. An individual cell measured approximately 0.5 micron in diameter. Strain 3 was a non-motile bacillus, often appearing in pairs, about 1.3 microns in length and 0.5 micron in width. Strain 4 was a motile bacillus, 0.8 micron in length and 0.4 micron in width. Strain 5 was a non-motile bacillus, 1.0 micron in length and 0.4 micron in width. All five strains stained Gram-negative.
There were no observable colonies under 40X magnification on any set of freshwater plates after five days of incubation at room temperature (22°C). Conversely, all five strains produced minute colonies clearly visible under 40X on the F4D saltwater agar streak plates and lawns of growth on the F4D saltwater agar spread plates.

2.3.2 Initial Enzyme Activities

The MUF assays, growth curves, and computed enzyme activities for the control, glucose, and cellobiose subcultures of Strain 1 are shown in Figures 11 to 13. Two sample readings are plotted at each hourly interval over the seven hour experiments. Regression lines are computed for enzyme activity readings of the three subcultures and slope analysis data are included in Figure 13. The same set of data for Strains 2 through 5 are shown in Figures 14 to 16, 17 to 19, 20 to 22, and 23 to 25, respectively. Table 8 shows the regression slope analyses of the enzyme activities produced by the cellobiose subculture and control subculture of each strain and the tests for significance of difference between the two slopes of each strain.

Strain 1 results show that this bacterial strain responded to the presence of cellobiose with the production of elevated beta-glucosidase activity (Figure 13) compared to no change in enzyme activity in the control subculture. Strain 1 also showed sensitivity to the presence of glucose
with a decrease in beta-glucosidase activity over the seven hour test (Figure 13).

Strain 2 results show no change in enzyme activity in either of the pulsed subcultures compared to the control culture (Figure 16), although it was capable of growth in F/4 medium supplemented with cellobiose as a sole carbohydrate source (Figure 15).

Strains 3, 4, and 5 all exhibit elevated beta-glucosidase activity in the presence of cellobiose (Figures 19, 22 and 25), while enzyme activities in control and glucose pulse subcultures remained unchanged.

The linear regression slope analyses and tests for significance of difference between slopes (Table 8) show that the cellobiose subcultures of Strains 1, 3, 4, and 5 produced slopes which were significantly different from the slopes of corresponding control subcultures. Strain 2 showed no significant difference between the slopes of its cellobiose and control subcultures.

2.3.3 Carbohydrate Analysis of the Strain 1 Medium

The results of the carbohydrate analyses of the Strain 1 subculture supernatants are shown in Figures 26 to 30. The two plots shown on each of Figures 26, 27, 29, and 30 represent the mean values plus and minus the standard deviations of the set of hydrolyzed samples and the set of unhydrolyzed samples in each experiment. Figure 26 shows the concentrations of glucose detected in the supernatant samples before and after hydrolysis of all carbohydrates in
Figure 11.
The moles of MUF assayed in the Strain 1 subcultures.

**MUF Assay Strain 1**

![Graph showing Moles MUF / 24 hr (X 10E-7) vs Hours with Control, Glucose, and Cellobiose markers.]

Figure 12.
Cell count per milliliter in the Strain 1 subcultures.

**Growth Curves Strain 1**

![Graph showing Cells ml (millions) vs Hours with Control, Glucose, and Cellobiose markers.]

59
Figure 13.
Enzyme Activities: Femtamoles (10^{-15} moles) of MUF hydrolyzed per cell by the Strain 1 subcultures in 24 hour assays.

Enzyme Activities Strain 1

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Slope Value (Activity/Hr)</th>
<th>R Value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.13</td>
<td>.18</td>
<td>16</td>
</tr>
<tr>
<td>Glucose</td>
<td>-1.08</td>
<td>.76</td>
<td>16</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>14.89</td>
<td>.98</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 14.
The moles of MUF assayed in the Strain 2 subcultures.

MUF Assay Strain 2

![Graph](image)

Figure 15.
Cell count per milliliter in the Strain 2 subcultures.

Growth Curves Strain 2

![Graph](image)
Enzyme Activities: Femtamoles (10^{-15} moles) of MUF hydrolyzed per cell by the Strain 2 subcultures in the 24 hour assays.

![Enzyme Activities Strain 2](image)

**Regression Slope Analysis**

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R Value:</th>
<th>N:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14</td>
<td>0.08</td>
<td>16</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.20</td>
<td>0.51</td>
<td>16</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.18</td>
<td>0.11</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 17.
The moles of MUF assayed in the Strain 3 subcultures.

**MUF Assay Strain 3**

![Graph showing MUF assay results for Strain 3.]

- Control
- Glucose
- Cellobiose

Figure 18.
Cell count per milliliter in the Strain 3 subcultures.

**Growth Curves Strain 3**

![Graph showing growth curves for Strain 3.]

- Control
- Glucose
- Cellobiose
Enzyme Activities: Femtmoles \(10^{-15}\) moles of MUF hydrolyzed per cell by the Strain 3 subcultures in the 24 hour assays.

**Regression Slope Analysis**

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R Value:</th>
<th>N:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.04</td>
<td>0.67</td>
<td>16</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.76</td>
<td>0.96</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 20.
The moles of MUF assayed in the Strain 4 subcultures.

**MUF Assay Strain 4**

![Graph showing MUF assay results for Strain 4](image)

- ▲ Control
- ○ Glucose
- □ Cellobiose

Figure 21.
Cell count per milliliter in the Strain 4 subcultures.

**Growth Curves Strain 4**

![Graph showing cell count results for Strain 4](image)

- ▲ Control
- ○ Glucose
- □ Cellobiose
Figure 22.

Enzyme Activities: Femtamoles \((10^{-15} \text{ moles})\) of MUF hydrolyzed per cell by the Strain 4 subcultures in the 24 hour assays.

**Regression Slope Analysis**

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R Value:</th>
<th>N:</th>
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<tbody>
<tr>
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<td>16</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.08</td>
<td>.52</td>
<td>16</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>5.67</td>
<td>.98</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 23.
The moles of MUF assayed in the Strain 5 subcultures.

**MUF Assay Strain 5**

![Graph showing MUF assay results for Strain 5]

*Legend: ▲ Control  ● Glucose  △ Celllobiose*

Figure 24.
Cell count per milliliter in the Strain 5 subcultures.

**Growth Curves Strain 5**

![Graph showing growth curves for Strain 5]

*Legend: ▲ Control  ● Glucose  △ Celllobiose*
Figure 25.
Enzyme Activities: Femtamoles \((10^{-15} \text{ moles})\) of MUF hydrolyzed per cell by the Strain 5 subcultures in the 24 hour assays.

![Enzyme Activities Strain 5](image)

### Regression Slope Analysis

<table>
<thead>
<tr>
<th>Subculture:</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R Value:</th>
<th>N:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02</td>
<td>.10</td>
<td>16</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.04</td>
<td>.31</td>
<td>16</td>
</tr>
<tr>
<td>Celllobiose</td>
<td>3.59</td>
<td>.97</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 8. Regression slope analyses of enzyme activities for Strains 1 to 5 and tests for significance of difference.

<table>
<thead>
<tr>
<th>Subculture:</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R</th>
<th>N</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose:</td>
<td>14.89</td>
<td>.98</td>
<td>10</td>
<td>9.98</td>
<td>16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Control:</td>
<td>0.01</td>
<td>.83</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose:</td>
<td>0.18</td>
<td>.11</td>
<td>16</td>
<td>0.055</td>
<td>28</td>
<td>0.4783</td>
</tr>
<tr>
<td>Control:</td>
<td>0.14</td>
<td>.08</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose:</td>
<td>0.76</td>
<td>.96</td>
<td>16</td>
<td>11.4</td>
<td>28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Control:</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose:</td>
<td>5.67</td>
<td>.98</td>
<td>16</td>
<td>16.6</td>
<td>28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Control:</td>
<td>0.05</td>
<td>.16</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose:</td>
<td>3.59</td>
<td>.97</td>
<td>16</td>
<td>14.5</td>
<td>28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Control:</td>
<td>0.02</td>
<td>.10</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the mixed pulse subculture samples. The higher levels of glucose detected in the hydrolyzed samples were due to the hydrolysis of cellobiose. Figure 27 gives the estimated glucose and cellobiose concentrations in the mixed pulse subculture using the difference in glucose levels between the hydrolyzed and unhydrolyzed samples to estimate the cellobiose contribution to the total carbohydrate levels. The seventh hour readings show a decline in glucose content compared to cellobiose content, indicating preferential uptake of glucose by the bacterial culture. Figure 27 shows the enzyme assays of the mixed pulse subculture, the cellobiose pulse subculture and the glucose pulse subculture. Induction of beta-glucosidase activity starts in the cellobiose subculture at the third hour reading, in the mixed pulse subculture at the fifth hour reading, but never appears in the glucose subculture. Figures 29 shows the carbohydrate determination of cellobiose, hydrolyzed and unhydrolyzed, in a pure cellobiose subculture, 2.5 mg cellobiose/liter. Figure 30 shows the carbohydrate determination of glucose, hydrolyzed and unhydrolyzed, in a pure glucose subculture, 2.5 mg glucose/liter.
The concentrations of glucose detected in the supernatant samples before and after hydrolysis of all carbohydrates in the mixed-pulse subculture, 2.5 mg glucose-cellobiose/liter. The mean plus and minus the standard deviation of each set of samples is plotted.

Mixed Substrate Subculture

Hydrolyzed readings of each sample are proportionately higher than unhydrolyzed readings due to the hydrolysis of cellobiose into two molecules of glucose. Both hydrolyzed and unhydrolyzed sample readings decline gradually over the seven hour experiment due to consumption of carbohydrate by the bacterial culture.
Figure 27.

The estimated glucose and cellobiose concentrations in the mixed-pulse subculture, 2.5 mg glucose-cellobiose/liter, using the difference in glucose levels between the hydrolyzed and unhydrolyzed samples to estimate the cellobiose contribution to the total carbohydrate levels. The mean plus and minus the standard deviation of each set of samples is plotted.

Mixed Substrate Subculture

The seventh hour readings of cellobiose and glucose concentrations indicate that glucose had declined substantially in the medium, whereas cellobiose had remained relatively high. This shows that glucose was preferentially metabolized.
Figure 28.

Moles of MUF hydrolyzed in the mixed-pulse subculture, the pure glucose pulse subculture and the pure cellobiose pulse subculture.

MUF ASSAY

Moles of MUF Hydrolyzed/24 hrs
(Times 10E-5)

Hours

--- 2.5mg/l cellob/gluc → 2.5mg/l glucose → 2.5mg/l cellobiose
The carbohydrate determination of cellobiose, hydrolyzed and unhydrolyzed, in the pure cellobiose subculture, 2.5 mg cellobiose/liter. The mean plus and minus the standard deviation of each set of samples is plotted.

The approximate 2-to-1 ratio between the hydrolyzed and unhydrolyzed cellobiose readings indicates that cellobiose was hydrolyzed into two molecules of glucose. The gradual decline in concentration over seven hours shows the consumption of cellobiose by the bacterial culture during that period.
Figure 30.

The carbohydrate determination of glucose, hydrolyzed and unhydrolyzed, in the pure glucose subculture, 2.5mg glucose/l. The mean plus and minus the standard deviation of each set of samples is plotted.

Glucose Substrate Subculture

The nearly equal readings for both hydrolyzed and unhydrolyzed samples indicates that only the glucose added to the medium at 2.5 mg/l was detected. There was little background carbohydrate from the original saltwater base of the F/4 medium showing in the hydrolyzed samples.
2.3.4 Strain 1 Induction and Repression Rate Results

The regression slope values representing the rate of induction of beta-glucosidase activity in Strain 1 subcultures pulsed with five different concentrations of cellobiose and the rate of repression of beta-glucosidase activity pulsed with five different concentrations of glucose are shown in Table 9. The slope values versus the pulse concentrations are plotted in Figure 31. Results indicate saturation kinetics in which increasing concentrations of cellobiose or glucose either induce or repress production of beta-glucosidase, respectively, to an asymptotic limit.

Table 9.

<table>
<thead>
<tr>
<th>Cellobiose Conc. (Activity/Hr)</th>
<th>Slope Value</th>
<th>Std. Err.</th>
<th>R Values</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mg/l</td>
<td>0.86</td>
<td>0.35</td>
<td>0.70</td>
<td>8</td>
</tr>
<tr>
<td>5.0 mg/l</td>
<td>2.24</td>
<td>0.45</td>
<td>0.94</td>
<td>5</td>
</tr>
<tr>
<td>15.0 mg/l</td>
<td>4.33</td>
<td>0.85</td>
<td>0.95</td>
<td>5</td>
</tr>
<tr>
<td>25.0 mg/l</td>
<td>4.22</td>
<td>0.68</td>
<td>0.94</td>
<td>5</td>
</tr>
<tr>
<td>50.0 mg/l</td>
<td>5.52</td>
<td>0.90</td>
<td>0.97</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose Conc. (Activity/Hr)</th>
<th>Slope Value</th>
<th>Std. Err.</th>
<th>R Values</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mg/l</td>
<td>-0.27</td>
<td>0.18</td>
<td>0.51</td>
<td>8</td>
</tr>
<tr>
<td>5.0 mg/l</td>
<td>-0.84</td>
<td>0.29</td>
<td>0.84</td>
<td>5</td>
</tr>
<tr>
<td>15.0 mg/l</td>
<td>-0.90</td>
<td>0.14</td>
<td>0.96</td>
<td>5</td>
</tr>
<tr>
<td>25.0 mg/l</td>
<td>-0.71</td>
<td>0.11</td>
<td>0.95</td>
<td>5</td>
</tr>
<tr>
<td>50.0 mg/l</td>
<td>-1.66</td>
<td>0.48</td>
<td>0.92</td>
<td>4</td>
</tr>
</tbody>
</table>

Note: Enzyme Activity = femtomoles (1 X 10\(^{-15}\) moles) MUF per cell in a 24 hour assay.
Figure 31. The regression slope values representing the rates of induction or repression of enzyme activities in Strain 1 subcultures pulsed with five different concentrations of cellobiose and glucose are plotted versus the pulse concentrations.

Note: Enzyme Activity = femtamoles (1 X 10^{-15} moles) MUF per cell in a 24 hour assay

INDUCTION and REPRESSION RATES
Enzyme Activity Regression Slope Values

---

---

- mg cellobiose/l
- mg glucose/l
2.3.5 Beta-Glucosidase Inhibition Studies

The results of the sensitivity of the beta-glucosidase activity produced by Strain 1 to gluconic acid and to glucose are compared in Figures 32, 33, and 34. Figure 32 shows the enzyme activities obtained with no chemical inhibitor added to the assay reaction mixtures. Figure 33 and 34 show the enzyme activities of the same culture after $2.5 \times 10^{-3}$ molar gluconic acid and $2.5 \times 10^{-3}$ molar glucose were added to the assay reaction mixtures, respectively. The same format is used to show the sensitivity of Strain 2 enzyme activity to the two inhibitors in Figures 35 to 37. The results for Strain 3 are presented in Figures 38 to 40, for Strain 4 in Figures 41 to 43, and for Strain 5 in Figures 44 to 46. Table 10 shows the regression slope analyses of enzyme activities and the tests for significance of difference between slopes comparing the control assays of the five isolated strains and the assays containing either gluconic acid or glucose.

The tests for significance of difference between slopes indicate the effect of gluconic acid or glucose on beta-glucosidase activity varies by strain. In Strain 1 the effect of gluconic acid on beta-glucosidase activity increases significantly over the five hour test period. The effect of glucose, in comparison, is constant. The effect of the two inhibitors on enzyme activities in Strains 2, 3 and 4 is constant, but to different degrees with respect to each strain. Strain 5 enzyme activity is increasing affected by both gluconic acid and glucose.
Figure 32.
Strain 1 with no chemical inhibitors added to enzyme assays.

Strain 1 Enzyme Activities
MUF-glu w/o GLN

Figure 33.
Strain 1 with gluconic acid added to the assays.

Strain 1 Enzyme Activities
MUF-glu + 2.5 X 10^{-3} M GLN

Figure 34.
Strain 1 with glucose added to the assays.

Strain 1 Enzyme Activities
MUF-glu + 2.5 X 10^{-3} M Glucose
Figure 35.
Strain 2 with no chemical inhibitors added to enzyme assays.

Strain 2 Enzyme Activities
MUF-glu w/o GLN

Figure 36.
Strain 2 with gluconic acid added to the assays.

Strain 2 Enzyme Activities
MUF-glu + 2.5 X 10^-3 M GLN

Figure 37.
Strain 2 with glucose added to the assays.

Strain 2 Enzyme Activities
Muf-glu + 2.5 X 10^-3 M Glucose
Figure 38.
Strain 3 with no chemical inhibitors added to enzyme assays.

Strain 3 Enzyme Activities
MUF-glu w/o GLN

Figure 39.
Strain 3 with gluconic acid added to the assays.

Strain 3 Enzyme Activities
MUF-glu + 2.5 X 10^-3 M GLN

Figure 40.
Strain 3 with glucose added to the assays.

Strain 3 Enzyme Activities
MUF-glu + 2.5 X 10^-3 M Glucose
Figure 41.
Strain 4 with no chemical inhibitors added to enzyme assays.

**Strain 4 Enzyme Activities**

MUF-gluc w/o GLN

![Graph](image)

Figure 42.
Strain 4 with gluconic acid added to the assays.

**Strain 4 Enzyme Activities**

MUF-gluc + 2.5 X 10^-3 M GLN

![Graph](image)

Figure 43.
Strain 4 with glucose added to the assays.

**Strain 4 Enzyme Activities**

Muf-gluc + 2.5 X 10^-3 M Glucose

![Graph](image)
Figure 44.
Strain 5 with no chemical inhibitors added to enzyme assays.

Strain 5 Enzyme Activities
MUF-glu w/o GLN

Figure 45.
Strain 5 with gluconic acid added to the assays.

Strain 5 Enzyme Activities
MUF-glu + 2.5 X 10^-3 M GLN

Figure 46.
Strain 5 with glucose added to the assays.

Strain 5 Enzyme Activities
MUF-glu + 2.5 X 10^-3 M Glucose
Table 10. Regression slope analyses of enzyme activities showing gluconic acid and glucose inhibition.

<table>
<thead>
<tr>
<th>Subculture:</th>
<th>Slope Value: (Activity/Hr)</th>
<th>R:</th>
<th>N:</th>
<th>t:</th>
<th>df:</th>
<th>p:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
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<td>.78</td>
<td>12</td>
<td>3.300</td>
<td>20</td>
<td>0.0018</td>
</tr>
<tr>
<td>Gluconic acid:</td>
<td>0.24</td>
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<td>12</td>
<td>0.456</td>
<td>20</td>
<td>0.3267</td>
</tr>
<tr>
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<td>12</td>
<td>0.456</td>
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2.3.6 Effect of Cyclic AMP on Strain 1 Enzyme Activity

The effect of cAMP on the beta-glucosidase activity produced by a glucose pulse subculture of Strain 1 is shown in Figures 47, 48 and 49. Figure 47 shows the hourly enzyme activities of the control subculture. Figure 48 shows the hourly enzyme activities of the subculture pulsed with 2.5 mg/l glucose without cAMP, and Figure 49 shows the results from the subculture pulsed with 2.5 mg/l glucose plus 1 X 10^{-4} molar cAMP.

The effect with 5.0 X 10^{-4} molar cAMP using the same procedure as above are shown in Figures 50, 51 and 52; results with 2.5 X 10^{-3} molar cAMP are shown in Figures 53, 54 and 55.

Results show that 1 X 10^{-4} molar cAMP had no effect on overcoming glucose repression of beta-glucosidase activity in Strain 1 (Figure 49). The higher concentrations of cAMP actually inhibited beta-glucosidase activity compared to control cultures (Figures 52 and 55).
Figure 47.
Strain 1 control subculture with no glucose pulse or cAMP.

Figure 48.
Strain 1 with a 2.5 mg/l glucose pulse, but no cAMP.

Figure 49.
Strain 1 with the glucose pulse plus 1 X 10\(^{-4}\) M cAMP added.
Figure 50.
Strain 1 control subculture with no glucose pulse or cAMP.

Strain 1 Enzyme Activities
Control Culture w/o Glucose Pulse

Figure 51.
Strain 1 with a 2.5 mg/l glucose pulse, but no cAMP.

Strain 1 Enzyme Activities
2.5mg/l glucose pulse w/o cAMP

Figure 52.
Strain 1 with the glucose pulse plus $5 \times 10^{-4}$ M cAMP added.

Strain 1 Enzyme Activities
2.5mg/l glucose + 5X10-4M cAMP
Figure 53.
Strain 1 control subculture with no glucose pulse or cAMP added.

Figure 54.
Strain 1 with a 2.5 mg/l glucose pulse, but no cAMP.

Figure 55.
Strain 1 with the glucose pulse plus 2.5 X 10^{-3} M cAMP added.
2.4 Discussion

The microscopic examination of the five isolated bacterial colonies shows morphological and mobility differences among them which indicates that they are separate strains (Sec. 2.3.1). All five morphologies are consistent with known marine microbial forms (Colombo, 1990). The growth of all five isolates on F4Dagar supplemented with cellobiose in a saltwater base compared to the lack of growth on the freshwater equivalent agar formula also suggests that all five strains require a saltwater-based medium.

Initial enzyme activity studies (Figures 13, 16, 19, 22, 25) show that four of the five strains respond to the presence of cellobiose as a sole carbohydrate source with elevated beta-glucosidase activity (the exception is Strain 2).

Within one hour of adding a 2.5 mg/l pulse of cellobiose to the Strain 1 subculture, enzyme activities began to rise (Figure 13) resulting in a change of approximately 60 enzyme activity units (femtamoles of MUF per cell) over the seven hour test period. There was rapid induction for the first three hours followed by a leveling off of enzyme activity over the remainder of the experiment. This could be due to early cellobiose depletion, and the levels of produced enzyme remaining relatively constant thereafter. This is viewed in comparison with the control culture (Figure 13) which showed no significant change in enzyme activity over the same time period. A test of significance of difference between two slope values (Edwards, 1973), comparing the cellobiose subculture regression slope and the control sub-
culture regression slope (Table 8), shows a significant difference \( (p < 0.0001) \), which indicates that the presence of cellobiose induced a significantly faster production of beta-glucosidase.

The plot of the induction rate of beta-glucosidase activity by different concentrations of cellobiose substrate in Strain 1 (Figure 31) indicates saturation kinetics similar to a plot of allolactose concentration versus repressor protein deactivation in E. coli which results in increased beta-galactosidase activity (Jacob and Monod, 1961). This suggests that cellobiose, or some derivative of cellobiose, interacts with the regulatory molecules governing the production of beta-glucosidase in Strain 1.

Another regulatory property observed in Strain 1 was a repression of beta-glucosidase activity caused by the 2.5 mg/l glucose pulse. There was a decrease of approximately 10 enzyme activity units over the seven hour test (Figure 13), compared to no change in the control subculture. There was a significant difference between the regression slope of the glucose subculture enzyme activities and the regression slope of the control culture enzyme activities \( (t=3.077, \ df=28, \ p=0.0023) \). This indicated an attenuation of beta-gluclusidase production after the addition of glucose. The rates of beta-glucosidase activity declined to an asymptotic limit opposite to that reached in the induction of beta-glucosidase with different concentrations of cellobiose (Figure 31). These results also parallel the effect of glucose on the production of beta-galactosidase in E. coli.
(Paigen et al. 1970). The repression of beta-galactosidase by glucose in E. coli is the result of glucose causing a decrease in intracellular concentrations of an intermediary regulatory molecule, cyclic AMP. Cyclic AMP, at an intracellular concentration of $1.0 \times 10^{-6}$ molar, is needed for the low level constitutive production of beta-galactosidase (Pastan and Adhya, 1976). By supplementing a culture of E. coli with cAMP, the glucose repression of beta-galactosidase production can be neutralized. In this study, however, the addition of $1.0 \times 10^{-4}$ molar cAMP to the culture medium (100X higher than the intracellular concentration in E. coli) did not neutralize the glucose repression in Strain 1 (Figures 48 and 49). Ammerman and Azam (1987) reported that less than fifteen percent of mixed bacterioplankton assemblages cultured in vitro actively absorb cAMP from the culture medium. In order to overcome the probable lack of cAMP active transport, higher concentrations of cAMP ($5 \times 10^{-4}$ molar and $2.5 \times 10^{-3}$ molar) supplemented with glucose were used, but these reduced enzyme activities (Figures 52 and 55) probably due to an inhibitory effect to beta-glucosidase, proportional to the concentration of cAMP. There were no cAMP tests done on the other isolated bacterial strains in this study because only Strain 1 showed a repression of beta-glucosidase activity by glucose.

The analysis of the medium of a mixed pulse subculture of Strain 1 (Figures 26 to 30) for its content of cellobiose versus glucose at hourly intervals during a seven hour, 2.5 mg CHO/liter experiment also indicated the regulation of
glucose and cellobiose metabolism by this marine bacterium. During the first five hours of the mixed pulse experiment the concentration of cellobiose dropped approximately 30% while the concentration of glucose remained stable (Figure 27). Some of the hydrolyzed cellobiose may not have been utilized immediately by the cells, as indicated by the increase in glucose levels between the third and fifth hours. The decrease in cellobiose concentration during this time period could be the effect of residual beta-glucosidase present in the starting culture similar to the enzyme activity shown in the Strain 1 control culture (Figure 13). In the sixth and seventh hour glucose declined approximately 60%, while cellobiose concentration leveled off. The increased rate of glucose utilization starting after the fifth hour coincided with increased enzyme activity in the mixed pulse subculture assay, shown as an increase in MUF hydrolysis (Figure 28). This relative increase in beta-glucosidase activity at a time when glucose concentration was declining suggests a relief from the glucose repression of enzyme production starting after the fifth hour in the mixed pulse subculture. Figure 28 also shows that induction of enzyme activity started earlier, at the third hour, in the subculture containing cellobiose only, but never occurred in the subculture which was pulsed with glucose alone. This is similar to the mechanism of glucose repression on beta-galactosidase production in *E. coli* (Paigen et al. 1970).

These results may relate to observations from diel dissolved carbohydrate studies *in situ* (Burney, 1986) which
report daily accumulation of polysaccharide in the afternoon-evening period following mid-day photosynthesis. Levels of monosaccharides do not accumulate during the day, probably due to bacterial uptake. One reason for the polysaccharide accumulation might be that released monosaccharides chemically inhibit the ability of any enzyme present to hydrolyze the polysaccharides, either competitively or allosterically (Chrost, 1989), until monosaccharide release rates decline. A second reason for the polysaccharide accumulation could be a repression of glucosidase production by the microbial community also caused by the presence of sufficiently high intracellular levels of monosaccharides which, in this second hypothesis, would repress the production of the required enzymes to hydrolyze polysaccharide until glucose in the environment was metabolized. The results of the present study show that glucose repressed the production of beta-glucosidase activity in the Strain 1 glucose subculture (Figure 13). It would be more energy-efficient for a microbe to repress production of a particular enzyme at the genetic level until it is needed. A third possibility could be a lag between the induction of enzyme synthesis and the expression of the hydrolytic enzymes ultimately resulting in growth and higher cell count. This is indicated in the comparison of the Strain 1 enzyme activities (Figure 13) and cell count (Figure 12). Induction of beta-glucosidase activity started in the first hour after administering the cellobiose pulse, but cell count did not rise until after the second hour.
The glucose determinations from supernatants of a pure cellobiose pulse subculture (Figure 29) and a pure glucose subculture (Figure 30) support the accuracy of the procedure to determine cellobiose and glucose concentrations in the supernatants of the mixed pulse subculture (Figure 27). The supernatant of the pure cellobiose subculture before and after hydrolysis showed a carbohydrate ratio of approximately two to one. This corresponds with a cellobiose molecule hydrolyzing into two glucose molecules. The analysis of the supernatant of the pure glucose subculture shows a ratio of approximately one to one between hydrolyzed and unhydrolyzed samples.

Strain 2 was the only isolated strain with a spherical coccus morphology and had by far the smallest cell volume. It was also the only strain not showing any regulation of beta-glucosidase production, but was still capable of growth in F/4 medium supplemented with cellobiose as a sole carbohydrate source (Figure 11). Its enzyme activity results show that beta-glucosidase activity is present in all treatments (Figure 16) but at relatively low levels of activity as compared to Strain 1 (Figure 13). None of the treatments induced significant rates of enzyme activity. None of the slopes were significantly different from zero (Table 8). The initial set of experiments utilized 2.5 mg CHO/liter pulses. A second set of experiments utilizing 50 mg CHO/liter instead of 2.5 mg/l also resulted in no change in levels of enzyme activities in either glucose and cellobiose
subcultures. This indicated that induction of beta-glucosidase did not occur with higher concentrations of cellobiose.

These two marine bacterial strains, isolated from the Gulf Stream off the coast of Florida, show a difference in their capacity to regulate beta-glucosidase activity. The regulatory ability of Strain 1 suggests that it would be highly competitive in pelagic environments in which carbohydrate nutrients are relatively scarce, i.e., being able to produce relatively abundant quantity of enzyme to readily digest beta-glucosides encountered. The continuous low-level constitutive production in Strain 2 suggests that it would probably utilize beta-glucosides best when in contact with a cellulosic particle.

A comparison of the slopes of the growth curves of Strain 1 and Strain 2 in the 2.5 mg/l cellobiose subcultures (Figure 56) shows that Strain 1 grew at more than twice the rate of Strain 2. The slopes were significantly different \((p < 0.0001)\) suggesting that Strain 1 would outperform Strain 2 in growth in marine environments which contained sparse levels of cellobiose and other cellulose derivatives. However, Strain 2 grew at 44% the rate of Strain 1, while producing about 15% of the total beta-glucosidase activity of Strain 1. This could be related to the smaller cell volume of Strain 2, which was 35% that of Strain 1, (Sec. 2.3.1).

Strains 3, 4 and 5, isolated from coastal seawater containing marine-snow-like macroaggregates, all exhibit
Figure 56. A comparison of the regression slopes of the growth curves of Strain 1 and Strain 2 in the cellobiose subcultures. Two samples from each subculture at each hour are plotted.

**Strains 1 and 2 Growth Curves**

2.5 mg/l cellobiose pulse

![Graph showing growth curves for Strains 1 and 2](image)

**Regression Slopes Analysis**

<table>
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<th>Strain</th>
<th>Slope Values (cells/ml/hr)</th>
<th>R</th>
<th>N</th>
<th>t</th>
<th>df</th>
<th>p</th>
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<td>.93</td>
<td>16</td>
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<td>.88</td>
<td>16</td>
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</table>
elevated beta-glucosidase activity in the presence of cello-
biose (Figures 19, 22 and 25), while enzyme activity in
control and glucose pulse treatments remained unchanged.
There were significant slope differences between each
strain's cellobiose subculture compared to its control
subculture (Table 8), indicating significant beta-glucosi-
dase induction in the presence of cellobiose. The lag be-
tween enzyme induction and resultant cell count increase,
discussed above with respect to Strain 1, was also observed
in these three strains. Strain 3 showed a lag of three hours
between onset of enzyme induction (Figure 19) and the rise
of cell count (Figure 18). Strains 4 and 5 both showed lags
of approximately two hours (Figures 21, 22, 24,
and 25).

This is further evidence for the hypothesis that the accumu-
lation of polysaccharide following mid-day photosynthesis in
situ (Burney, 1986) might be the result of a lag between the
synthesis of glucosidases within bacterial cells and their
expression demonstrated as bacterial cell growth.

There was no correlation between the relative cell
volumes of the cells of these three strains (Sec. 2.3.1) and
the induced rates of beta-glucosidase activity observed.
Strain 3, the largest bacillus isolated in this study,
actually shows the lowest rate of induction among the three
coastal water isolates (Table 8). Herndl (1988) reported
that elevated enzyme activity in bacteria associated with
marine snow compared to free-living bacteria in ambient
waters was not because the attached bacteria had a larger
surface area, since the attached and free-living bacteria which he studied did not differ significantly in size.

The observed difference in glucose repression of beta-glucosidase activity between Strain 1 and the other four isolated strains (Figures 13, 16, 19, 22, and 25) may also suggest variability in this trait among marine bacteria generally. Reports on repression of glucosidases by glucose from other researchers also vary. Chrost (1989) reported repression of glucosidase production by glucose in limnetic studies, whereas Karner and Herndl (1992) were not able to detect such a regulatory mechanism among marine bacteria associated with marine snow. Much of the discrepancy may result from the mixed-bacterial populations. Isolation of individual bacterial strains in these studies and the determination of enzyme activities would determine whether or not this was the case.

The addition of glucose and gluconic acid, a known inhibitor of beta-glucosidase activity (Boschker and Cappenberg, 1994), to the enzyme assays caused an inhibition of MUF-glu hydrolysis to MUF by varying degrees in all five bacterial strains tested (Figures 32 to 46). This is evidence that true beta-glucosidase enzyme activity was being measured in these assays.

The individual differences in the effect of the beta-glucosidase inhibitors, gluconic acid and glucose, on the MUF produced during enzyme assays suggest that the five bacterial strains were each producing different beta-glucosidase enzymes. Figures 32 to 34 show that gluconic acid and
glucose had different inhibitory effects on beta-glucosidase activity in Strain 1. There was a significant difference (t=3.72, df=20, p=0.0007) between the slopes of the gluconic acid and glucose treatments. The slopes of the gluconic acid and control treatments were also significantly different (Table 10), but the slope in the glucose treatment was not significantly different than the control. Gluconic acid apparently bound to the beta-glucosidase enzyme more strongly than MUF-glu, resulting in an increasing degree of competitive inhibition over time. In contrast, the inhibitory effect of glucose caused a proportional lowering of the enzyme activity by approximately 30% throughout the five hour test (Figures 32 and 34). This suggests that the glucose-enzyme complex was less stable and more reversible than the gluconic acid-enzyme binding, and therefore showed no cumulative inhibition.

The effect of both inhibitors on Strain 2, 3 and 4 (Figures 35 to 43) was similar to that of glucose in Strain 1, causing a constant proportional decrease in enzyme activity compared with the controls. The slope tests showed no significant differences (Table 10). This suggests that both gluconic acid and glucose compete reversibly with MUF-glu on the beta-glucosidase enzymes produced by Strains 2, 3 and 4 with no cumulative binding by either inhibitor.

Strain 5 (Figures 44 to 46) showed yet another pattern of inhibition. Both gluconic acid and glucose decreased overall enzyme activities, but showed significantly lower slopes than the control (Table 10). This suggests that the
beta-glucosidase enzyme produced by Strain 5 binds both gluconic acid and glucose more irreversibly at the active site, not allowing MUF-glu to compete during the enzyme assay.

The differences among the five isolated strains in their individual beta-glucosidase sensitivity to gluconic acid and glucose inhibitors suggests differences in their respective enzyme composition and structure, i.e., amino acid sequences and active sites on the enzymes. This correlates with the observations of Rath and Herndl (1994) who separated beta-glucosidase isoenzymes in samples taken from ambient seawaters and marine snow of the Adriatic Sea by anion exchange and size exclusion chromatography. They showed that one isoenzyme of beta-glucosidase was present in ambient waters, while two isoenzymes were present in marine snow. The different isoenzymes could be beta-glucosidases from different constituent bacterial strains. A combination of the enzyme isolation procedures used by Rath and Herndl, mentioned above, with the marine bacteria isolation procedures used in this study would allow a determination of the amount of a particular enzyme in a given seawater sample plus a determination of which marine bacterium produced the particular enzyme. Furthermore, the regulation of the enzyme by the source bacterial strain could be studied.

The chemical inhibition of all five strains’ beta-glucosidases by glucose (Figures 34, 37, 40, 43, and 46) may be compared with the results of glucose repression of beta-glucosidase production among the five strains (Figures 13,
16, 19, 22 and 25). Strain 1 was the only isolate showing evidence of glucose repression. This suggests variability among strains in mixed-bacterial assemblages between regulation of beta-glucosidase production and inhibition of beta-glucosidase activity by glucose. The isolation of individual constituent strains and enzyme analysis of each isolate would provide more information on how glucose was affecting beta-glucosidase activity in particular marine environments.

Evidence that four of the isolated marine bacterial strains from Gulf Stream and Florida coastal waters can induce elevated levels of beta-glucosidase in response to a particular carbohydrate substrate suggests that this regulatory capacity may be common among many strains of marine bacteria. This supports the hypothesis of Cho and Azam (1988) that marine bacteria attached to particulate organic matter (POM) exhibit "hyperproduction" of enzymes resulting in the solubilization of POM to dissolved organic matter (DOM) which then becomes available to the planktonic bacterial community.

The results of this study also contribute to the understanding of differing amounts of enzyme activity among marine bacteria along eutrophic gradients studied in the Caribbean Sea (Rath et al., 1993) as well as the differences in quantity of enzyme activity in seawater samples taken inside and outside of marine snow (Karner and Herndl, 1992; Rath and Herndl, 1994). Karner and Herndl proposed two possible hypotheses to explain the observed differences. There could be different strains of bacteria inside and
outside of marine snow, producing different amounts of beta-glucosidase constitutively. Conversely, identical strains could be present in the two environments with the ability to regulate their production of beta-glucosidase depending upon the beta-glucosides present. The enzyme activities produced by the five isolated strains in this study show variability in regulation of beta-glucosidase production. If there are differences among the various strains in the mixed bacterial assemblages in response to changing carbohydrate environments, there will be differences in growth rates among the strains and resulting changes in the relative population sizes among the various strains composing the assemblages.

During the past decade it has been determined that marine bacteria are a vital component of the base of the food chain in the ocean, i.e., the microbial loop (Azam et al. 1983). Much of the primary production is concentrated in particular geographic areas such as coastal regions (Crouse et al. 1993). These coastal regions are being affected by commercial development, chemical pollution, and community debris. The survivability of marine bacteria to changing nutrient environments will be due, in part, to their capacity to regulate their metabolic pathways. Preferential growth of particular bacterial strains in changing chemical environments as a result of pollution could alter the natural balance of microbial populations constituting the base of the food chain in marine ecosystems.

Regulation of production of beta-glucosidase among terrestrial bacteria is a field of active research (Spear et
al. 1993). The comparison of terrestrial bacteria with marine bacteria in the regulation of beta-glucosidase activity will contribute to our knowledge of this process in procaryotic cells generally. The procedures developed in this study to isolate and culture in vitro individual marine bacterial strains under carbohydrate-limiting conditions may also be applied in the study of strains with other carbohydrate metabolic properties of interest.
3. REFERENCES


