



Physiological diversity of an attached marine bacterial population  
by Ace Melton Baty III

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in  
Microbiology

Montana State University

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Abstract:

This research characterizes chitinolytic gene expression and the partitioning of bacterial biomass between the surface and bulk aqueous phase on a spatial and temporal scale, at the single cell level during chitin degradation. The relationship between chitinolytic gene expression and extracellular chitinase activity was evaluated in individual cells of the marine bacterium *Pseudoalteromonas* sp. strain S91 attached to solid chitin. Green Fluorescent Protein (GFP) reporter genes under the control of the *chiA/chiB* and *chiA* promoters were used to evaluate chitinase gene expression. Furthermore, this work describes the synthesis of a new ELF®-97 enzyme substrate for chitinase activity that permitted the identification of single cells participating in the production of extracellular enzyme during chitin degradation.

Upon initial attachment to a pure chitin surface, the *chiA/chiB* genes of the marine bacterium, *Pseudoalteromonas* sp. strain S91, became highly up-expressed in discrete clusters that represented less than 20% of the total colonized surface area. During bacterial reproduction, 96.8% of the total bacterial production (BP) generated on the surface of pure chitin detached to form free living bacterial biomass. The resulting high detachment rates suggest detachment may play a major role in the dispersion and survival strategies of marine bacteria. Furthermore, 96% of the bacterial biomass that detached to form the free living population were completely down-expressed for *chiA/chiB* gene activity, while the highly up-expressed subpopulation remained associated with the chitin surface.

Evaluation of *chiA* expression and ELF®-97 crystal location at the single cell level revealed two physiologically distinct subpopulations of S91 on the chitin surface: one that was chitinase-active and remained associated with the surface and another that was chitinase-inactive and released daughter cells into the bulk aqueous phase. Following detachment from the chitin surface, cells appeared to enter a starvation survival mode of existence. The ability of a population to coordinate chitinase activity, cell reproduction and surface detachment among different cells on a surface may enhance their ability to locate new sources of nutrients in the pelagic marine environment.

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POPULATION

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of the requirements for the degree

of

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in

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APPROVAL

of a thesis submitted by

Ace Melton Baty III

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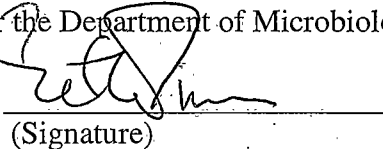
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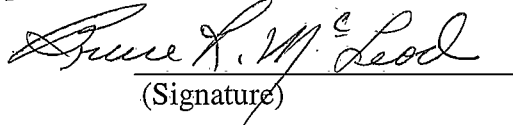
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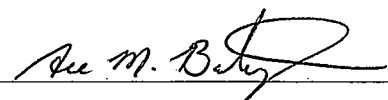
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This work is dedicated to Kristi, Ellie, and Madigan

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## ABSTRACT

This research characterizes chitinolytic gene expression and the partitioning of bacterial biomass between the surface and bulk aqueous phase on a spatial and temporal scale, at the single cell level during chitin degradation. The relationship between chitinolytic gene expression and extracellular chitinase activity was evaluated in individual cells of the marine bacterium *Pseudoalteromonas sp.* strain S91 attached to solid chitin. Green Fluorescent Protein (GFP) reporter genes under the control of the *chiA/chiB* and *chiA* promoters were used to evaluate chitinase gene expression. Furthermore, this work describes the synthesis of a new ELF<sup>®</sup>-97 enzyme substrate for chitinase activity that permitted the identification of single cells participating in the production of extracellular enzyme during chitin degradation.

Upon initial attachment to a pure chitin surface, the *chiA/chiB* genes of the marine bacterium, *Pseudoalteromonas sp.* strain S91, became highly up-expressed in discrete clusters that represented less than 20% of the total colonized surface area. During bacterial reproduction, 96.8% of the total bacterial production (BP) generated on the surface of pure chitin detached to form free living bacterial biomass. The resulting high detachment rates suggest detachment may play a major role in the dispersion and survival strategies of marine bacteria. Furthermore, 96% of the bacterial biomass that detached to form the free living population were completely down-expressed for *chiA/chiB* gene activity, while the highly up-expressed subpopulation remained associated with the chitin surface.

Evaluation of *chiA* expression and ELF<sup>®</sup>-97 crystal location at the single cell level revealed two physiologically distinct subpopulations of S91 on the chitin surface: one that was chitinase-active and remained associated with the surface and another that was chitinase-inactive and released daughter cells into the bulk aqueous phase. Following detachment from the chitin surface, cells appeared to enter a starvation survival mode of existence. The ability of a population to coordinate chitinase activity, cell reproduction and surface detachment among different cells on a surface may enhance their ability to locate new sources of nutrients in the pelagic marine environment.

## CHAPTER 1

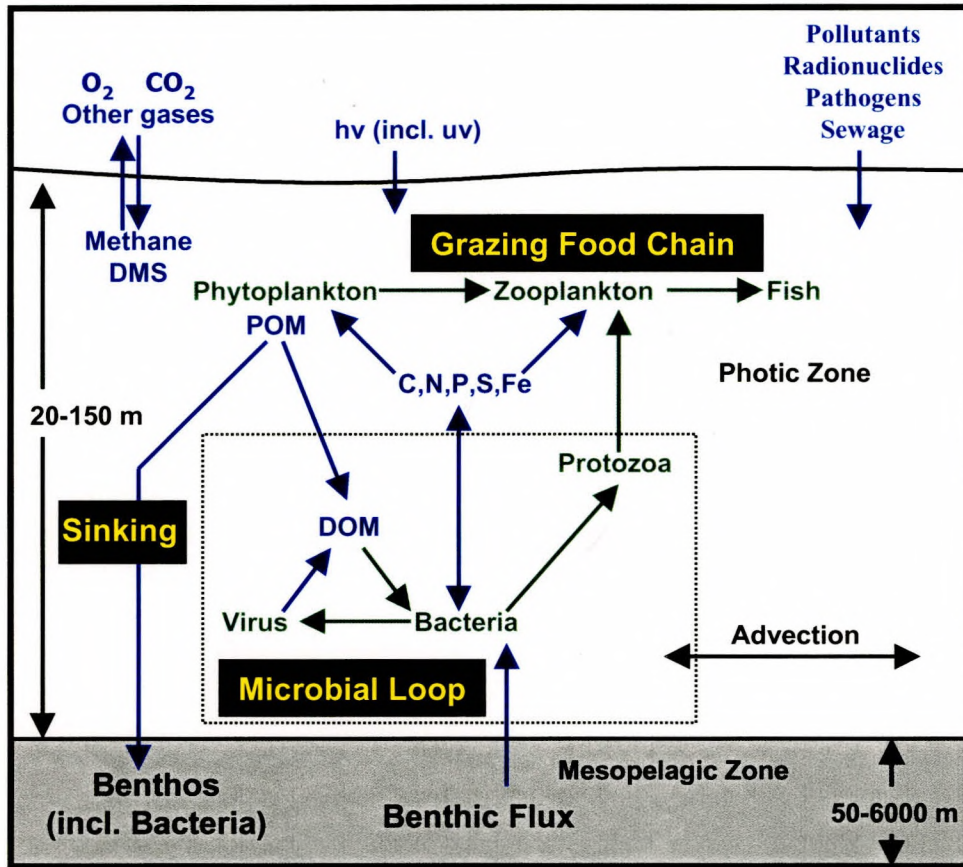
## GENERAL INTRODUCTION

The Role of Bacteria in the Pelagic Marine Environment

Bacteria were once thought to play only a trivial role in organic matter fluxes in the pelagic marine environment. It was believed almost all primary production was utilized by herbivores and passed on to the grazing food chain (Steele 1974; Thurman 1981). These views were a direct reflection of culturing methods used to quantify bacterial biomass and growth rates of pelagic marine bacteria. Direct enumeration of bacteria by epifluorescence microscopy revealed that bacterial abundance had been underestimated by at least an order of magnitude (Hobbie et al. 1977). Furthermore, new techniques to quantify bacterial secondary production (BSP) and bacterial growth rates, based on frequency of dividing cells and radioactive tracer studies, suggested that one-half of all the carbon biomass produced during primary production was required to support the high rates of BSP observed (Hagstrom et al. 1979; Fuhrman and Azam 1980). The bacterial biomass in the photic zone is estimated to be 10-50% of all plankton biomass (Fuhrman and Azam 1982). [<sup>3</sup>H]Thymidine and autoradiography studies showed that 30-80% of these bacteria were actively growing (Tabor and Neihof 1982; Fuhrman and Azam 1982). This bacterial biomass is subsequently preyed upon by protozoans and zooplankton and passed on to the grazing food chain (Hollibaugh et al.

1980; Fenchel 1982). The flux of organic matter through bacteria and the subsequent production of new bacterial biomass are now known as the "Microbial Loop" (Fig 1). Bacteria are considered to be a dynamic integral component involved in organic matter fluxes. The "Microbial Loop" is now accepted as one of the cornerstones of the food web in the pelagic marine environment (Pomeroy 1974; Azam et al. 1983; Azam 1998).

The importance of bacteria in nutrient cycling has raised new questions concerning the biochemistry of bacteria organic matter relationships. However, directly quantifying bacteria organic matter interactions has been impeded by the complexity of organic matter in the pelagic marine environment. Even the smallest particles of organic detritus in the open ocean possess complex and varied nutrient "patches" present in micron scale microbial niches (Blackburn et al. 1998). As a result, carbon flux into bacteria and subsequent bacterial biomass production are highly variable. Actual carbon fluxes into bacteria can vary between 0 and 100% of local primary production, depending on the nature of the organic matter, the location, depth in the water column, and time of year (Pomeroy et al. 1991). This variability and the lack of techniques to address bacterial behavior and physiological responses at the micron scale have provided a major obstacle in the synthesis of unifying concepts for bacteria organic matter interactions in the pelagic marine environment. New techniques are needed to localize and quantify micron scale bacteria organic matter biochemical interactions (Azam et al. 1993). It is essential to evaluate these processes at the scale in which they occur to understand how bacteria regulate ecosystem dynamics through control of organic matter fluxes.



**Figure 1.** Schematic of the pelagic marine food web depicting the “microbial loop”. Detrital POM created in the photic zone settles through the water column and is rapidly hydrolyzed by bacteria. Bacterial biomass is preyed upon by protozoans and re-enters the grazing food chain (Azam 1998).

### Organic Matter in the Marine Environment

Organic matter exists in many forms in the pelagic marine environment, including suspended and sinking particulate organic matter (POM) as well as dissolved organic matter (DOM). Concentrations of DOM in the open ocean are very low and average  $100 \text{ ug L}^{-1}$  (Biddanda 1985; Kirchman 1993). The source of DOM is controversial and is highly variable depending upon the location, time of year and depth in the water column. However, most DOM is thought to originate from phytoplankton excretion, viral lysis of bacteria, and solubilization of detrital POM by bacterial extracellular enzymes (Williams and Druffel 1988). There is little known about the composition of DOM. However, it has been estimated that generally 15% can be identified as combined amino acids and carbohydrates, 1-2% as lipids, and 20-50% as "humic substances" (Williams and Druffel 1987; Thorn et al. 1987). Currently, it is hypothesized that most bacterial biomass production in the pelagic marine environment is a result of growth on DOM (Cho and Azam 1988; Azam 1998). However, it is also hypothesized that much of this DOM is derived from POM, through the action of ectohydrolytic enzymes produced by POM associated bacteria.

A large fraction of organic matter in the pelagic marine environment is in the form of POM. The total mass of suspended POM in the pelagic marine environment is estimated to be on the order of  $10^{13} \text{ kg}$  or  $10\text{-}20 \text{ ug L}^{-1}$ , at any given time (Lal 1977). However, the actual POM content can vary 20-40 fold depending upon the geographic location and time of year. POM is comprised of living biomass as well as detrital organic

matter resulting from plankton mortality and to a lesser extent other higher animals and plant matter, and is generally formed from coagulation processes due to turbulent mixing in the photic zone (Alldredge and Gotschalk 1988). Much of the resulting suspended POM exists as particles that are 0.5 mm or larger in diameter and are known as "marine snow" (MacIntyre et al. 1995). Mucous formed by the release of extracellular polymeric materials by diatoms and other microorganisms forms the matrix of most marine snow (Alldredge et al. 1993). As a result marine snow contains distinct chemical microenvironments within their interior and can provide a rich source of nutrients to the local bacterial flora (Alldredge and Cohen 1987). The organic detritus within POM is also colonized by a diverse array of microbial life including bacteria, protozoans and phytoplankton (Finenko and Zaika 1977). As would be expected from such a diverse aggregation of organic matter, POM composition is biochemically diverse containing many different carbohydrates (40%) and amino acids (60%) (Parsons and Strickland 1962). Detrital POM serves as a rich source of energy and nutrients (Shanks and Trent 1979) for bacteria, which, in turn are preyed upon by protozoans and other zooplankton as well as consumed by herbivores feeding on the particles themselves (Alldredge and Silver 1988). Therefore, detrital POM provides the energetic basis for the existence of many other trophic levels.

Detrital POM created in the photic zone is transported to deeper water through sedimentation. Measured settling velocities for sinking detrital POM in the open ocean range between 1-150 m d<sup>-1</sup> (Shanks and Trent 1980; Lampitt 1985; Asper 1986). At an average settling velocity of  $74 \pm 39$  m d<sup>-1</sup> and a mean ocean depth of 3.8 km, many of

these aggregates will take approximately 50 days to settle out of the water column (Aldredge and Gotschalk 1988). However, POM associated bacteria rapidly consume the majority of these aggregates between 100m and 500m during their journey to the ocean floor. As a result, very little POM reaches the ocean floor (Suess 1980; Martin et al. 1987).

While there remains a distinction between POM and DOM, other classes of organic matter are being discovered, including colloids, submicrometer particles, and transparent polymer particles (Koike et al. 1990; Aldredge et al. 1993; Chin et al. 1998). These classes of organic matter are replacing the traditional POM versus DOM concept with the concept of an organic matter continuum (Azam et al. 1993).

#### Extracellular Hydrolytic Enzymes

Much of the organic matter that comprises detrital POM consists of high molecular weight biopolymers that cannot be directly utilized by bacteria. Many bacteria are capable, however, of synthesizing extracellular hydrolytic enzymes that can act on POM associated, high molecular weight, organic matter. These extracellular enzymes convert high molecular weight biopolymers into soluble oligomers and monomers for incorporation into the cell. Extracellular enzymatic hydrolysis is considered to be the rate limiting step in the conversion of high molecular weight polymers, of detrital POM origin, into bacterial biomass (Button 1985). Extracellular enzymes may be located on the cell surface or released as free enzymes into the environment (Wetzel 1992).

The extracellular enzyme activity associated with epiphytic marine bacteria was first recognized in culturable bacteria isolated from the surfaces of marine plants. Corpe and Winters (1972) identified a variety of hydrolytic enzymes, including proteases,  $\alpha$ -amylase,  $\beta$ -D-glucosidase, glucosaminidase, and phosphatase that were secreted into the medium during growth in batch culture. Hoppe (1983) established that many of these enzymes were present as free enzymes in bulk water samples free of POM. Smith et al. (1992) reported a variety of hydrolytic enzymes associated with POM and POM free seawater, including a variety of proteases, phosphatases,  $\beta$ -glucosidase, lysozyme, chitinases, and  $\alpha$ -glucosidase. Smith et al. (1992) and Karner and Herndl (1992) then revealed the importance of attached bacteria by demonstrating that enzymatic hydrolysis rates associated with POM were 100 (glucosidases) to 1000 (proteases) times higher than the surrounding bulk aqueous phase. Therefore, POM associated bacteria exhibited higher extracellular enzymatic activity than their free living counterparts. However, the fate of DOM derived from POM, following enzyme hydrolysis, was less clear. Hoppe et al. (1993) demonstrated a tight coupling between POM hydrolysis and uptake of hydrolysate by bacteria in natural aggregates collected in the North Atlantic, while, others have shown that rapid POM hydrolysis results in uncoupled POM hydrolysis and uptake of hydrolysate (Smith et al. 1992). It was hypothesized that a mechanism of uncoupled hydrolysis with hydrolysate uptake, was a biochemical mechanism for the large scale transfer of insoluble POM to the DOM pool for utilization by free living bacteria. However, this issue remains controversial and unresolved. Nevertheless, the intense hydrolytic enzyme activity associated with detrital POM explains the rapid rates of

particle hydrolysis observed as detrital POM sinks to the ocean floor. More recently, Rassoulzadegan (1995) measured free- and particle-bound  $\alpha$ -glucosidase,  $\beta$ -glucosidase and aminopeptidase activity and showed strong diurnal variations. Increases in enzyme activity were observed following increases in primary production, emphasizing the importance primary production plays in fueling the microbial loop.

### Bacterial Secondary Production

Although a variety of methods have been used (Karl 1986, for a review), estimates of BSP are generally calculated based on [ $^3\text{H}$ ]thymidine and [ $^3\text{H}$ ]leucine uptake methods and are reported in units of  $\text{mgC m}^{-3} \text{d}^{-1}$  (Kirchman 1982; Ducklow and Kirchman, 1983; Kirchman et al. 1985). BSP varies as a function of depth in the water column, time of day and year, and geographic location. Reported BSP rates in the literature range over seven orders of magnitude, from  $0.0004 \text{ mgC m}^{-3} \text{d}^{-1}$  in McMurdo Sound Antarctica to over  $2000 \text{ mgC m}^{-3} \text{d}^{-1}$  in a eutrophic bay along the Ivory Coast (Fuhrman and Azam 1980; Torreton et al. 1989). However, average estimates of total BSP range between  $2\text{-}119 \text{ mgC m}^{-3} \text{d}^{-1}$  (Ducklow et al. 1992).

BSP of POM associated bacteria and free living bacteria in the pelagic marine environment are distinguished by fractionation of seawater samples after incubation with [ $^3\text{H}$ ]thymidine. In a variety of studies throughout the Atlantic and Pacific oceans the contribution of attached bacteria to total BSP ranged from 1.8-10% (Allredge et al. 1986; Griffith et al. 1994; Turley and Mackie 1994). Therefore, because only a small

fraction of the total BSP was associated with detrital POM compared to the amount of free living BSP it has been concluded attached bacteria contribute little to the direct production of bacterial biomass in the pelagic marine environment (Alldredge et al. 1986; Karner and Herndl 1992; Griffith et al. 1994; Turley and Mackie 1994). Calculations based on these rates of BSP associated with POM, have estimated it would take months to years to consume an aggregates carbon load (Ducklow et al. 1982; Karl et al. 1988; Simon et al. 1990). This low rate of carbon uptake cannot account for the apparent disappearance of particles with depth and has been called the "particle decomposition paradox" (Karl et al. 1988).

Growth rates of POM associated bacteria, measured by [<sup>3</sup>H]thymidine incorporation, do not differ significantly from free living bacteria (Alldredge et al. 1986; Iriberry et al. 1990; Karner and Herndl 1992; Muller-Niklas et al. 1994). However, when radiolabeled substrates such as glucose, acetate and amino acids were used, attached bacteria were more active than free living bacteria (Hodson et al. 1981; Kirchman and Mitchell 1982; Pearl and Merkel 1982; Simon 1985). Why growth rates of attached bacteria do not reflect the higher uptake rates of organic compounds relative to free living bacteria remains a mystery. One possible explanation is that attached bacteria need more of these substrates to synthesize extracellular materials used in attachment and the production of ectohydrolytic enzymes (Fletcher and Floodgate 1973; Kirchman and Mitchell 1982).

Reconciling the "Particle Decomposition Paradox"?

Due to high observed rates of ectohydrolytic enzyme activity and the apparent low rate of POM associated BSP, it has been hypothesized that POM associated bacteria generate more hydrolysate than they take-up (Azam and Cho 1987; Cho and Azam 1988; Azam 1998). This scenario suggests that attached bacteria incorporate more substrates than their free living counterparts to fuel increased production of ectohydrolytic enzymes. The over production or hyperactivity of those enzymes results in the release of DOM into the bulk aqueous phase for uptake by free living bacterial populations. The free living bacterial populations utilize this source of DOM for BSP. However, this remains speculative, since it is difficult to determine what fraction of the low molecular weight DOM, resulting from ectohydrolytic enzyme solubilization of POM, is directly taken up by bacteria on the particle surface and what fraction is released into the bulk aqueous phase (Hoppe et al. 1988). While it is likely that attached bacteria release some DOM during POM solubilization, it is unlikely they would have such inefficient substrate uptake mechanisms to release the amount of DOM that would be necessary to support a significant portion of free living BSP (Kirchman 1993). Little is known about what controls the rates of POM hydrolysis and even less is known about what controls the abundance of attached and free living bacterial populations in the pelagic marine environment. The inability to identify fluxes of organic matter into bacteria remains one of the largest problems facing marine microbial ecologists. Considering the multitude of carbon, nitrogen and energy sources available to bacteria in the natural environment, it is

almost impossible, using current methods, to assess whether bacteria are controlled by resource availability. This is primarily due to the difficulty in assessing which carbon, nitrogen and energy sources bacteria are using in these microenvironments.

### A Problem of Methods?

The recycling of carbon, nitrogen, energy, and other nutrients that remain sequestered within detrital POM is dependent on POM associated bacterial activity. The most frequently reported indicators of bacterial activity associated with detrital POM are rates of BSP, measured by [<sup>3</sup>H]thymidine incorporation, and enzyme activities using fluorogenic enzyme substrates. Fluorogenic enzyme substrates use fluorophores such as methylumbelliferone, methylcoumarinylamide,  $\beta$ -naphthylamide or methyl-fluorescein to detect specific enzyme activities (Hoppe 1991). These fluorophores are conjugated to an organic molecule via an ester linkage specific for the enzyme activity being detected. There are two major drawbacks to this technique. The first is that these enzyme substrates are all membrane permeable and are hydrolyzed by extracellular enzymes as well as periplasmic enzymes. Therefore, these substrates are not specific for extracellular hydrolytic enzymes, but report all enzymatic activity. In a report by Okutani (1978), 100% of bacterial seawater isolates could utilize the monomer of chitin, N-acetyl- $\beta$ -D-glucosamine, but only 10% could produce extracellular chitinase. It is unknown how many of those bacteria unable to produce extracellular chitinase, possessed enzymes to hydrolyze chitin oligomers in the periplasm. Thus, the use of these fluorescent probes

could misrepresent actual rates of POM hydrolysis. The second drawback is that the fluorophores are soluble and readily diffuse away from sites of enzyme action. Therefore, enzyme activity can only be related to total numbers of cells. In spite of these drawbacks these methods have been used in a variety of environments to yield a qualitative understanding of the distribution of enzyme activities in the pelagic marine environment. New technology is needed to assess enzyme activities associated with specific bacterial populations during POM solubilization. The location and distribution of those extracellular hydrolytic enzymes will most likely influence the solubilization of POM as well as influence the amount of DOM released into the bulk aqueous phase. It has been hypothesized that extracellular hydrolytic enzymes that remain bound to the cell surface result in the generation of DOM that is directly incorporated into the cell (Hoppe 1991). In contrast, extracellular hydrolytic enzymes that are released from the cell generate DOM that enters the DOM pool of the surrounding water. To date, this has not been tested since there are no methods available to detect extracellular hydrolytic enzymes associated with individual cells.

While the [ $^3\text{H}$ ]thymidine method has been widely adopted to provide rates of BSP and estimates of bacterial growth rates associated with POM, their accuracy remains controversial (Riemann and Bell 1990). The [ $^3\text{H}$ ]thymidine method involves incubation of a seawater sample, fractionation of the sample into a "particulate" and a "bulk aqueous phase", followed by a trichloroacetic acid extraction to recover DNA that has assimilated [ $^3\text{H}$ ]thymidine. The amount of radioactivity is then measured ( $\text{pmoles m}^{-3} \text{d}^{-1}$ ) in the DNA extraction. Much of the controversy concerning the accuracy of this method has

centered on the use of conversion factors for translating uptake rates ( $\text{pmoles m}^{-3} \text{ d}^{-1}$ ) into cell ( $\text{cells m}^{-3} \text{ d}^{-1}$ ) and carbon based rates ( $\mu\text{g C m}^{-3} \text{ d}^{-1}$ ). Kirchman et al. (1982) showed that if [ $^3\text{H}$ ]thymidine incorporation is tightly coupled with growth, accurate conversion factors can be derived. However, if [ $^3\text{H}$ ]thymidine is catabolized it becomes difficult to derive these conversion factors accurately. Assuming that conversion factors can be derived and the resulting BSP rates are accurate, specific growth rates ( $\text{hr}^{-1}$ ) can be estimated by dividing the BSP by the number of bacteria incorporating the [ $^3\text{H}$ ]thymidine.

An additional problem that has received less attention is the disruptive nature of the technique. The [ $^3\text{H}$ ]thymidine method only captures BSP associated with POM at a specific point in time. This method is unable to detect the dynamic partitioning of bacterial biomass between POM and the bulk aqueous phase through the processes of attachment and detachment. Current estimates of POM associated BSP and bacterial growth rates are based on the assumption that no detachment or exchange of bacterial biomass occurs between the surface of POM and the bulk aqueous phase, or at least assumes it is insignificant. This could lead to a significant underestimation of BSP and specific bacterial growth rates associated with POM.

### Detachment

Detachment of POM associated bacteria can contribute to free living bacterial populations. Karner and Herndl (1992) found the contribution of attached bacteria to

total BSP ranged between 25-50%, during seasonal fluctuations. Likewise, Jacobsen and Azam (1984) found that aggregates and copepod fecal pellets displaced bacteria into the surrounding water that accounted for approximately 40-90% of the total bacterial biomass present in the surrounding bulk aqueous phase. Jacobsen and Azam hypothesized that detrital POM may serve as "baby machines", where reproducing cells on the surface seeded daughter cells into the bulk aqueous phase. They admitted this would cause serious errors in calculating POM associated BSP and growth rates. However, detachment was not directly observed in these studies. Bacteria were only recovered in the bulk aqueous phase and it was assumed that they detached since bacterial numbers of the copepod fecal pellet were decreasing and those in the bulk aqueous phase were increasing. There are no reported detachment rates for attached bacteria in the pelagic marine environment, and the effect this process has on our estimates of BSP are currently unknown. Without a fundamental understanding of potentially dynamic processes, like detachment, our understanding of carbon fluxes within the pelagic marine environment will remain incomplete.

### Model Systems

The variability in POM composition and the lack of techniques to address bacterial behavior and physiological responses at the micron scale have proven to be major obstacles in the synthesis of unifying concepts for bacteria organic matter interactions. Efforts have been made to generate model detrital POM for more controlled

laboratory experiments. Krank and Milligan (1980) attempted to make model detrital POM by blending organic matter from fish and plankton, and Biddanda (1985) formed model detrital POM by growing bacteria on ground seaweeds. In a more sophisticated approach Shanks and Edmundson et al. (1989) generated model detrital POM by conditioning unfiltered seawater in a rolling tank until aggregates formed. Unanue et al. (1998) created model POM with a more defined biochemical composition by using defined seawater and inoculating it with known populations of bacteria and diatoms and used the same rolling tank configuration as Shanks and Edmundson. This form of model detrital POM was successfully used with a natural inoculum in microcosm experiments by Agis et al. (1998) to demonstrate that particle hydrolysis and release of hydrolysate was a highly variable process over time. It was subsequently shown, using the same model POM and microcosms, that different enzyme activities peaked at different times within the microcosms suggesting successional changes may be important during POM decomposition (Unanue et al. 1998). These model systems provided well defined conditions for examining the metabolic activities of marine bacterial assemblages. However, the complexity of these model systems still prevented direct observation of interactions between detrital POM and microbial communities. Metabolic activities are still defined in terms of undefined bacterial communities. It is essential to design systems to evaluate these processes at the micron scale in an effort to understand how bacteria influence ecosystem dynamics through control of organic matter fluxes. This will not only contribute to our understanding of bacteria organic matter interactions, but, also to

our understanding of inter- and intra-species bacterial relationships within these diverse microenvironments.

### Goals

There were three main goals in this study. The first was to quantify the rate of BSP and growth rate of the marine bacterium *Pseudoalteromonas sp.* strain S91 during the degradation of solid chitin. To achieve this goal, the contribution of chitin associated BSP to free living bacterial biomass via the processes of reproduction and detachment was evaluated over time. The second goal was to assess chitinolytic gene expression of S91 at the single cell level during the processes of bacterial attachment to, reproduction on, and detachment from the surface of chitin. To this end, chitinolytic gene expression was assessed in individual cells on the chitin surface and in the bulk aqueous phase over time. The third goal was to localize chitinolytic gene expression and relate it to extracellular chitinase activity at the single cell level during chitin degradation. To this end, a new precipitating enzyme substrate was developed to localize extracellular chitinase activity in individual bacterial cells of a population on the surface of chitin. Chitinase activity was also assessed in cells that detached to form the free living population. Extensive methods development was required to overcome the obstacles associated with observing single cell activities on relevant surfaces, in real time, in a flowing seawater environment. This work provides an example of the physiological

diversity that exists within a population of marine bacteria during the degradation of chitin.

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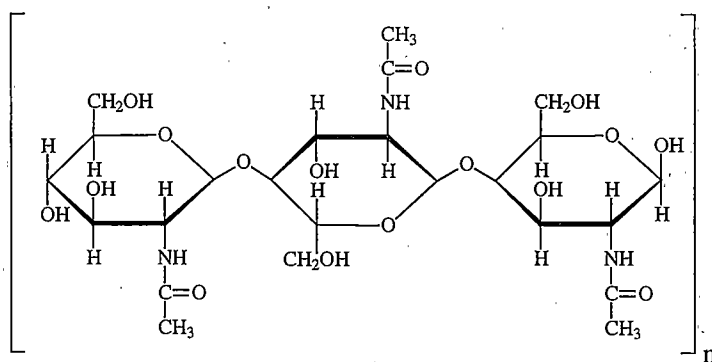
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## CHAPTER 2

## PREPARATION OF CHITIN THIN FILMS FOR THE EVALUATION OF BACTERIAL COLONIZATION

Introduction

Chitin, an insoluble, homopolymer of  $\beta$ -1,4-linked N-acetyl- $\beta$ -D-glucosamine (Fig 2), occurs commonly as an exoskeletal and endoskeletal material in many marine organisms including Mollusca, Coelenterata, Protozoa, Fungi and Crustacea (Purchase and Braun 1946; Gooday 1979). Chitin is also produced as an extracellular polymer in many diatoms (Blackwell et al. 1967). The production of chitin from krill alone ranges from 1.6-10 million metric tons annually (Clarke 1980), making it the largest single carbon sink in the pelagic marine environment (Place 1996).

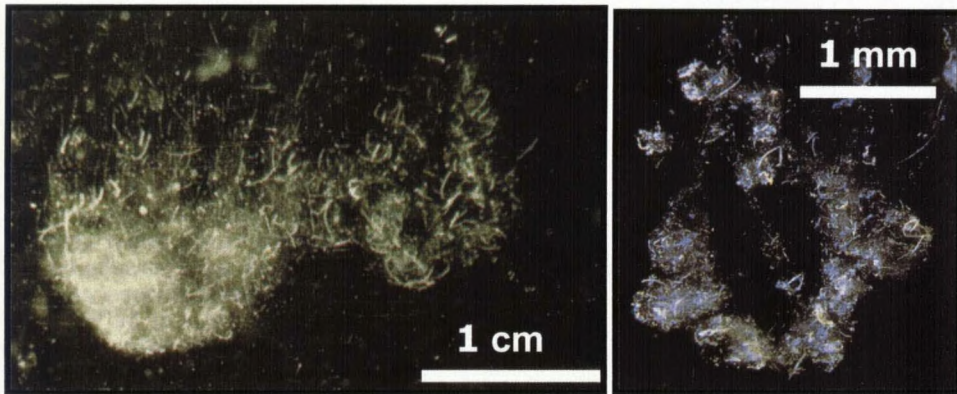


**Figure 2.** A subunit of the homopolymer chitin consisting of  $\beta$ -1,4-linked N-acetyl- $\beta$ -D-glucosamine monomers.

Bacteria are the principle mediators of chitin degradation, and their ability to mediate extracellular hydrolysis of chitin is an essential step in recycling carbon and other nutrients in the pelagic marine environment (Smucker and Kim 1993). However, due to the difficulty in overcoming the material properties of chitin, the dynamic relationship between the production of bacterial biomass and chitin hydrolysis has never been observed at the cellular level. Therefore, chitin hydrolysis has only been evaluated at the population or community level. The observation and quantification of microbial activities at the cellular level on the surface of solid chitin is essential to define the role bacteria play in the dissolution of chitin at the micron scale. This will not only contribute to our understanding of chitin solubilization, but, ultimately to our understanding of inter- and intra-species bacterial behavior during chitin degradation.

There are three problems typically associated with direct observation and quantification of bacterial activities associated with natural chitin surfaces. The first is the high degree of surface roughness that prevents the direct observation of bacterial cells participating in chitin degradation (Fig 3). The second problem, and one that is more significant, is the fact that chitin possesses a broad spectrum fluorescence under ultraviolet light illumination, rendering the natural surface unsuitable for visualizing bacterial activities reported by fluorescent probes. The third problem that arises when trying to define the activities of bacterial populations attached to natural chitin is the multitude of carbon, nitrogen and energy sources associated with the natural material. Chitin is rarely found in nature in its pure form and is generally associated with proteins, glucans or pigments, making it difficult to associate microbial activity to the degradation

of chitin (Muzzarelli 1977). However, all of these problems can be overcome by the use of thin polymer films of pure chitin.



**Figure 3.** Natural detrital POM, containing chitin particles (Alldredge 1996).

The deposition of thin polymer films can be achieved by means of four different techniques: solution deposition, vacuum evaporation, plasma polymerization and sputtering (Ruckenstein and Gourisankar 1986). Solution deposition by spin casting provides a fast, economical way to make stable thin films from high molecular weight polymeric materials. Spin casting polymer films from solution creates ultra-smooth thin films of the pure polymer. Furthermore, the typical UV-induced fluorescence that is associated with many polymers is below the detection limits of most charge coupled device (CCD) cameras when prepared as thin films. Polymer thin films are also pure, allowing the evaluation of bacterial behavior with respect to a single carbon and nitrogen source. Spin-cast chitin thin films overcome the problems associated with natural chitin

and offers a relevant solid substratum on which microbial activities can be quantified at the single cell level during the processes of bacterial attachment, reproduction and detachment from the surface.

This work describes the preparation and characterization of chitin thin films to study single cell behavior of chitinolytic bacteria during chitin degradation. The fluorescence of the thin films was evaluated using epifluorescence microscopy. Differential interference contrast (DIC) microscopy and profilometry were used to evaluate the continuity of the chitin thin films. Surface chemistry was quantified spatially using x-ray photoelectron spectroscopy (XPS). The density of the chitin thin films was determined using thickness measurements obtained by profilometry and mass measurements determined by quartz crystal microbalance (QCM). Finally, chitin thin films were evaluated in the presence of chitinolytic marine bacteria engineered with a green fluorescent protein (*gfp*) reporter gene for chitinase expression, demonstrating that these surfaces can be used to resolve individual bacterial cell activity at the micron scale.

## Materials and Methods

### Chitin Thin Film Preparation.

Chitin thin films were cast onto silicon coupons for the evaluation of bacterial gene expression, XPS analysis, and profilometry. In addition chitin thin films were cast onto AT-cut quartz crystals for parallel profilometry and mass determination by QCM. Silicon coupons, 1cm x 1cm square (Harrick Scientific, Ossining, NY), were cleaned in

“Pirhana” which consisted of a 70:30 mixture of concentrated sulfuric acid and 30.0% hydrogen peroxide [**WARNING:** Piranha solution reacts violently, even explosively with organic materials (Dobbs et al. 1990)]. The silicon coupons were then rinsed in dH<sub>2</sub>O and dried under a stream of pure dry nitrogen. Quartz crystals were cleaned with a cotton tipped applicator successively in water, methanol, and chloroform and dried under a stream of dry nitrogen. Methods for casting chitin films from chitosan solutions were adapted and modified for use with spin-casting techniques (Bae and Hudson 1997; Rathke and Hudson 1994; Qin 1993). In an effort to determine the optimum chitin thin film thickness, films were cast from six different concentrations, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0%, of high molecular weight chitosan (1.0% solution in 1.0% acetic acid: 800-2000 cps, Aldrich, Milwaukee, WI) prepared in aqueous solutions of 2.0% acetic acid. The chitosan solutions were filtered through a 0.2 µm syringe filter to remove any insoluble debris. With exception to the cleaning procedure, the spin casting protocols for the silicon and quartz crystals were identical. All thin films prepared for this work were cast at 23°C. To spin-cast a chitin thin film on a silicon coupon a clean, dry coupon was affixed to the middle of a centrifuge rotor using double-sided sticky tape, and scrubbed with methanol followed by chloroform using cotton tipped applicators. Approximately, 300 µL of one of the chitosan solutions was placed on the silicon coupon and the surface was spun with rapid acceleration at 4500 rpm for 5 minutes. While the surface was still spinning the chitosan thin film was washed successively with 1.0 mL of 5.0% NaOH, 10.0 mL of dH<sub>2</sub>O, and 1.0 mL of methanol. The chitosan thin films were then dried in a desiccating chamber overnight, prior to N-acetylation to generate the chitin thin film. To

prepare the chitin thin films, the chitosan thin films were N-acetylated by placing the coupons in 10.0 mL of ice cold, dry methanol and adding 2.5 mL of ice-cold acetic anhydride while stirring. The reaction was held for 18 hrs at 4°C. The chitin thin films were then rinsed in methanol 4x and placed on the spin caster where they were rinsed with an additional 10.0 mL of methanol while spinning at 3000 rpm. The films were dried overnight in a desiccating chamber.

#### Atomic Composition of Thin Films by XPS

To insure the surface chemistry of the chitin thin films were homogeneous, a spatial characterization of the atomic composition was performed using XPS. This technique irradiates a sample with an x-ray source and induces the ejection of photoelectrons from the surface (Carlson 1975; Briggs 1977). It quantitatively measures atomic concentrations and valence states and is ideal for quantifying spatially related chemistries on surfaces. XPS spectra were obtained with a Physical Electronics instrument Model 5600 spectrometer (Physical Electronics, Eden Prairie, Minnesota). A 5 eV flood gun was used to offset charge accumulation on the samples. An aperture of 30  $\mu\text{m}$  was used with a monochromatized aluminum  $K\alpha$  x-ray source at 350 Watts and a pass energy of 11.750 eV. Survey spectra and high resolution spectra were gathered at a take-off angle of 45°. Abundance of carbon, nitrogen and oxygen were calculated from high resolution C1s, N1s, and O1s peak areas. The binding energy scale was referenced by setting the CHx peak maximum in the C1s spectrum to 285.0 eV (Ratner and Castner 1994). The atomic composition of three 30  $\mu\text{m}$  spots on each of three separately

prepared chitin thin films (n=9) were evaluated. The atomic composition is presented as percent carbon, nitrogen, and oxygen with the standard error.

#### Film Thickness by Profilometry

Thin film thickness was determined directly on chitin thin films cast onto quartz crystals and on silicon substrata using profilometry. The use of profilometry to measure surface topography is well known in the semiconductor industry (Sommargren 1987; Bobroff 1993). Historically, profilometry has been based on dragging a stylus over a surface, but the optical profilometer used in this case utilizes white light interferometry (Deck et al. 1995; Demarest 1998). White light (200 nm) is simultaneously reflected from the test surface and a reference surface and is recombined to produce a fringe pattern. A vertical scanning piezoelectric transducer synchronized with a CCD camera and frame grabber generates interferograms of the surface and a computer translates these interferograms into a quantitative 3-D image of surface topography. To get a film thickness from the 3-D profile of the surface, the chitin thin films were scored with a 27-gauge needle down to the substratum. Because the chitin thin films were transparent, it was necessary to coat the surfaces with approximately 150 Å of gold using a sputter coater. The depth of the score was then measured using a Zygo NEW View 200 profilometer (Zygo Middlefield, CT) to obtain the film thickness. A 20x objective was used to obtain data with a vertical resolution of 0.1 nm and a lateral resolution of 0.64 μm. Data were collected at a scan rate of 2.4 μm s<sup>-1</sup> and analyzed using Zygo MetroPro

software™. For practical use in preparing chitin thin films the data were plotted as; chitin thin film thickness (nm) versus % of chitosan in the casting solutions.

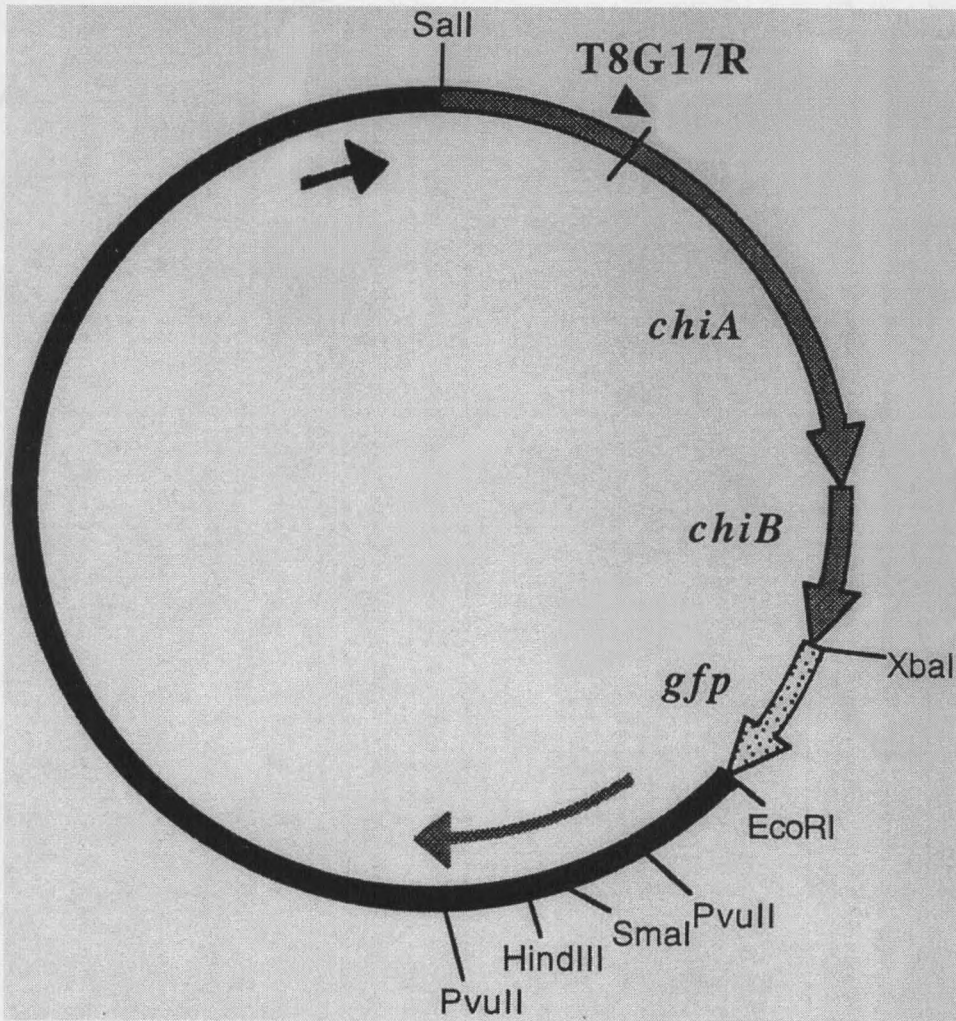
#### Film Mass by Quartz Crystal Microbalance

Film mass was measured using QCM. The QCM is an ultra-sensitive weighing device based on a piezoelectric, electromechanical oscillator (Bruckenstein 1985; Henry 1996; Stellenberger et al. 1997). The QCM comprises a thin quartz crystal sandwiched between two excitation electrodes. A shear acoustic standing wave is generated in the crystal by the application of a sinusoidal voltage across the electrodes at the crystal's fundamental resonance, in this case 10 MHz. This resonance frequency varies linearly with the mass deposited on the surface, according to the Sauerbrey equation,  $df/dm = 0.23 \text{ Hz cm}^2 \text{ ng}^{-1}$  (Sauerbrey 1959). The QCM, therefore, directly measures the aerial mass on the surface. Chitin thin films were prepared on AT-cut quartz crystals with a nominal frequency of 10 MHz (International Crystal Manufacturing, Oklahoma City OK). The quartz crystals were 20 mm in diameter and 0.167 mm thick, with 100 Å-thick chromium (to enhance gold adhesion), and 1800 Å-thick gold bilayer electrodes. The electrodes on each side of the crystal were of different size and were plated prior to purchase. The ground electrode had a diameter of 7 mm; the excited electrode had a diameter of 5 mm. The ground electrode was additionally coated with 1000 Å of silicon dioxide to match the surface chemistry of the solid silica substrata. The crystals were mounted in a spring mount HC-48/u base. A standard laboratory crystal oscillator, model #035360 (International Crystal Manufacturing, Oklahoma City OK), was used to excite

the quartz crystal. The oscillator was equipped with a BNC output to a frequency counter. A Hewlett Packard 5314A frequency counter (Hewlett Packard, Palo Alto CA) was used to measure the resonant frequency changes. The mass of each chitin thin film was calculated from the change in frequency of the crystal resonance before and after thin film deposition. The mass measurements were combined with the thickness measurements obtained by profilometry to report the mass per unit volume or density of the chitin thin films.

#### Bacterial Cultivation in a Flowing Seawater System

A green fluorescent protein reporter gene (*gfp*) construct for chitinase expression was used to visualize the chitinase gene expression of *Pseudoalteromonas sp.* strain S91 during the degradation of the chitin thin films. Strain S91 contained plasmid pDSK519 with a complete chitinase gene (*chiA*) and a truncated chitin binding protein gene (*chiB*) (Fig 4) (Keen et al. 1988). Strain S91 is streptomycin (Sm) resistant and the plasmid confers kanamycin (Km) resistance. The gene for *gfp* is under control of both the *chiA/chiB* promoters and a set of fully functional genes resides on the chromosome (Stretton and Goodman 1998; Techkarnjanaruk and Goodman 1999). For the growth of S91, when the induction of the *chiA/chiB* genes was desired, 0.05M N-acetyl- $\beta$ -D-glucosamine was added to the defined seawater as the sole carbon, nitrogen and energy source. Growth of S91 under conditions of *chiA/chiB* down-expression was achieved by supplementing the defined seawater with 0.05M glutamic acid as the sole carbon, nitrogen and energy source. The defined seawater consisted of 201.1 mM NaCl,



**Figure 4.** Diagrammatic representation showing construction of the plasmid containing the *chiA/chiB-gfp* reporter gene (Techkarnjanaruk 1998).

4.8 mM  $\text{H}_3\text{BO}_4$ , 27.5 mM  $\text{Na}_2\text{SO}_4$ , 2.4 mM  $\text{NaHCO}_3$ , 88.5 mM  $\text{KCl}$ , 8.4 mM  $\text{KBr}$ , 54.1 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 1.5 mM  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$  suspended in 0.05M Sigma 7-9 buffer using once distilled Millipore water and adjusted to pH 8.0. Following autoclave sterilization, 0.008 mM  $\text{FeCl}_3$ , 0.04 mM  $\text{K}_2\text{HPO}_4$ , 2.0 mM  $\text{CaCl}_2$  were each added separately by filter sterilization through a 0.2  $\mu\text{m}$  pore size Millipore syringe filter. To insure retention of the plasmid by the bacterial cells  $\text{Km}$  was added to a concentration of 600  $\mu\text{g mL}^{-1}$ . In an effort to minimize contamination of the culture during long term starvation and the flow-cell experiment,  $\text{Sm}$  was added to a concentration of 200  $\mu\text{g mL}^{-1}$ .

A Teflon<sup>®</sup> laminar flow cell (LFC) was used to monitor surface associated *chiA/chiB* gene expression in association with the chitin thin films (Davies and Geesey 1995). Glass viewing ports in the LFC allowed direct microscopic examination of attached S91 without disturbing the flowing system. Prior to inoculation of the LFC, S91 was starved in artificial seawater with no carbon, nitrogen or energy sources. The chitin LFC was inoculated with a 400 hr starved population of S91 for a period of 1 hr at a flow rate of 0.5  $\text{cm}^3 \text{min}^{-1}$ . After the inoculation, the bulk aqueous phase flowing through the LFC was switched to sterile defined seawater. The LFCs and the sterile defined seawater were maintained at 20°C throughout the experiment. The flow rate was maintained at 0.5  $\text{cm}^3 \text{min}^{-1}$  allowing the growth of S91 on the chitin thin film for a period of 200 hrs. The chitin thin film served as the only added source of carbon, nitrogen and energy within the LFC. The chitin thin films were imaged by differential interference contrast (DIC) microscopy and the expression of the *chiA/chiB-gfp* genes was monitored using epifluorescence microscopy.

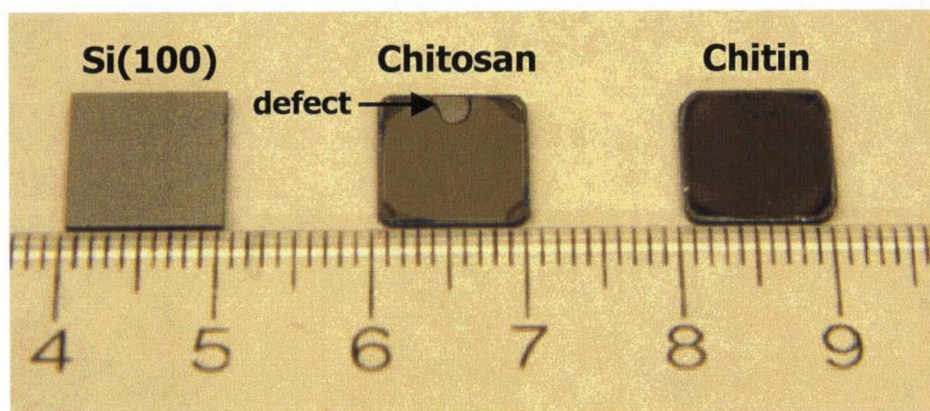
### Microscopy and Image Analysis

At 0, 50, 100, and 200 hrs post-inoculation total cells, *chiA/chiB* up-expressed cells were imaged directly on the chitin thin film surface in the LFC without removing the coupon or stopping the seawater flow. Images were acquired using an Olympus B-max 60 microscope (Olympus Optical Co., Tokyo, Japan) with both reflected DIC and epifluorescence optics. All images were acquired using a Nikon infinity corrected, 40x, water immersion objective (Nikon Inc., Torrance, CA) and a mercury lamp (Chiu Technical Corporation, Kings Park, NY). Digital images were gathered using a Photometrics Imagepoint™ cooled CCD camera (Photometrics, Tucson, Az). Fluorescence images of *chiA/chiB-gfp* reporter gene expression were acquired using an excitation of 481 nm and an emission of 507 nm. All epifluorescence images were gathered using an exposure time of 6 seconds and a gain of 4. Images were analyzed with Image-Pro Plus™ software (Media Cybernetics, Silver Springs, MD). The only image manipulation performed on the DIC images was a background correction and in a few cases minor adjustment of the gray scale contrast.

The UV-induced fluorescence of the chitin thin films was monitored at the excitation (481) and emission (507) wavelengths of GFP. The relative luminosity of the thin films was compared to natural squid pen chitin and batch cultures of S91 that were up- and down-expressed for *chiA/chiB-gfp*.

### Results and Discussion

The chitosan solution spin-casting procedure developed here provided a fast, simple method of constructing high molecular weight, pure chitin thin films. The quality of the chitin thin films decreased with increasing chitosan casting solution concentrations. Chitin thin films prepared from solutions containing more than 4% chitosan could not be constructed due to the high viscosity of the chitosan solutions. Chitin thin films prepared from solutions containing more than 2% chitosan trapped air bubbles during casting, resulting in usable films but with visually observable film defects. Based on visual observation, 1% chitosan-cast chitin thin films appeared to be continuous and evenly distributed (Fig 5). The most critical step in the construction of these films is the cleaning of the silicon substratum. The slightest residue, translates into major defects in the thin film (Fig 5).

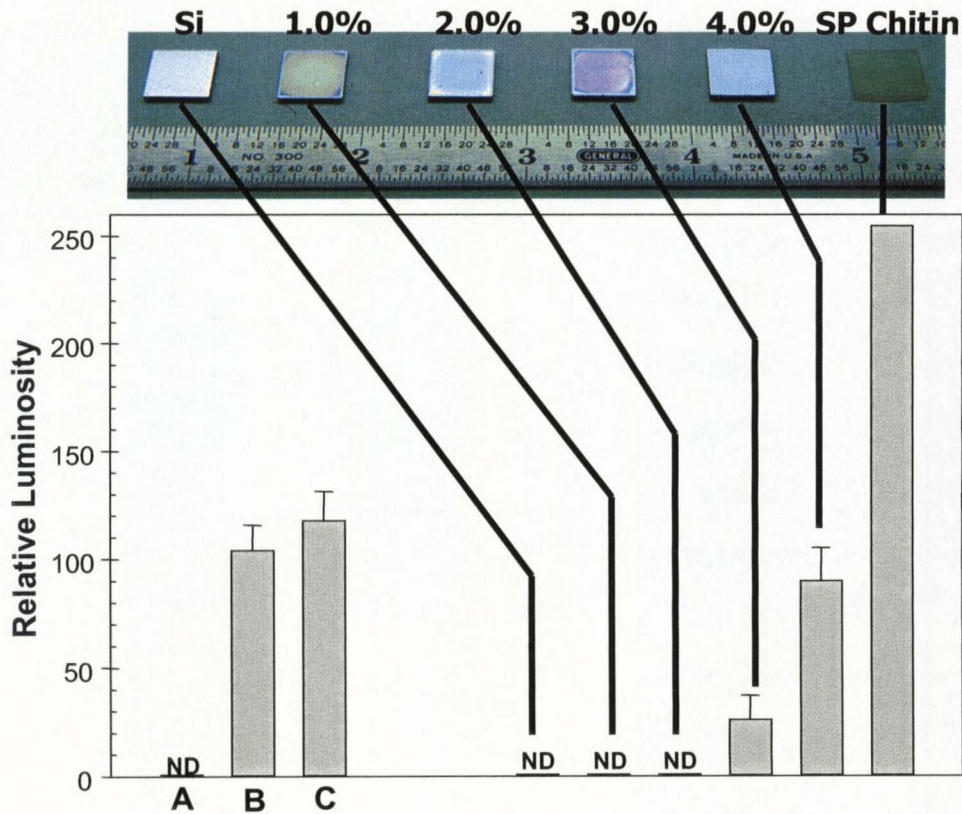


**Figure 5.** Chitosan and chitin thin films prepared from a 1.0% chitosan casting solution. The chitin thin film is evenly spread across the silicon coupon. The defect in the chitosan film was due to an incorrectly cleaned surface.

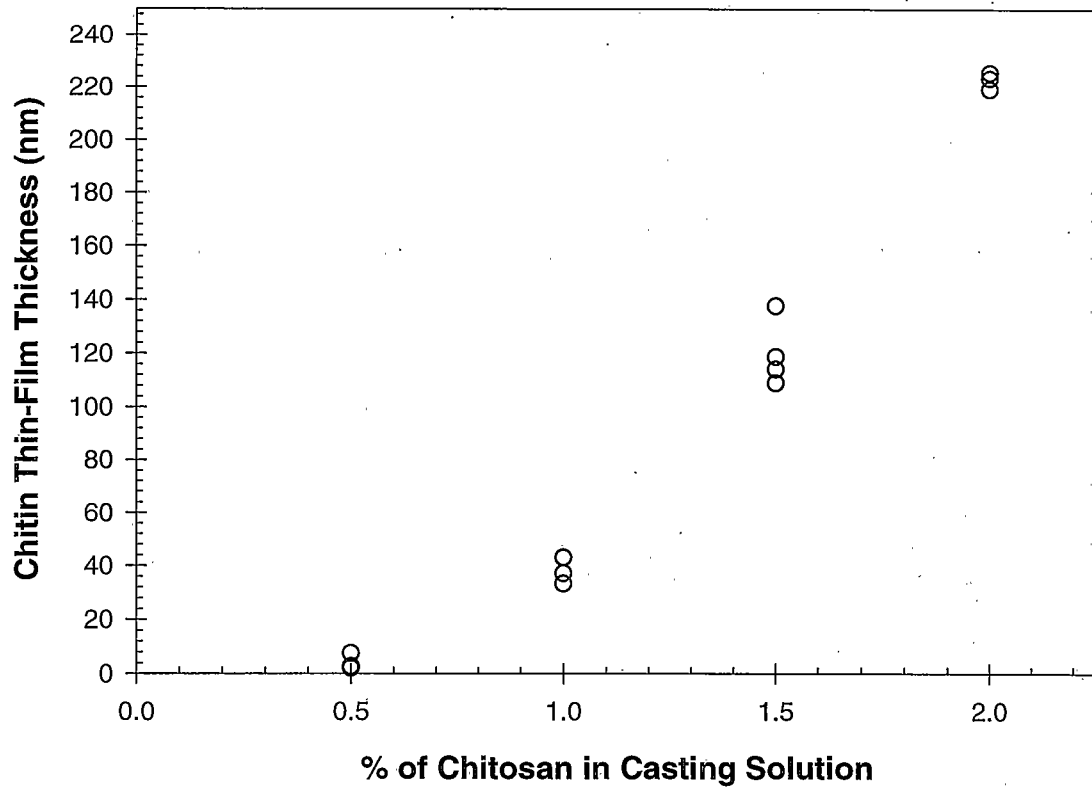
However, this was recognized immediately upon spin casting the chitosan solution by visual observation. The only other flaw was the slight accumulation of material near the corners of the silicon coupons, as can be seen by the darker color. Upon N-acetylation of the chitosan films, the resulting chitin film always appeared slightly darker in color.

No detectable fluorescence was observed for the 1 and 2% chitosan-cast chitin thin films, at an exposure of 6 sec and a gain of 4 using our CCD camera (Fig 6). However, the 3% chitosan-cast chitin thin film showed an increase in fluorescence intensity and the 4% chitosan-cast chitin thin film showed a dramatic increase. The fluorescence of these films was very low compared to natural squid pen chitin. However, the fluorescence was enough to provide a considerable amount of background relative to the levels of fluorescence emission displayed by the GFP in cells of S91. Only chitin thin films prepared from  $\leq 2\%$  chitosan solutions were characterized further.

Chitin thin film thickness was quantified on the silicon coupons using 0.5, 1.0, 1.5, and 2% chitosan casting solutions. Chitin film thickness ranged from approximately 3.6 nm for the 0.5% chitosan casting solution to 230 nm for the 2% solution (Fig 7). The 0.5% and 1.0% chitosan-cast chitin films were very thin and would most likely be rapidly degraded by chitinolytic bacteria and were disregarded for further study. The 2.0% solution was difficult to cast consistently due to the high viscosity of this concentration of chitosan in solution. Therefore, the 1.5% chitosan-cast chitin films were chosen for further characterization. The average film thickness for chitin thin films cast from 1.5% chitosan casting solutions onto the QCM electrodes was  $120.0 \pm 6.3$  nm. The average mass of these films was  $24.6 \pm 0.8$   $\mu\text{g cm}^{-2}$ . The density of these films calculated



**Figure 6.** Relative luminosity at an excitation of 481 nm and an emission of 507 nm of chitin thin films cast from 1, 2, 3, and 4% chitosan casting solutions: The relative luminosity of the chitin thin-films cast from 1 and 2% chitosan casting solutions displays no observable luminosity when compared to the luminosity of natural squid pen (SP) chitin. The luminosity of the thin films were compared to the luminosity of down- (A) and up-expressed (B) S91 with the *chiA/chiB-gfp* reporter gene. S91 attached to the chitin thin-film surface (C) shows this form of chitin is able to induce the *chiA/chiB-gfp* chitinolytic genes. ND = Not Detectable.



**Figure 7.** Thickness of chitin thin films cast using different concentrations of chitosan in the spin casting solution.

from QCM mass data and profilometry data gathered directly on the quartz crystals was  $2.05 \text{ g cm}^{-3}$ . There was some variability in film thickness, which may have been dependent on environmental conditions (i.e. temperature) at the time the films are spin-cast. Preliminary results suggest film thickness may increase as much as 30-40% for every  $5^\circ\text{C}$  decrease in temperature. This is may be caused by an increase in viscosity of the chitosan solutions at lower temperatures.

The surface chemistry of the chitin thin films was evaluated using XPS. The atomic concentrations of carbon, nitrogen and oxygen of the chitin thin films were very similar to expected theoretical concentrations for pure chitin (Table 1).

**Table 1.** Atomic concentration of chitosan and chitin thin films.

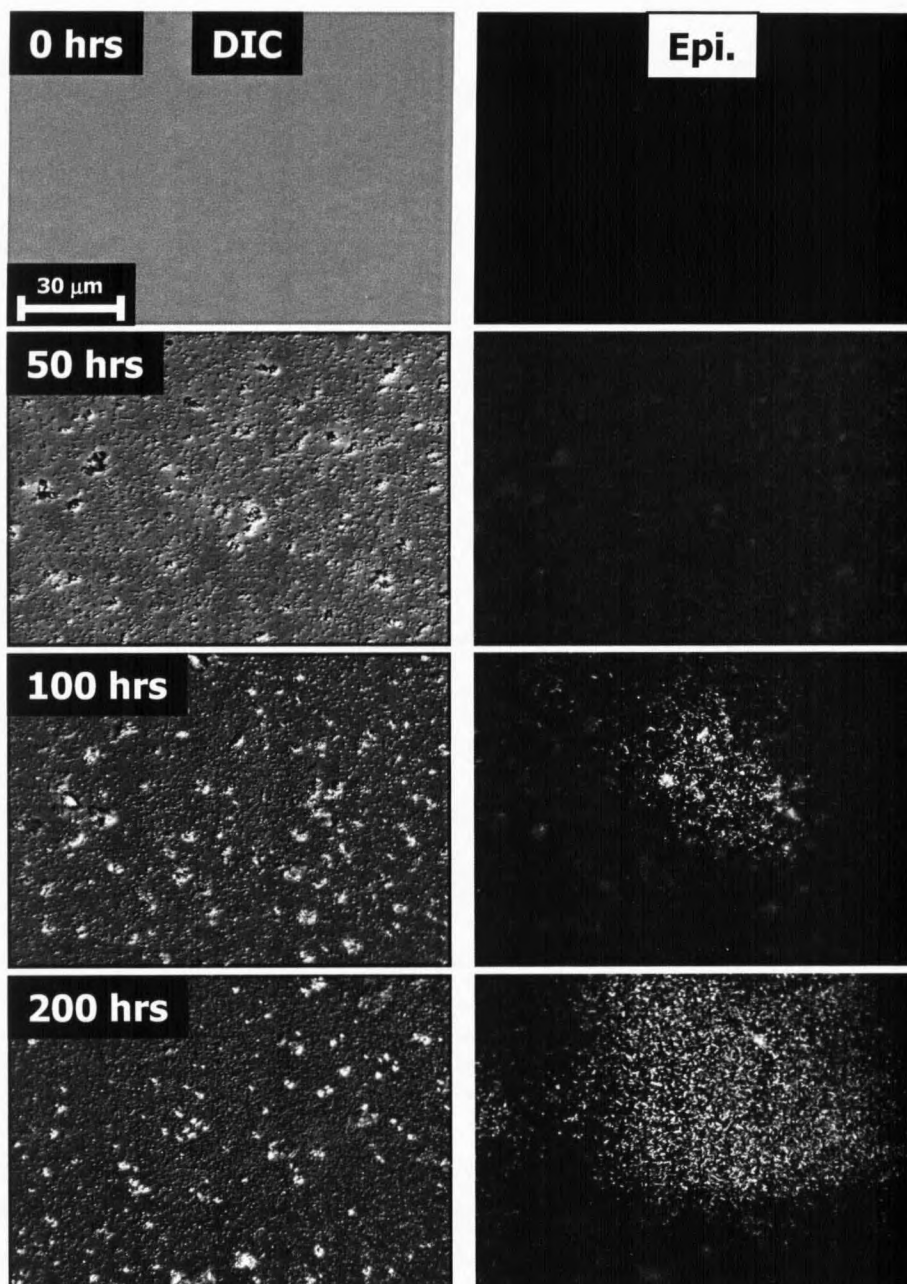
	Atomic Concentration (%)		
	Carbon	Nitrogen	Oxygen
Chitosan Thin Film	$46.19 \pm 1.12$	$5.56 \pm 0.91$	$48.25 \pm 1.01$
Chitin Thin Film	$62.19 \pm 1.23$	$6.18 \pm 0.74$	$31.63 \pm 0.89$
Chitin (Theoretical)	57.14	7.14	35.7

The difference between the actual and the theoretical concentrations for pure chitin is most likely due to ubiquitous hydrocarbon contamination of the prepared surfaces. Since these chitin thin films were synthetically prepared it was essential to determine the degree of chemical heterogeneity present within the films. The standard error in atomic concentration displayed between a total of 9 different  $30 \mu\text{m}$  spots on three separately prepared chitin thin films ranged from 0.74-1.23%. These standard errors were within

the range of anticipated reproducibility for XPS. Therefore, these atomic concentrations were representative of a chemically homogeneous surface.

The chitin thin films were placed in a LFC and colonized by the chitinolytic marine bacterium, *Pseudoalteromonas sp.* strain S91. Before inoculation of the chitin surface, the DIC image revealed that the chitin thin films were very smooth with no observable defects (Fig 8). The epifluorescence image shows no detectable UV-induced fluorescence at an excitation of 481 nm and an emission of 507 nm. At 50 hrs post inoculation, the cells had proliferated across the surface and were expressing detectable levels of GFP. At 100 and 200 hrs the attached cells displayed increased levels of *chiA/chiB* gene expression as was evidenced by the increase in GFP luminosity of the attached cells. The relative luminosity of the *chiA/chiB-gfp* up-expressed cells was equal to the luminosity of the up-expressed batch control (Fig 6). These images show that these chitin thin films can be used to detect single cell gene expression directly associated with a chitin surface.

Chitin is found in nature in three crystalline forms depending on whether the polymer chains are arranged in parallel ( $\alpha$ -form), anti-parallel ( $\beta$ -form) or a mixture of parallel and anti-parallel ( $\gamma$ -form) (Muzzarelli 1977). The  $\alpha$ -chitin form is the most abundant, followed by the  $\beta$ -chitin and  $\gamma$ -chitin. Bacteria are known to use these forms of chitin at different rates (Svitil et al. 1997). The crystal structure of the chitin thin films characterized here is unknown. Thin film x-ray diffraction was performed and the results were inconclusive. Preliminary work using Fourier Transform Infrared Spectroscopy (FT-IR) indicates these films may be of the  $\beta$ -form. However, more work is needed in



**Figure 8.** DIC and epifluorescence (Epi.) micrographs of *Pseudoalteromonas sp.* strain S91 attached to chitin thin film surfaces at 0 (un-inoculated), 50, 100, and 200 hrs post-inoculation. Epifluorescence micrographs show up-expression of *chiA/chB* chitinolytic genes reported by GFP.

characterizing these films before a crystal structure can be defined. The inability to assign a crystal structure to these chitin thin films does not impact the interpretation of results in this manuscript. However, if a natural inoculum were used instead of a pure culture, the chitin surface would most likely "select" those chitinolytic bacteria that could most efficiently degrade that specific form of chitin. Therefore, this issue must be resolved if these chitin thin films were to be used in the field.

In summary these data show that these chitin thin films can be used to detect single cells directly associated with a chitin surface. Furthermore, these thin films allow the use of fluorescent probes to evaluate single cell activity in association with the chitin surface. This approach will enable the observation and quantification of bacterial activities at the single cell level during chitin degradation.

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## CHAPTER 3

## PREPARATION OF A NEW FLUOROGENIC CHITINASE SUBSTRATE THAT YIELDS PRECIPITATES AT SITES OF ENZYME ACTIVITY

IntroductionChitin Enzymology

Chitinoclastic bacteria capable of hydrolyzing high molecular weight chitin were first reported by Zobell (1937), and were shown to be widely distributed in sediments, on the surface of plants, in the gut of marine mammals and associated with POM. These bacteria synthesize and export extracellular chitinase enzymes that can cleave the  $\beta(1-4)$  linkage of the homopolymer chitin. High molecular weight chitin is hydrolytically cleaved into its constituent carbohydrate by the concerted action of a variety of different chitinase enzymes that include N-acetylglucosaminidases, chitobiases and endo- and exochitinases (Brurberg et al 1996). The solubilization of high molecular weight chitin first begins with the breakdown of high molecular weight polymers into shorter chitin oligomers (less than five monomers in length) by extracellular endochitinase. The resulting chitin oligomers can then be acted upon by chitobiase to produce dimers of chitin that can be incorporated into the periplasm of bacteria. Finally in the periplasm these dimers are cleaved into monomers by N-acetylglucosaminidases. In addition, extracellular exochitinases cleave single sugars off the reducing ends of the polymer

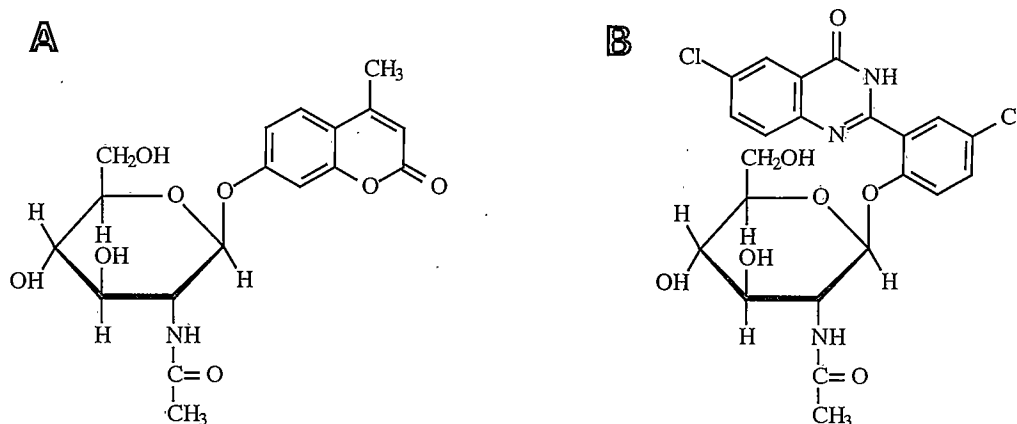
chains. Extracellular N-acetylglucosaminidase can also be secreted from most chitinoclastic bacteria to cleave small oligomers into monomers outside the cell. A single chitinolytic bacterium may express all of these enzymes in an effort to efficiently utilize high molecular weight solid chitin.

Detection of extracellular enzyme activity is essential for the analysis of biochemical processes associated the conversion of POM to DOM. It has been hypothesized that excess extracellular enzyme activity by POM associated bacterial communities supports the growth and reproduction of free living bacterial populations by releasing excess DOM (Azam and Cho 1987; Cho and Azam 1988; Azam 1998). However, the distribution of enzyme activity between attached cells, free living cells, or enzymes that may have been released from cells has never been directly observed.

#### Detection of Chitinase Activity

Traditionally, tests for chitinase activity relied on conventional plate methods utilizing colloidal chitin as a substrate (Shihamara and Takiguchi 1988). These methods employed colloidal chitin incorporated into an agar base. Zones of clearing within the agar were attributed to chitinase activity. These methods were time consuming and could only detect those bacteria that would grow on the medium selected. Currently, methods utilizing fluorogenic enzyme substrates are preferred for measuring hydrolytic enzyme activities and have been proven to be extremely sensitive (Hoppe 1991; Hoppe 1993). The most widely used fluorogenic enzyme substrates are those utilizing the 4-methylumbelliferone fluorophore (Fig 9A). These fluorogenic substrates consist of the

fluorophore, 4-methylumbelliferone (MUF) conjugated to one or more natural molecules (e.g. N-acetyl- $\beta$ -D-glucosamine for measuring N-acetylglucosaminidase activity).



**Figure 9.** (A) The fluorogenic substrates 4-methylumbelliferone-N-acetyl- $\beta$ -D-glucosaminide and (B) ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide

The fluorescence is observed after hydrolysis of the complex molecule. A variety of 4-methylumbelliferone enzyme substrates have been used to detect chitinase activity, including MUF probes conjugated to mono-, di-, tri-, and tetramers of N-acetyl- $\beta$ -D-glucosamine (GlcNAc) (Hoppe 1983; O'Brien and Colwell 1987; Robbins et al. 1988). These enzyme substrates can be used in combination to distinguish between different types of chitinases. For example, N-acetylglucosaminidase and exochitinases will cleave all of the MUF probes, while endochitinases cannot cleave MUF-GlcNAc and chitobiase only cleaves MUF-diGlcNAc (Robbins et al. 1988; Cottrell et al. 1999). However, a major limitation of these enzyme substrates is that they cannot be used to spatially resolve enzyme activity, as the fluorescent product after enzyme cleavage is soluble and readily diffuses away from the site of enzyme action. Therefore, microbial ecologists

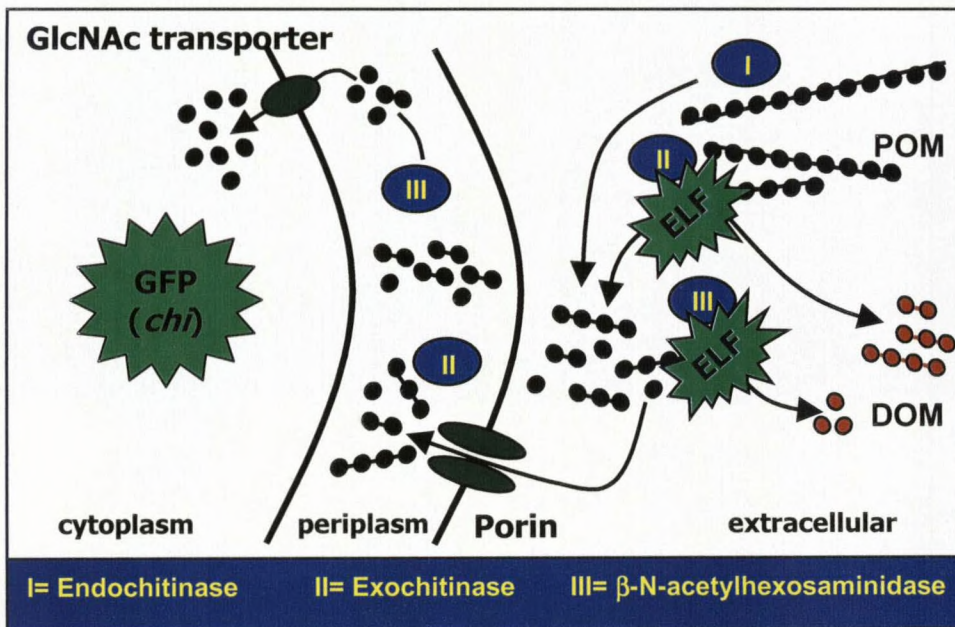
lack the appropriate tools to localize enzyme activities at the micron scale, and *in situ* measurements of enzyme activities can only be defined at the population or community level.

#### A New Enzyme Substrate to Detect Chitinase Activity

Recently, precipitating enzyme linked fluorescent (ELF<sup>®</sup>-97) substrates for phosphatase and  $\beta$ -glucuronidase activity have been used in histochemical applications for localizing enzyme activities within eukaryotic cells (Paragas et al. 1997). These ELF<sup>®</sup>-97 enzyme substrates are cell impermeable and soluble in water when conjugated to one or more natural molecules. However, the fluorescent product is insoluble in water after enzyme cleavage and crystallizes at the site of enzyme action (Haugland et al. 1991). ELF<sup>®</sup>-97- $\beta$ -glucuronidase has been used to detect and localize  $\beta$ -glucuronidase activity in solutions, gels and at the micron scale in plant tissues (Zhou et al. 1996). ELF<sup>®</sup>-97-phosphate has been used to localize the distribution of different tissue in zebra fish retina by sequentially applying primary antibody, biotinylated secondary antibody, streptavidin, biotinylated alkaline phosphatase and the ELF<sup>®</sup>-97-phosphate enzyme substrate (Larison et al. 1995). More recently, Van Omen Kloeke and Geesey (1999) used ELF<sup>®</sup>-97-phosphate in combination with fluorescence *in situ* hybridization probes (FISH) to localize phosphatase activity to specific phylogenetic groups of bacteria in an activated sludge system.

In order to spatially resolve enzyme activity at the micron scale, a new ELF<sup>®</sup>-97 enzyme substrate for chitinase activity was synthesized. This new enzyme substrate permits the detection of single cells participating in the production of extracellular enzyme during chitin degradation. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide (Fig 9B) is soluble in water, bacterial cell impermeable and reacts with  $\beta$ -N-acetylhexosaminidase and exochitinases to yield the fluorophore ELF<sup>®</sup>-97. Endochitinases are responsible for the initial solubilization of chitin, however,  $\beta$ -N-acetylhexosaminidase and exochitinases are essential in generating chitin monomers that are incorporated into the cell. Therefore,  $\beta$ -N-acetylhexosaminidase and exo-chitinase enzymes must be produced concomitantly with endochitinases to generate a usable carbon, nitrogen and energy source from high molecular weight solid chitin. Figure 10 gives a schematic representation of the chitinase activities detected by the ELF<sup>®</sup>-97 enzyme substrate.

The reaction of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide is evaluated in the presence of pure *Streptomyces griseus* chitinase enzyme and in the presence of a chitinolytic, marine bacterium containing a green fluorescent protein (*gfp*) reporter gene for visualizing chitinase gene expression. The distinct spectral properties of ELF<sup>®</sup>-97 and GFP allow simultaneous detection of chitinase activity and chitinase gene expression (Fig 10).



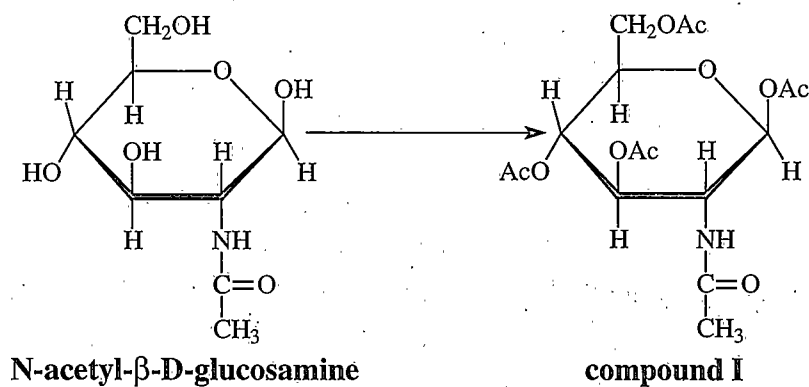
**Figure 10.** Schematic depicting the reaction of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide with  $\beta$ -N-acetylhexosaminidase and exochitinase. Sites of chitinase activity, revealed by ELF, are directly related to chitinase gene expression, using GFP, simultaneously at the single cell level.

## Materials and Methods

### Synthesis of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide

All organic chemicals were purchased from Aldrich (Milwaukee, WI), except for 5-chloro-2-aminobenzoic acid, which was purchased from Arcos Organics (Pittsburgh, PA). NMR spectra were obtained on a Bruker YLIV370.040.

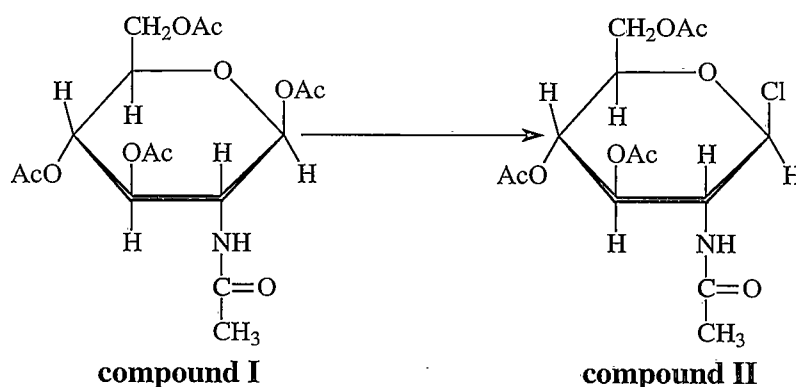
Preparation of 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-1,3,4,6-tetraacetate (Compound I). The O-acetylation and halogenation of N-acetyl- $\beta$ -D-glucosamine was performed using a modification of the methods of Inabe et al. (1984). N-acetyl- $\beta$ -D-glucosamine (20 g, 90.4 mmol) was dried and triturated and slowly dissolved in a mixture of acetic anhydride (100 mL) and concentrated sulfuric acid (13.0 mL) on ice.



The mixture was held for 24 hrs at room temperature and then poured into a cold solution of anhydrous sodium acetate (82 g, 1 mol) in water (520 mL) while stirring. The solution was then extracted with chloroform (3 x 100 mL). The chloroform extracts were combined and washed successively in ice-cold water (2 x 200 mL), ice-cold saturated

sodium bicarbonate (3 x 200 mL), and ice-cold water (2 x 200 mL). The chloroform was removed using a rotary evaporator and dried over anhydrous sodium sulfate. The product was then further dried under reduced pressure over phosphorous pentoxide. The final product, 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-1,3,4,6-tetraacetate (compound I), was then redissolved and recrystallized from ethyl acetate.

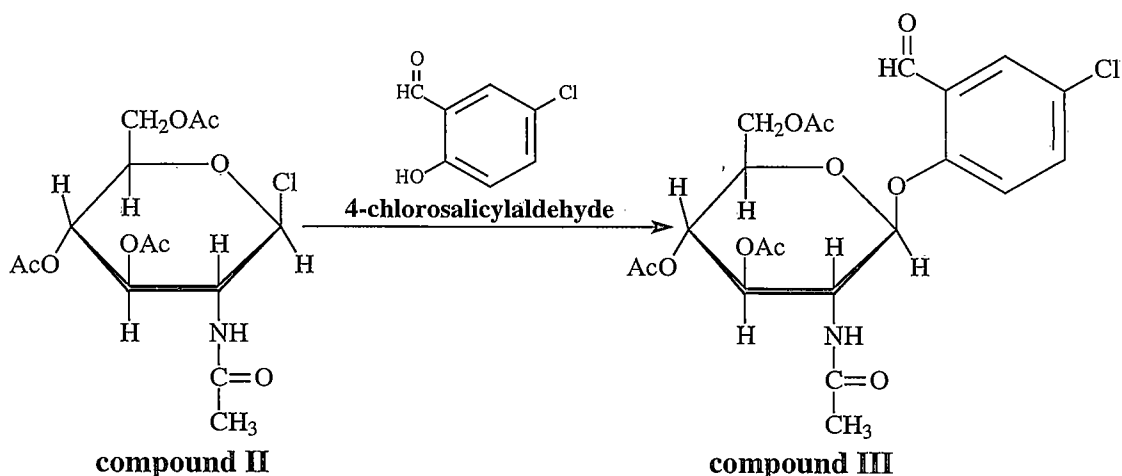
Preparation of 1-chloro-2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate (Compound II). Compound I (10.0 g, 25.6 mmol) was dissolved in a solution of acetic acid (120 mL) and acetic anhydride (1.0 mL) and cooled to 4° C.



The mixture was then saturated with dry hydrochloride gas using a fine glass air stone while stirring on ice. The reaction mixture was then tightly sealed and held at room temperature for 48 hrs while stirring. The reaction mixture was poured into chloroform (400 mL) and washed successively in ice-cold water (2 x 200 mL), ice-cold saturated sodium bicarbonate (3 x 200 mL), and ice-cold water (2 x 200 mL). The chloroform was removed with a rotary evaporator to a volume of 10-12 mL. The remaining solvent was removed under a gentle stream of dry nitrogen. The product was dried over anhydrous

sodium sulfate and then further dried under reduced pressure over phosphorous pentoxide. The final product, 1-chloro-2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate (compound II), was re-dissolved and recrystallized from chloroform-diethyl ether.

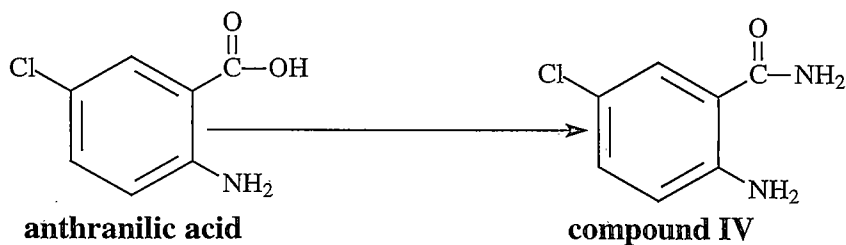
Preparation of 4-Chloro-2-formylphenyl-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate) (Compound III). Compound II (3.7 g, 10 mmol) and 4-chlorosalicylaldehyde (2.5 g, 16 mmol) were dissolved in anhydrous dichloromethane (50 mL).



To the solution were added at once  $\text{Ag}_2\text{CO}_3$  (4.4 g, 16 mmol) and 3 Å molecular sieves (5 g). 2,4,6-Collidine (2.1 mL) was added, drop-wise, with stirring. The resulting mixture was stirred in the dark, at room temperature for 4 days under dry argon protection, and then diluted with chloroform (150 mL). The mixture was filtered through a pad of Celite™ diatomaceous earth and the residue was washed with chloroform (3 × 50 mL).

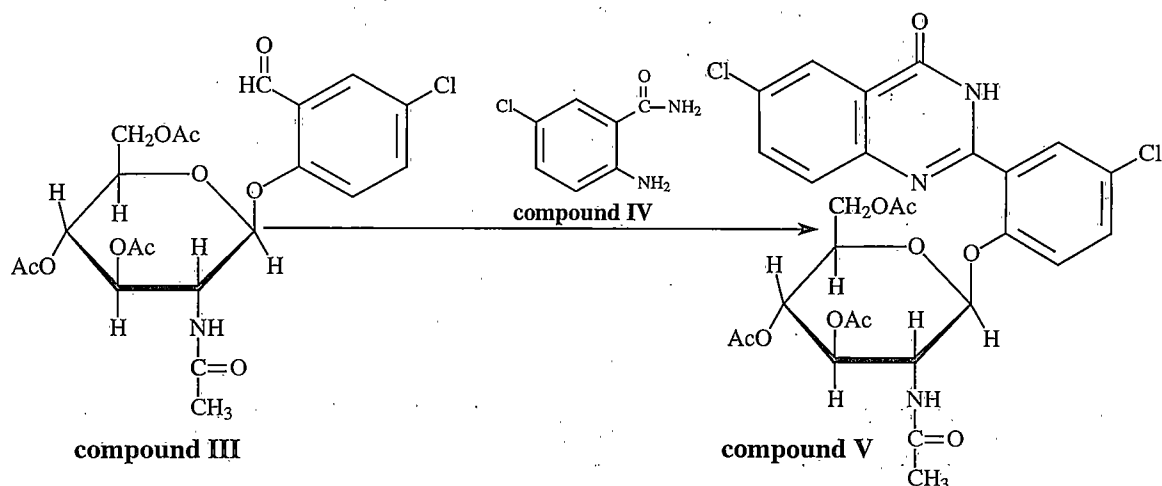
The combined filtrates were washed successively with 1 M HCl solution ( $2 \times 250$  mL), water ( $1 \times 250$  mL), 0.1 M  $\text{Na}_2\text{S}_2\text{O}_3$  solution ( $2 \times 250$  mL), saturated  $\text{NaHCO}_3$  solution ( $2 \times 250$  mL) and water ( $2 \times 250$  mL). The organic layer was dried over anhydrous  $\text{MgSO}_4$ , and evaporated *in vacuo* to give an off-white solid. The crude material was purified on a silica gel column using a gradient elution of chloroform and acetonitrile to yield the final product, 4-chloro-2-formylphenyl-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate) (compound III), a colorless solid (3.7 g, yield: 84%).

Preparation of 4-chloroanthranilamide (Compound IV). 4-chloroanthranilamide was prepared using methods described previously (Diwu et al. 1997). 5-Chloro-2-aminobenzoic acid (anthranilic acid) (5.0 g: 36.4 mmol) was dissolved in tetrahydrofuran (100 mL).



The mixture was cooled to  $0^\circ\text{C}$  and saturated with dry ammonia gas for 2 hrs while stirring. The reaction mixture was then allowed to warm to room temperature and was stirred overnight. The tetrahydrofuran was removed by rotary evaporation. The product, 4-chloroanthranilamide (compound IV), was recrystallized from toluene.

Preparation of 4-chloro-2-[2'-(6"-chloro-4(3H)-quinazolinonyl)]-phenyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate (Compound V). Compound III (741 mg, 1.7 mmol), compound IV (297 mg, 1.7 mmol) and *p*-toluenesulfonic acid (20 mg, 0.1 mmol) were dissolved in anhydrous methanol (30 mL) and stirred at room temperature for 2 hrs.

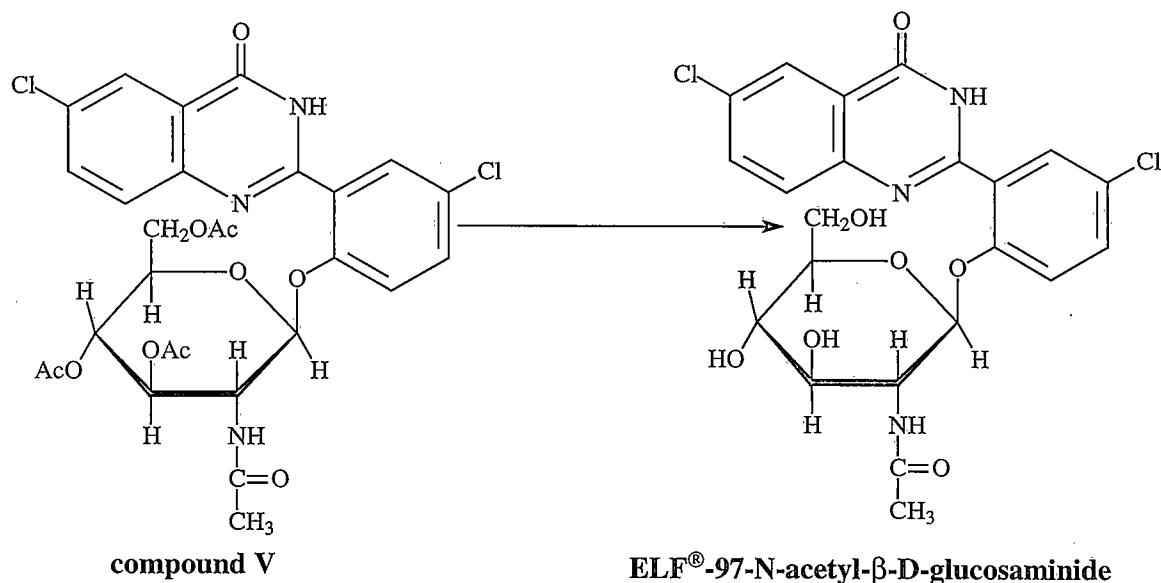


The reaction mixture was then refluxed for another hour. The resulting solution was cooled to 0 °C and 2,3-dichloro-4,5-dicyano-1,4-benzoquinone (386 mg, 1.7 mol) was added in 3 portions over the course of 30 min. The mixture was stirred at 0°C for 2 hrs, and evaporated *in vacuo* to give a brown solid. The solid was washed with benzene (5  $\times$  50 mL) to remove the hydroquinone formed during the reaction. The resulting residue was redissolved in methanol and precipitated by ether. The crude material was purified on silica gel using a gradient elution of chloroform and methanol to yield the final product, 4-chloro-2-[2'-(6"-chloro-4(3H)-quinazolinonyl)]-phenyl-2-acetamido-2

deoxy- $\beta$ -D-glucopyranose-3,4,6-tetraacetate (compound V), a colorless solid, (874 mg, yield: 76%).

Preparation of 4-Chloro-2-[2'-(6"-chloro-4(3H)-quinazolinonyl)]-phenyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranose (ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide).

Compound V was deprotected to yield the enzyme substrate ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide. Compound V (800 mg, 2.2 mmol) was dissolved in anhydrous methanol (10 mL) and cooled to 0° C.



Freshly prepared 0.1 M sodium methoxide in anhydrous methanol ( $3 \times 0.1$  mL, 0.03 mmol) was slowly added to the solution in 3 portions over 2 hrs under dry Ar protection. The solution was stirred at 0° C for 2 hrs, warmed to room temperature and stirred for 4 hrs. The reaction mixture was then diluted with methanol (50 mL), and acidified (pH 6.0) with Amberlite<sup>™</sup> IRC-50 ion exchange resin (H<sup>+</sup> form). The mixture was filtered,

the resin was washed with methanol ( $2 \times 25$  mL). The combined filtrate was evaporated *in vacuo* to give an off-white solid. The crude material was redissolved in methanol, and precipitated by ether to afford the final product, ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide, an off-white solid (1.4 g, yield: 94%); <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>): 12.20 (1H, s); 8.1 (1H, s); 7.85, 7.28 (2H, dd); 7.70, 7.47 (2H, dd); 7.60 (1H, s); 5.10 (1H, m); 5.00 (1H, m); 4.92 (1H, m); 4.60 (1H, m); 4.30 (1H, m); and 3.10-3.90 ppm (9H, m).

#### Enzymatic Hydrolysis with Pure Chitinase Enzyme

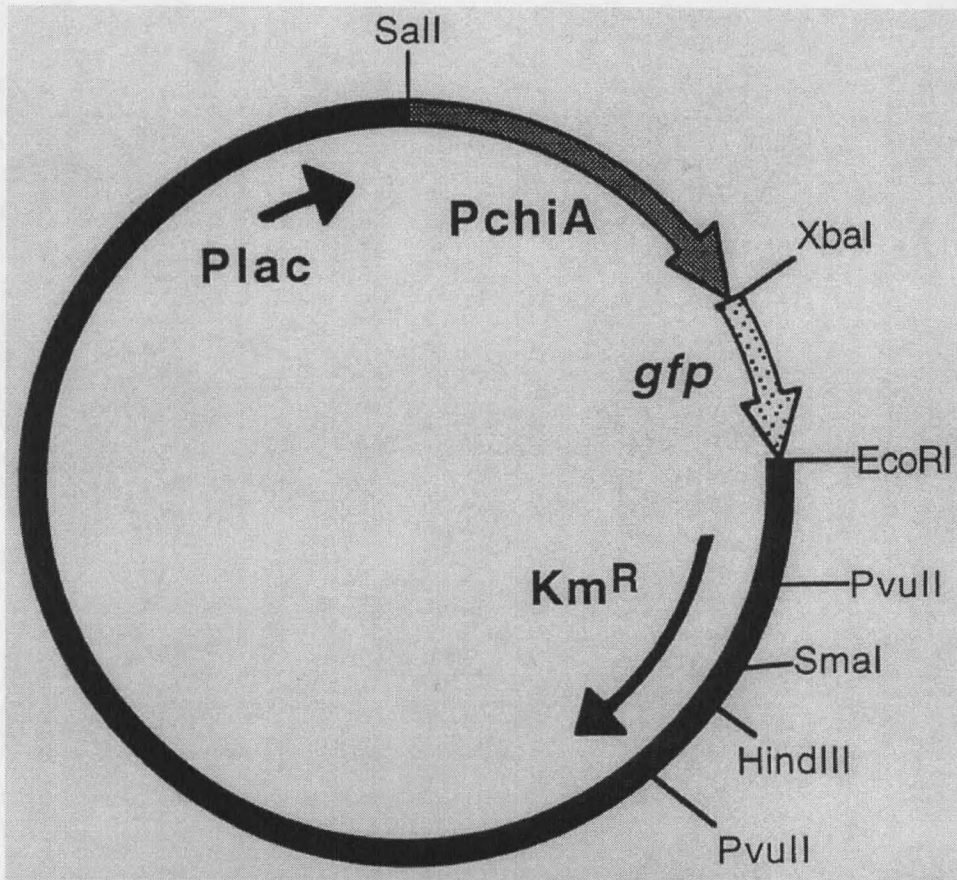
The enzymatic hydrolysis of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide to yield the fluorescent fluorophore, ELF<sup>®</sup>-97 was tested with pure *Streptomyces griseus* chitinase [EC 3.2.1.14] (Fluka, #22725). A 17.8  $\mu$ M solution (1 mg mL<sup>-1</sup> of *S. griseus* chitinase was prepared in 0.01 M Trizma HCL buffer at pH 4.4, the optimal pH for this enzyme. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was dissolved in dimethyl sulfoxide at a concentration of 10 mM. The mixture was filtered through a 0.2  $\mu$ m PTFE Millipore filter to remove any residual ELF<sup>®</sup>-97 crystals. A 50  $\mu$ M solution of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was prepared by adding a 10  $\mu$ l volume of the 10 mM stock solution to 2.0 mL of the 17.8  $\mu$ M solution of *S. griseus* chitinase. The reaction mixture was allowed to incubate at room temperature for 1 hr. Precipitated ELF<sup>®</sup>-97 was separated from the reaction mixture using a Sorvall RC-5B centrifuge at 16,000 g for 10 min then washed 3x in sterile water. The precipitated ELF<sup>®</sup>-97 crystals were then resuspended in sterile water and a 20  $\mu$ l drop was placed on a microscope slide. Fluorescence of

ELF<sup>®</sup>-97 was observed using at an excitation of 360 nm and an emission of 540 nm using an Olympus U-MWU filter set with an excitation filter of 330-385 nm, a dichroic mirror at 400 nm and a barrier filter at 420 nm. Images were acquired using an Olympus B-max 60 microscope as described previously.

### Bacterial Cultivation in a Flowing Seawater System

Enzymatic hydrolysis of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide to yield the fluorophore, ELF<sup>®</sup>-97, was tested with whole cells of the chitinolytic marine bacterium *Pseudoalteromonas* sp. strain S91. A green fluorescent protein reporter gene (*gfp*) was used to visualize chitinase gene expression of S91 and chitinase activity was visualized using the ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide enzyme substrate, during the degradation of solid chitin. The gene for *gfp* was under control of the *chiA* promoter on a plasmid in the bacterium and a fully functional *chiA* gene resided on the chromosome (Fig 11) (Stretton and Goodman 1998; Techkarnjanaruk and Goodman 1999).

Teflon<sup>®</sup> laminar flow cells (LFCs) with glass viewing ports were used for direct microscopic examination of surface associated S91 without disturbing the flowing system (Davies and Geesey 1995). Three separate LFCs were used to monitor surface associated *chiA* gene and ELF<sup>®</sup>-97 activity. The first LFC contained two chitin thin films cast onto silicon coupons. The second LFC contained clean silicon coupons and served as nonnutritional control surfaces, and the third LFC contained clean silicon coupons and was used as an abiotic control. The chitin and silicon LFCs were simultaneously



**Figure 11.** Diagrammatic representation showing construction of the plasmid containing the *chiA-gfp* reporter gene (Techkarnjanaruk 1998).

inoculated with a 400 hr starved population of S91 for a period of 1 hr at a flow rate of  $0.5 \text{ cm}^3 \text{ min}^{-1}$ . The abiotic control was inoculated with sterile defined seawater. After the inoculation, the bulk aqueous phase flowing through the flow cells was switched to sterile defined seawater as previously described (Chapter 2). The LFCs and the sterile defined seawater were maintained at  $20^\circ\text{C}$  throughout the experiment. The flow rate was maintained at  $0.5 \text{ cm}^3 \text{ min}^{-1}$  allowing the growth of S91 on solid chitin and the silicon control surface for a period of 100 hrs.

#### Visualizing Chitinase Active Sites with ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide

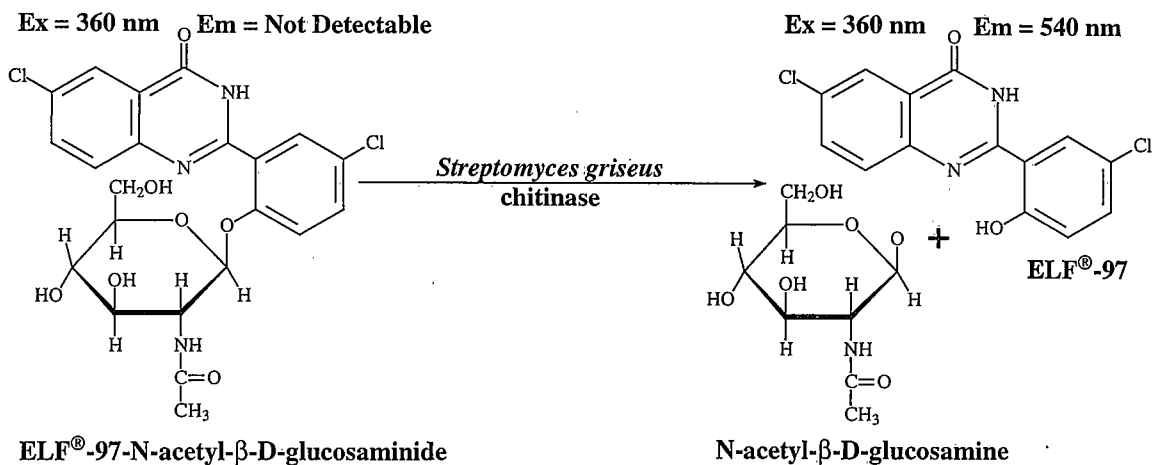
At 100 hrs post-inoculation the LFCs were incubated with ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide for a period of 1 hr in an effort to spatially resolve chitinase activity using ELF<sup>®</sup>-97 fluorescence. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was dissolved in dimethyl sulfoxide at a concentration of 10 mM. A 50  $\mu\text{M}$  solution of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was prepared by adding a 10  $\mu\text{l}$  volume of the 10 mM stock solution to 2.0 mL of defined seawater. The mixture was filtered through a 0.2  $\mu\text{m}$  PTFE Millipore filter to remove any residual ELF<sup>®</sup>-97 crystals and injected into the LFCs. The LFCs were allowed to incubate in the dark at  $20^\circ\text{C}$  for 1 hr. The ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide-seawater solution was then rinsed out of the flow cells and the ELF<sup>®</sup>-97 activity was assessed using epifluorescence microscopy and image analysis.

At 100 hrs post-inoculation total cells, *chiA* up-expressed cells and sites of ELF<sup>®</sup>-97 activity were directly detected and enumerated on both the chitin and silicon surfaces. Images were acquired using an Olympus B-max 60 microscope as described previously (Chapter 2). Fluorescent images of *chiA-gfp* reporter gene expression were acquired using an excitation of 481 nm and an emission of 507 nm. Fluorescence images of ELF<sup>®</sup>-97 were acquired at an excitation of 360 nm and an emission of 540 nm using the previously described filter set. All images were analyzed with Image-Pro Plus<sup>™</sup> software (Media Cybernetics, Silver Springs, MD). The only image manipulation performed on the DIC images was a background correction and in a few cases minor adjustment of the gray-scale contrast. Enzyme activity was assessed by measuring the relative luminosity of the precipitated ELF<sup>®</sup>-97 fluorophore in the same field of view, every 2 min for the first 20 min, and then at 1 hr post-innoculation of the enzyme substrate. The images of *gfp* expression were pseudocolored green and the images of ELF<sup>®</sup>-97 activation were pseudocolored red. Total cells, *chiA* up-expressed cells and sites of ELF<sup>®</sup>-97 activity are presented as triple image overlays.

### Results and Discussion

The enzymatic hydrolysis of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide with pure *Streptomyces griseus* chitinase yielded a bright yellow-green precipitate with the excitation (360 nm) and emission (540 nm) wavelengths of the fluorophore, 2-[2'-hydroxy-5'-chlorophenyl)-6-chloro-4(3H)-quinazolinone) or ELF<sup>®</sup>-97 (Fig 12) (Larison

et al. 1995). The excitation spectrum of ELF<sup>®</sup>-97 was distinct from the excitation spectrum used to excite GFP. This enabled the simultaneous use of a *gfp* reporter gene to detect *chiA* up-expressed cells and the ELF<sup>®</sup>-97 enzyme substrate to detect chitinase activity. The enzymatic hydrolysis of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide by intact *chiA* up-expressed cells of *Pseudalteromonas sp.* strain S91 also yielded a bright yellow-green precipitate with the fluorescence properties of the fluorophore, ELF<sup>®</sup>-97.

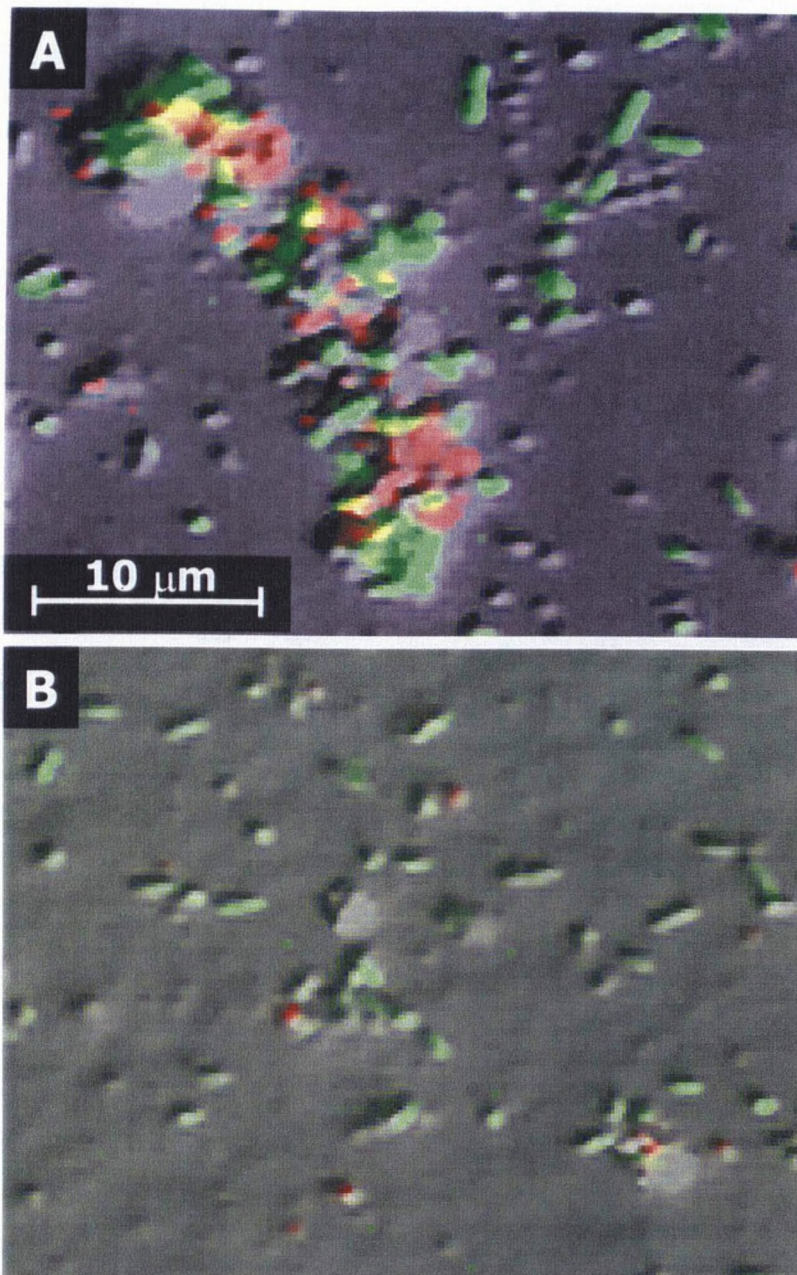


**Figure 12.** (A) Reaction of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide with chitinase enzyme yields the ELF<sup>®</sup>-97 fluorophore and N-acetyl- $\beta$ -D-glucosamine.

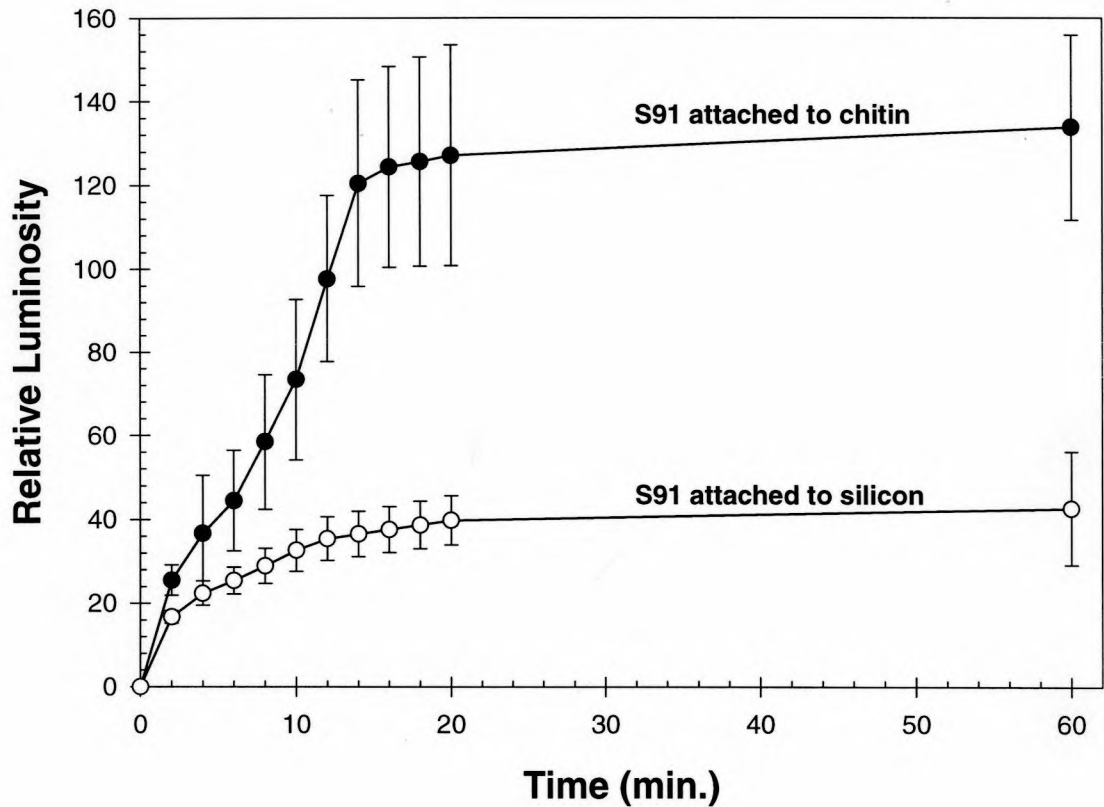
To better define the relationship between total cells and cells actively involved in chitin degradation, *chiA* up-expressed cells and sites of chitinase activity were evaluated at the single cell level, in the same field of view using image overlays (Fig 13). These images show chitinase activity was closely linked to *chiA* up-expressed cells on the chitin surface. Chitinase activity appeared directly associated with *chiA* up-expressed cells or

directly adjacent to *chiA* up-expressed cells. However, not all cells that were *chiA* up-expressed produced active chitinase. In contrast, cells attached to a silicon surface displayed little chitinase activity when compared to cells attached to the chitin surface.

It is difficult to quantify enzyme activity based on ELF<sup>®</sup>-97 fluorescence due to the precipitating nature of the probe. However, enzyme activity can be assessed semi-quantitatively based on luminosity of the surface as ELF<sup>®</sup>-97 accumulates as a result of enzyme activity. Epifluorescence microscopy and image analysis were used to monitor the relative rate of conversion of the enzyme substrate to ELF<sup>®</sup>-97 by measuring the relative luminosity of the enzyme active sites on chitin, silicon and the abiotic silicon control (Fig 14). These data show that enzyme activity can be detected in as little as two minutes within a surface associated population of S91 actively involved in chitin degradation. Sites of ELF<sup>®</sup>-97 accumulated over the course of 20 min, as depicted by the increase in relative luminosity of the surface. In comparison a chitin-free silicon surface colonized by the same bacteria showed little chitinase activity. The reactivity of the ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide chitinase substrate then slowed considerably at 20 min post inoculation of the enzyme substrate. It is uncertain why this sharp decrease was suddenly observed. It was possible that the precipitating enzyme substrate is forming a physical barrier around the active site of the chitinase as ELF<sup>®</sup>-97 accumulates. This would limit the mass transport of substrate to the enzyme, inhibit enzyme activity, and prevent any further accumulation of ELF<sup>®</sup>-97. However, this most likely aids in the resolution of enzyme activity, since enzyme that continued to generate ELF<sup>®</sup>-97 would



**Figure 13.** DIC, epifluorescence image overlays showing *chiA* up-expression and ELF<sup>®</sup>-97 activity associated with *Pseudoalteromonas sp.* strain S91 on chitin (A) and silicon (B) surfaces. *chiA* up-expression is depicted in green and sites of enzyme activity, as reported by the ELF<sup>®</sup>-97 enzyme product are depicted in red.



**Figure 14.** Reaction of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide with attached *Pseudoalteromonas sp.* strain S91 on chitin and silicon surfaces. The reaction was monitored using epifluorescence microscopy and image analysis by measuring the relative luminescence of ELF<sup>®</sup>-97 following hydrolysis of the enzyme substrate. No ELF<sup>®</sup>-97 crystal formation was observed in the abiotic control.

accumulate over the surface to the extent that it is no longer possible to achieve single cell resolution of enzyme activity.

Another explanation for the decrease in enzymatic activity may be due to DMSO inactivation of the enzyme. The final concentration of DMSO was 0.05% during the incubation. This possibility could be addressed in a separate experiment by assessing the effect increasing concentrations of DMSO has on pure chitinase enzyme using the MUF-GlcNAc enzyme substrate and measuring the enzyme reaction spectrophotometrically. However, due to the limited solubility of the ELF<sup>®</sup>-97 enzyme substrate in aqueous solution, some amount of DMSO (or a similar organic solvent) must be used to solubilize the substrate.

In summary, the synthesis of a new ELF<sup>®</sup>-97 enzyme substrate for chitinase activity enables the detection of cells participating in the production of active extracellular enzyme during chitin degradation. This offers a new tool for evaluating extracellular chitinase enzyme activity at the micron scale.

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## CHAPTER 4

SPATIAL AND TEMPORAL VARIATIONS IN CHITINOLYTIC GENE  
EXPRESSION AND BACTERIAL BIOMASS PRODUCTION DURING CHITIN  
DEGRADATIONIntroduction

The enzymatic release of dissolved organic matter (DOM) from particulate organic matter (POM) is the first step in recycling high molecular weight biopolymers into living bacterial biomass (Vetter and Deming 1999). It is hypothesized that DOM released by the action of extracellular hydrolytic enzymes, produced by POM associated bacteria, supports maintenance and reproduction of free-living bacteria in the pelagic marine environment (Cho and Azam 1988; Azam 1998). POM associated bacteria are thought to participate more in the synthesis of extra cellular enzymes that mediate the release of DOM (Karner and Herndl 1992; Smith et al. 1992), and less in the direct production of bacterial biomass than free living bacterial populations (Azam and Hodson 1977; Simon et al. 1990; Griffith et al. 1994; Turley and Mackie 1994). However, Jacobsen and Azam (1984) recognized that bacteria associated with copepod fecal pellets were displaced into the surrounding water, resulting in the production of free living bacteria derived directly from attached populations. While these researchers recognized the potential importance of detachment as a process that contributes to free living

bacterial production (BP), the magnitude of this process is currently unknown in the pelagic marine environment.

In order to quantify bacterial detachment from the surface of POM, bacteria derived from attached populations must be distinguished from those derived from free living populations. However, when both attached populations and free living populations are reproducing in the same system it is difficult to distinguish free living cells generated on surfaces from those generated in the bulk aqueous phase. Detachment can be directly quantified in flowing systems where cells that detach into the bulk aqueous phase are removed faster than they can reproduce, thus, eliminating free living BP from the system. Total BP can then be directly evaluated by enumerating those bacteria remaining on the surface and those that are displaced into the bulk aqueous phase.

Natural particle surfaces possess properties that preclude the nondestructive visualization of single cells over time. Chitin, a dominant form of particulate carbon, nitrogen and energy in the pelagic marine environment, displays surface roughness that generally prevents the visualization of single cells. In addition, chitin fluoresces under epi-illumination preventing the use of fluorogenic compounds that are used to visualize individual cells. In this study, surfaces comprised of pure chitin thin films were used to overcome the obstacles associated with natural chitin surfaces.

Chitin is a high molecular weight biopolymer of  $\beta$ -1,4-linked N-acetyl-D-glucosamine and is used by organisms that can produce essential extracellular chitinase enzymes. Bacteria are the principle mediators of chitin degradation (Gooday 1990), and since chitin is an insoluble biopolymer, the bacterial populations that contribute to the

dissolution of this material are primarily surface associated. However, activities of chitin associated bacteria are difficult to evaluate nondestructively, in real time at the cellular level. In order to link chitin degradation to chitin associated BP, a green fluorescent protein (GFP) reporter was used to identify those cells expressing chitinase genes. This approach led to the discovery of bacterial heterogeneity in chitinase gene expression within a population of cells attached to a chitin surface and the importance of detachment during chitin associated BP to total BP in the system.

### Materials and Methods

#### Bacterial Strains and Media

A green fluorescent protein reporter gene (*gfp*) was used to visualize chitinase gene expression of *Pseudoalteromonas* sp. strain S91, during the degradation of solid chitin. Strain S91 was derived from the wild type strain S9, a chitinolytic marine bacterium isolated from the surface waters of Botany Bay, New South Wales, Australia in 1981 (Humphrey et al. 1983). Strain S91 contains plasmid pDSK519 with a complete chitinase gene (*chiA*) and a truncated chitin binding gene (*chiB*) (Chapter 2) (Keen et al. 1988). Strain S91 is streptomycin (Sm) resistant and the plasmid confers kanamycin (Km) resistance. The *gfp* gene is under control of both the *chiA/chiB*, promoters and a set of fully functional genes resides on the chromosome (Stretton et al. 1998; Techkarnjanaruk et al. 1999). The expression of these chitinase genes is essential for utilization of high molecular weight chitin as a carbon, nitrogen and energy source

(Techkarnjanaruk et al. 1997). The *gfp* gene is the GFPmut2 derivative of the wild type gene and confers a 30-fold increase in GFP fluorescence intensity (Cormack et al. 1996).

### Substratum Preparation

The physical complexity and chemical heterogeneity found in natural detrital POM was minimized, in this system, by using a substratum consisting of the natural biopolymer chitin. Thin films of pure chitin and natural squid pen chitin served as the only added sources of carbon, nitrogen and energy during chitin degradation. Silicon coupons, 1 cm x 1 cm square and 1.5 mm thick (Harrick Scientific, Ossining, NY), were used as nonnutritional controls. The methods used in the preparation of pure chitin thin films were adapted from previously published methods for spin-casting chitin films from chitosan solutions (Bae and Hudson 1997; Rathke and Hudson 1994; Qin 1993). Briefly, the thin films were cast onto the silicon substrates at 4500 rpm from a 1.5% solution of high molecular weight chitosan (Aldrich, Milwaukee, WI) in an aqueous solution of 2.0% acetic acid. These thin films of chitosan were N-acetylated to chitin using a 20% solution of acetic anhydride in methanol for 18 hrs at 4°C. The N-acetylation of the chitosan film was monitored using Fourier transform, infrared spectroscopy (FT IR) and the chemical purity and homogeneity was assessed with small spot x-ray photoelectron spectroscopy (XPS) (data not shown). Film thickness and surface coverage was assessed using profilometry. The average dry density of these films calculated from quartz crystal microbalance (QCM) mass data and profilometry data was 2.05 g cm<sup>-3</sup>. The exact thickness of the thin films used is uncertain, as there was some variability that was

dependent on environmental conditions (temperature and humidity) at the time the films were spin-cast. However, these chitin thin films are approximately 230 nm thick. These chitin thin films were ultra smooth, continuous, and nonfluorescent. These physical and chemical properties permitted spatial quantification of attached bacteria at the single cell level during chitin degradation. For experiments involving natural squid pen chitin the pen from a *Loligo* sp. squid was used as the substrate. Squid pens were dissected and stored at  $-40^{\circ}\text{C}$ . Prior to use, the squid pens were sterilized by autoclave, in artificial seawater, cut into 1 cm x 1cm squares and then rinsed in 70 % ethanol and placed in the flow cells. Squid pen chitin consists of approximately 40% chitin and 60% protein (Gooday 1990).

#### Media Preparation

*Pseudoalteromonas* sp. strain S91 was grown in a defined seawater medium. The defined seawater consisted of 201.1 mM NaCl, 4.8 mM  $\text{H}_3\text{BO}_4$ , 27.5 mM  $\text{Na}_2\text{SO}_4$ , 2.4 mM  $\text{NaHCO}_3$ , 88.5 mM KCl, 8.4 mM KBr, 54.1 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 1.5 mM  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$  suspended in 0.05M Sigma 7-9 buffer using once distilled Millipore water and adjusted to pH 8.0. Following autoclave sterilization, 0.008 mM  $\text{FeCl}_3$ , 0.04 mM  $\text{K}_2\text{HPO}_4$ , 2.0 mM  $\text{CaCl}_2$  were each added separately by filter sterilization through a 0.2  $\mu\text{m}$  pore size Millipore syringe filter. To insure retention of the plasmid by the bacterial cells Km was added to a concentration of  $600 \mu\text{g mL}^{-1}$ . In an effort to minimize contamination of the culture during long term starvation and flow cell experiments, Sm was added to a concentration of  $200 \mu\text{g mL}^{-1}$ . The defined seawater was used without

any added carbon, nitrogen or energy sources for the starvation of S91. For the growth of S91, when the induction of the *chiA/chiB* genes was desired, 0.05M N-acetyl-D-glucosamine was added to the defined seawater as the sole carbon, nitrogen and energy source. Growth of S91 under conditions of *chiA/chiB* down-expression was achieved by supplementing the defined seawater with 0.05M glutamic acid as the sole carbon, nitrogen and energy source.

### Bacterial Cultivation

Stock cultures of S91 were grown to exponential phase in MB2216 (Difco, Detroit, MI). Approximately 5  $\mu$ L of this culture was used to inoculate 50 mL of the defined seawater medium supplemented with glutamic acid as the sole carbon and nitrogen source. At a culturable cell density of approximately  $6.0 \times 10^7$  cfu mL<sup>-1</sup> the cells were pelleted in a Sorvall RC-5B centrifuge at 16,000 g for 10 min then washed and repelleted 3 times in the defined seawater starvation media. The cells were resuspended in 10 mL of defined seawater and added to 1.0 L of defined seawater in an air sparged, continuously stirred tank reactor (CSTR) maintained at 18°C. The defined seawater was supplemented with Km and Sm but lacked added utilizable carbon, nitrogen and energy sources. The CSTRs (Knick Machining, Bozeman, MT) were constructed of 1.0 L fluted glass reaction kettles (Lab Glass, Buena, NJ) and 304L stainless steel headplates. All reactor ports were constructed of Swagelock<sup>®</sup> gas fittings with Teflon<sup>®</sup> ferrules (Idaho valve and Fitting, Idaho Falls, Idaho). To determine the number of culturable cells in the

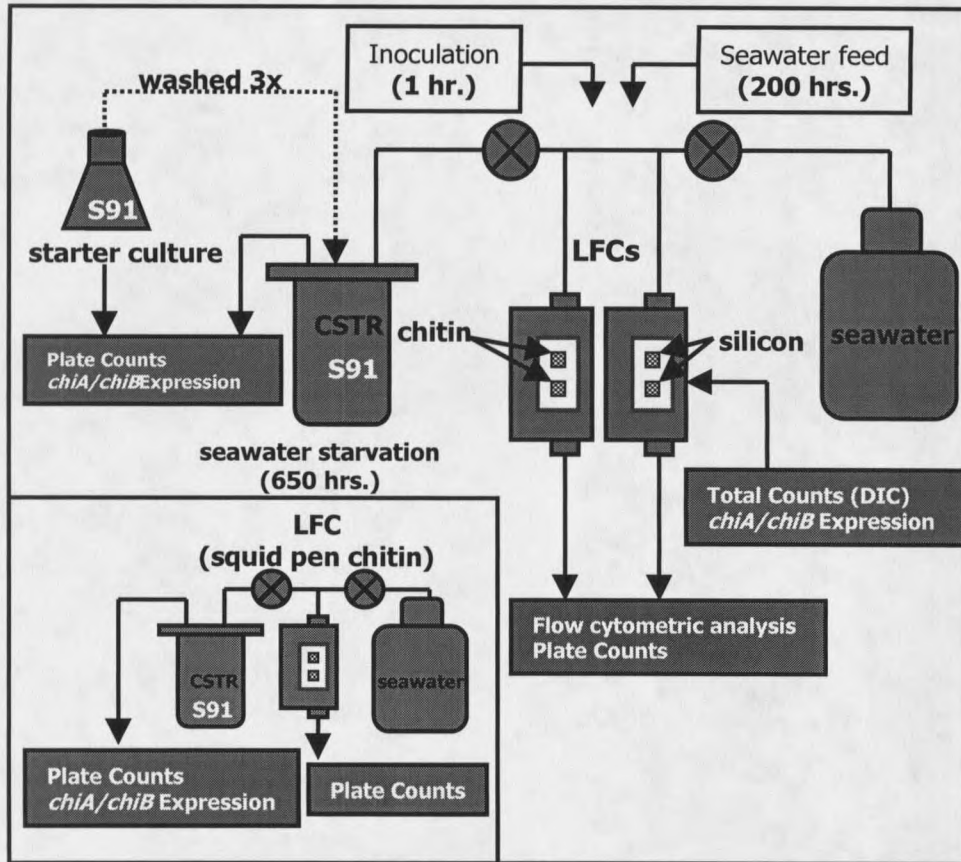
starved cell suspension, samples were withdrawn from the CSTR every 24 hrs and culturability was assessed using plate counts on MB2216 agar (Difco, Detroit, MI).

### Flow Cell Operation

Flow cell experiments were conducted to observe bacterial attachment, reproduction and detachment during chitin degradation under a constant seawater flow. Teflon<sup>®</sup> laminar flow cells (LFCs) with glass viewing ports were used to allow direct microscopic examination of the bacteria associated with the chitin and silicon surfaces over time. The LFCs were of a modified design to that used previously to monitor reporter gene expression on surfaces (Davies and Geesey 1995). The LFCs were constructed of virgin Teflon<sup>®</sup> (McMaster Carr, Los Angeles, CA) and had a flow channel that was 0.8 mm deep, 12 mm wide and 48 mm in length (Knick Machining, Bozeman, MT). Two 1 cm x 1 cm squares were recessed, in series, into the floor of each LFC to allow the placement of silicon, chitin or squid pen chitin coupons. A #2 coverslip (24 x 60 mm) was used as the viewing window and was sealed against the Teflon<sup>®</sup> using an oversized viton gasket and an aluminum coverplate. Teflon<sup>®</sup> influent and effluent lines were connected with Chemflour<sup>®</sup> PTFE gas fittings (Cole Palmer, Vernon Hills, IL). The LFCs were connected with platinum cured silicon tubing (Cole Palmer, Vernon Hills, IL) to both the air sparged CSTR and a 20 L carboy that contained defined seawater (Fig 15). The 20 L carboy, Teflon LFCs, glass cover slips and silicon coupons were cleaned in "Pirhana" which consisted of a 70:30 mixture of concentrated sulfuric acid and 30.0%

hydrogen peroxide, rinsed in double distilled water (ddH<sub>2</sub>O), and baked dry at 120°C prior to assembly of the system [**WARNING:** Piranha solution reacts violently, even explosively with organic materials (Dobbs et al. 1990)]. All influent lines were cleaned by flushing with 95% ethanol at a flow rate of 1 cm<sup>3</sup> min<sup>-1</sup> for 60 min, followed by ddH<sub>2</sub>O to remove residual organic carbon on the surface of the tubing. Three separate LFCs were used to monitor surface associated bacterial activity. The first LFC contained two chitin thin films cast onto silicon coupons. This LFC was used to evaluate bacterial activity during the degradation of pure chitin. The second LFC contained clean silicon coupons and served as nonnutritional control surfaces, and a third LFC contained natural squid pen chitin. Any deviation from the cleaning procedure described above resulted in increased growth in the silicon control LFC. Glass flow breaks placed in line between the media feed and the LFCs prevented back contamination of the sterile seawater feed. All influent lines were fed through a Buchler 12 roller Multistatic<sup>®</sup> pump (Cole Palmer, Vernon Hills, IL) for inoculation with the starved cell suspension of S91 and subsequent delivery of the sterile seawater feed.

The chitin and silicon LFCs were simultaneously inoculated with a 400 hr starved population from the CSTR at a cell density of  $3.2 \times 10^6$  cfu cm<sup>-3</sup>. The squid pen chitin LFC was inoculated with a second 400 hr starved population at a cell density of  $2.3 \times 10^6$  cfu cm<sup>-3</sup>. All three LFCs were inoculated for a period of 1 hr at a flow rate of 0.5 cm<sup>3</sup> min<sup>-1</sup>. After the inoculation, the bulk aqueous phase flowing through the flow cells was switched to the sterile defined seawater containing Km and Sm but no added carbon, nitrogen or energy sources. The LFCs and the sterile defined seawater were maintained



**Figure 15.** Schematic representation of the experimental design showing: laminar flow cells (LFCs), continuously stirred tank reactor (CSTR), peristaltic pumps ( $\otimes$ ). The silicon and chitin LFCs were run in tandem and the LFC containing the squid pen chitin was run independently.

at 18°C throughout the experiment. The flow rate was maintained at 0.5 cm<sup>3</sup> min<sup>-1</sup> for the remaining 200 hrs of the experiment, providing the surface associated cells with a continuous seawater flow.

### Microscopy and Image Analysis

Total cell densities during attachment and reproduction of S91 on the pure chitin and silicon surfaces were quantified over a period of 200 hrs, following inoculation of the surfaces. Images were acquired using an Olympus B-max 60 microscope (Olympus Optical Co., Tokyo, Japan) with both reflected differential interference contrast (DIC) and epifluorescence optics. All images were acquired using a Nikon infinity corrected, 40x, water immersion objective (Nikon Inc., Torrance, CA) and a mercury lamp (Chiu Technical Corporation, Kings Park, NY). Digital images were gathered using a Photometrics Imagepoint™ cooled CCD camera (Photometrics, Tucson, Az). The LFCs were imaged every 10 min during inoculation of the coupon surfaces with the starvation culture for a period of 1 hr. Following the switch to the sterile seawater feed, the surfaces in each LFC were imaged at 1, 2, 3, 5, 7 and 11 hrs, and every 12 hrs for the remainder of the 200 hr experiment. A minimum of six random fields on both coupons in each flow cell were imaged using both DIC and epifluorescence microscopy for every time point. Fluorescence images of *chiA/chiB-gfp* reporter gene expression were acquired using an excitation of 481 nm and an emission of 507 nm (Cormack et al. 1996). In an effort to compare relative fluorescence intensities between images, all fluorescence images were acquired at an exposure of 6 seconds using a gain of 4 and analyzed with Image-Pro

Plus™ software (Media Cybernetics, Silver Springs, MD). The only image manipulation performed on the DIC images was a background correction and in a few cases minor adjustment of the gray scale contrast. No image manipulations were performed on the epifluorescence images. The software was used to count individual cells in all images using manually adjusted threshold values for each individual image. The intensity of GFP fluorescence was measured using Image-Pro Plus software™ by measuring the relative luminosity of *chiA/chiB-gfp* expressing cells in each image.

#### Flow Cytometric Analysis

The *chiA/chiB-gfp* expression of cells that detached from the surfaces over time was quantified using flow cytometric analysis. LFC effluent samples were gathered at the same time points that the coupons were imaged and analyzed using a Becton Dickinson FACSCalibur™. A maximum of 50,000 counts were acquired for each sample analyzed and *chiA/chiB-gfp* expression was monitored at an excitation of 481 nm and an emission of 507 nm. Batch cultures of S91 grown on glutamic acid and N-acetyl- $\beta$ -D-glucosamine were used as controls to assess down- and up-expression of *chiA/chiB-gfp*, respectively.

#### Surface Associated Growth Rates

Growth rates for the surface associated bacteria were calculated for two time periods during the LFC experiments. The sum of the total cells on the surface,

enumerated by DIC microscopy, and the total  $\text{cfu cm}^{-3}$  that detached into the effluent, enumerated by plate counts, were used to calculate the total specific growth rates of the surface associated population. Since no aggregations of cells were observed in effluent samples it was assumed that the culturable cell counts, in  $\text{cfu cm}^{-3}$ , were equivalent to actual cells  $\text{cm}^{-3}$ . The first specific growth rate was calculated for the time period coincident with the initial proliferation of the attached bacteria between 20 and 70 hrs post-inoculation. The second specific growth rate was calculated between 100 and 200 hrs post-inoculation. Specific growth rates associated with the squid pen chitin surface could not be calculated because the total cells on the surface could not be accurately enumerated as a result of excessive surface roughness and auto-fluorescence of the natural substratum.

#### Detachment Rates and Bacterial Production

Total bacterial biomass generated by the surface associated populations was calculated by integrating the total cells throughout the 200 hr experiment. Total cells for each time point were calculated by adding the cells on the surface to the cells that detached into the bulk aqueous phase since the last evaluation period. BP was calculated by multiplying the average carbon biomass ( $\text{mg C cell}^{-1}$ ) and the total integrated rate of biomass accumulation ( $\text{cells cm}^{-3} \text{ min}^{-1}$ ). The units were converted to report BP as  $\text{mg C m}^{-3} \text{ d}^{-1}$ . The estimated carbon biomass per cell was calculated using the average volume of the cells and previously published equations. Images were acquired at 1000x and resolved with 32 bits of memory per pixel. The length and width of forty randomly

chosen bacteria in each of five images ( $n = 200$ ) were measured in order to obtain an average length and width for all surface associated bacteria. Likewise, the length and width of bacteria that detached into the effluent were also measured. The conversion to cell volume was calculated based on the equation [1],

$$v = \left( w^2 \cdot \frac{\pi}{4} \right) \cdot (l - w) + \left( \pi \cdot \frac{w^3}{6} \right) \quad [1]$$

where  $v$  is volume and  $l$  and  $w$  are length and width respectively (Felip et al. 1995).

Equation [2] was used to equate biovolume to carbon biomass,

$$C = 0.12 \cdot V^{0.72} \quad [2]$$

where  $v$  is the volume in  $\mu\text{m}^3$  and  $C$  is in picograms of carbon per cell (Norland 1993).

The detachment rates of the attached bacteria were calculated as the number of culturable cells displaced into the effluent of the flow cells per  $\text{cm}^3$  per min ( $\text{cfu cm}^{-3} \text{min}^{-1}$ ). The percentage of the total population that detached over time was calculated by dividing the free living cells that accumulated in the effluent by the total bacteria generated by the surface associated population (surface associated + free living).

### Flow Cell Hydrodynamics

In order to characterize the flow conditions experienced by the attached bacteria; the residence time, average fluid velocity, and Reynolds number (Re) were calculated from the flow rate and flow cell geometry. Re was calculated based on relationship [4],

$$\text{Re} = \left( \frac{D_e \cdot V \cdot \rho}{\mu} \right) \quad [4]$$

where  $D_e$  is a characteristic length that depends on the system geometry,  $V$  is the fluid velocity,  $\rho$  is the density of the fluid and  $\mu$  is the absolute viscosity (Lindeburg 1992).  $D_e$  was calculated based on the equation for a full flowing rectangular conduit in cross section. The density and kinematic viscosity of artificial seawater was used to calculate the absolute viscosity ( $\mu$ ) using equation [5],

$$\mu = (0.01 \cdot v \cdot \rho) \quad [5]$$

where  $v$  is the kinematic viscosity in centipose and  $\rho$  is the density in  $\text{g cm}^{-3}$  (Weast 1987).

The shear stress at the point of cell attachment was calculated using the equation for parabolic flow velocity between two plates as described by equation [6],

$$v = V_{\max} \left[ 1 - \left( \frac{x}{y} \right)^2 \right] \quad [6]$$

where  $x$  is the distance from the centerline of the flow cell in cm and  $y$  is 1/2 the flow channel height in cm (Siedlecki 1995). The shear stress ( $\tau$ ) was calculated by substituting equation [6] into equation [7] and solving to yield equation [8].

$$\tau = -\mu \cdot \frac{dv}{dx} \quad [7]$$

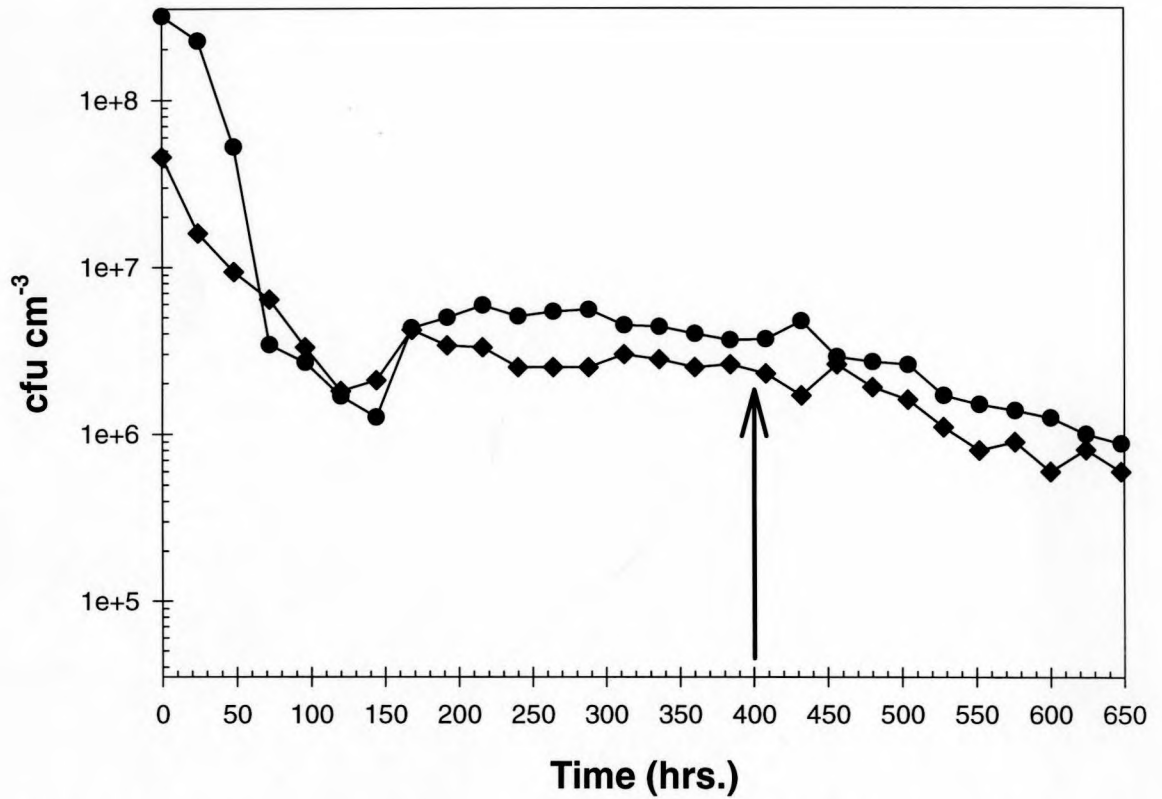
$$\tau = 2\mu \cdot V_{\max} \frac{x}{y^2} \quad [8]$$

The units were converted to  $\text{pN } \mu\text{m}^{-2}$  and represent the shear stress at the surface of the silicon and chitin coupons in the LFCs.

## Results

### Starvation Response of S91

Following transfer from nutrient rich to nutrient deprived artificial seawater, S91 experienced an initial rapid loss of cell culturability followed by a slower but steady rate of decline after 150 hrs (Fig 16). The starved populations then slowly declined over the remaining time the starved cell suspensions were monitored. Despite having initial cell densities that differed by almost an order of magnitude, the starved populations of S91, in replicate starvation experiments, had culturable cell densities of  $4.2 \times 10^6$  cfu  $\text{cm}^{-3}$  and  $4.3 \times 10^6$  cfu  $\text{cm}^{-3}$  at 170 hrs of starvation. Similar cell densities are found in natural

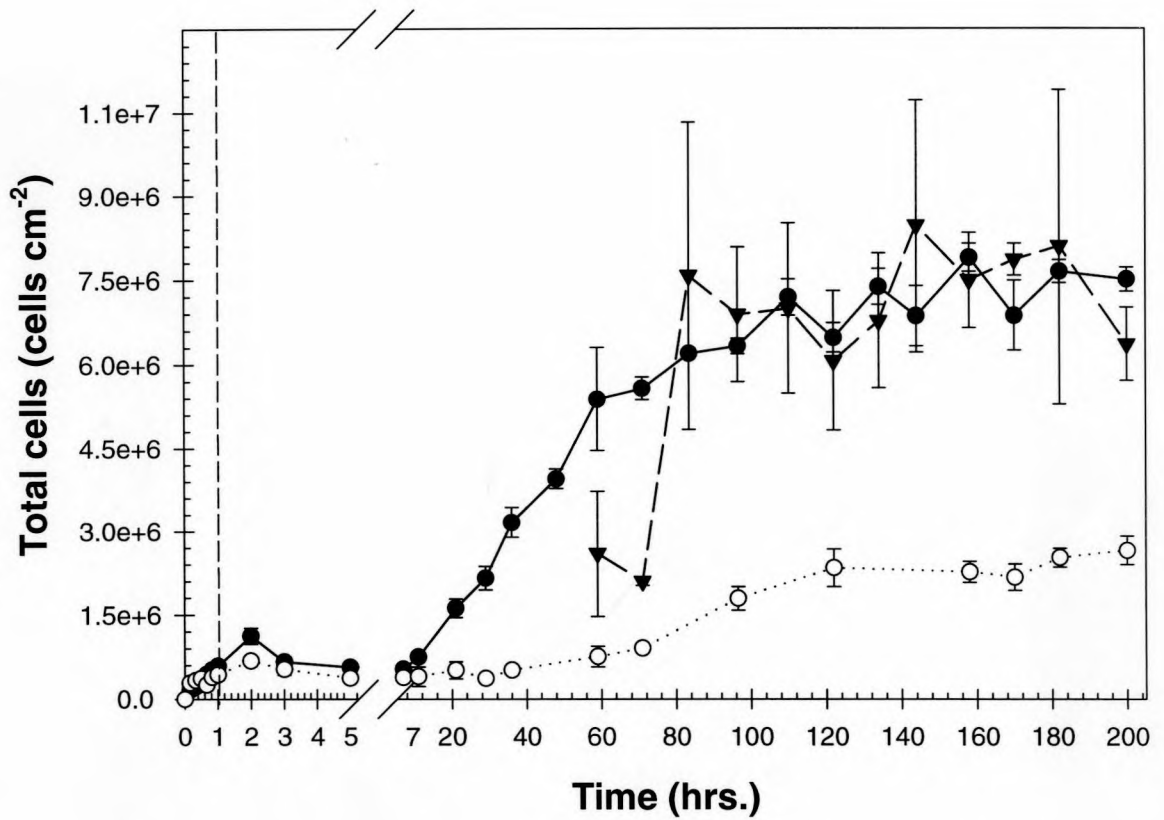


**Figure 16.** Culturable cells of S91 in seawater during a starvation period of 650 hrs: (◆) culture used to inoculate LFC containing natural squid pen chitin, (●) culture used to inoculate the LFCs containing the silicon and chitin surfaces. The arrow depicts the time at which the LFCs were inoculated.

seawater where direct counts of bacteria range between  $10^4$ - $10^6$  cells  $\text{cm}^{-3}$  (Watson et al. 1977; Fuhrman and Azam 1980; Fuhrman 1981). After 170 hrs, the cells began to decrease in size and appeared as coccoid forms similar in appearance to many bacteria recovered from natural seawater. After 300 hrs there were no detectable rod forms of S91 remaining in the populations. At a time of 400 hrs, the starved cell suspensions represented 5.0% and 1.2% of the original cell populations in the replicate experiments. The population of S91 cells exhibited no detectable *chiA/chiB* expression at any time during the 400 hrs of starvation.

#### Surface Colonization

The silicon and pure chitin surfaces were rapidly colonized by cells that had been starved for 400 hrs (Fig 17). After 1 hr exposure to the flowing culture of starved cells, the chitin surface accumulated  $7.4 \times 10^5$  cells  $\text{cm}^{-2}$ , while the silicon surface accumulated  $5.6 \times 10^5$  cells  $\text{cm}^{-2}$ . Thus, surface colonization rates were independent of the nutritional value of the surface. On the chitin surface, cell densities increased linearly at a rate of  $3.79 \text{ d}^{-1}$  after 11 hrs (Table 2), achieving a plateau of  $7.1 \times 10^6$  cells  $\text{cm}^2$  after 80-100hrs. Specific growth rates could not be calculated for the attached population associated with natural squid pen chitin since surface roughness and auto-fluorescence prevented the observation and enumeration of single cells on the surface.



**Figure 17.** Total cell counts of S91 on the silicon surface (○), the chitin surface (●), and in the *chiA/chiB* up-expressed clusters on the chitin surface (▼). The vertical dashed line represents the time point at which the 1 hr inoculation ended and the sterile seawater flow was initiated.

**Table 2.** Specific growth rates for S91 attached to silicon and, chitin surfaces in a flowing seawater environment.

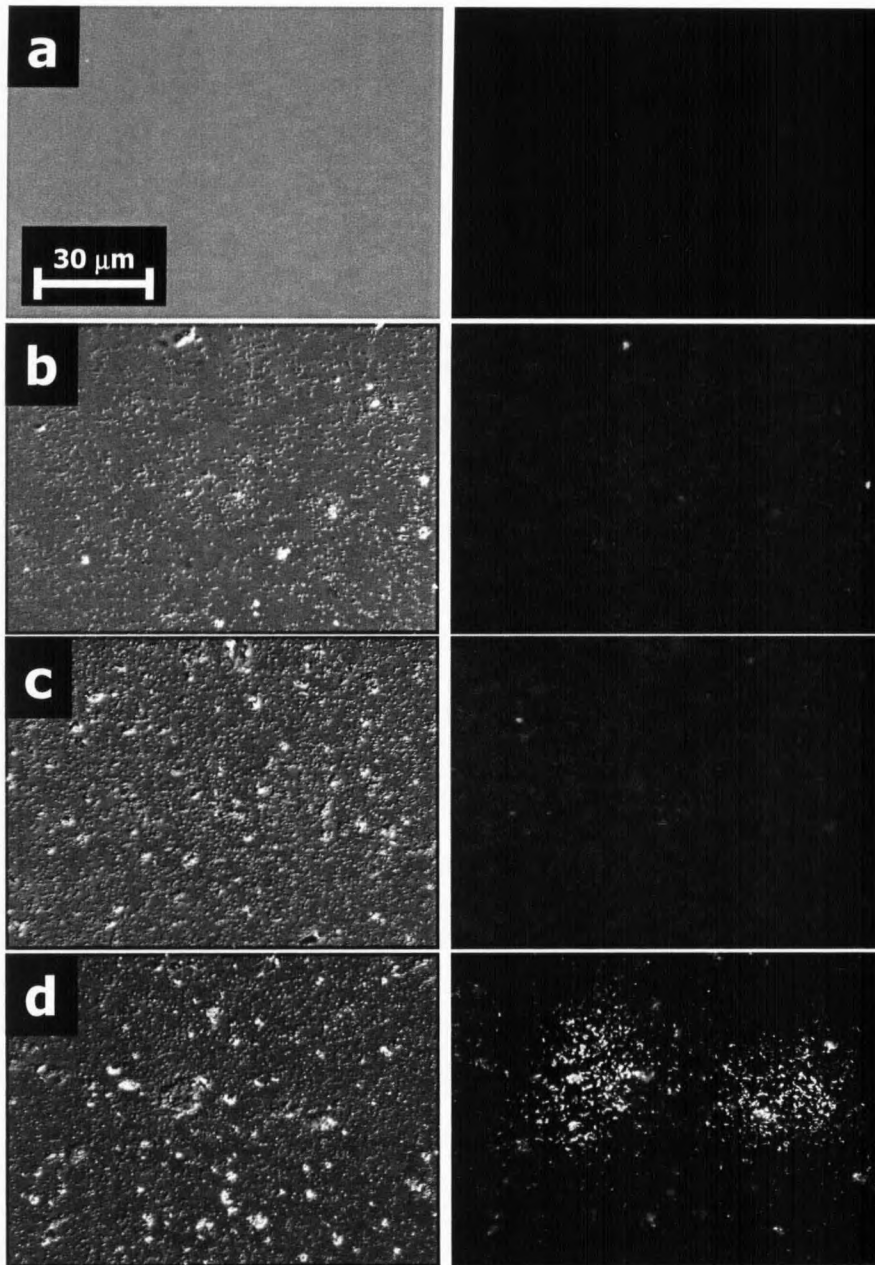
<u>Surface</u>	<u>Growth Rate (d<sup>-1</sup>)</u>	
	<u>21-70 hrs</u>	<u>100-200 hrs</u>
Silicon	2.83	0.79
Chitin	3.79	1.08

On the silicon surface cell densities increased at a rate of 2.83 d<sup>-1</sup> after 21 hrs (Table 2), and achieved a plateau cell density after 120 hrs of 2.2 x 10<sup>6</sup> cells cm<sup>-2</sup>. Thus, the chitin surface supplied essential nutrients for attached cell growth and replication that were not available on the silicone surface. However, cells attached to the nonnutritional silicon surface were still able to grow and replicate.

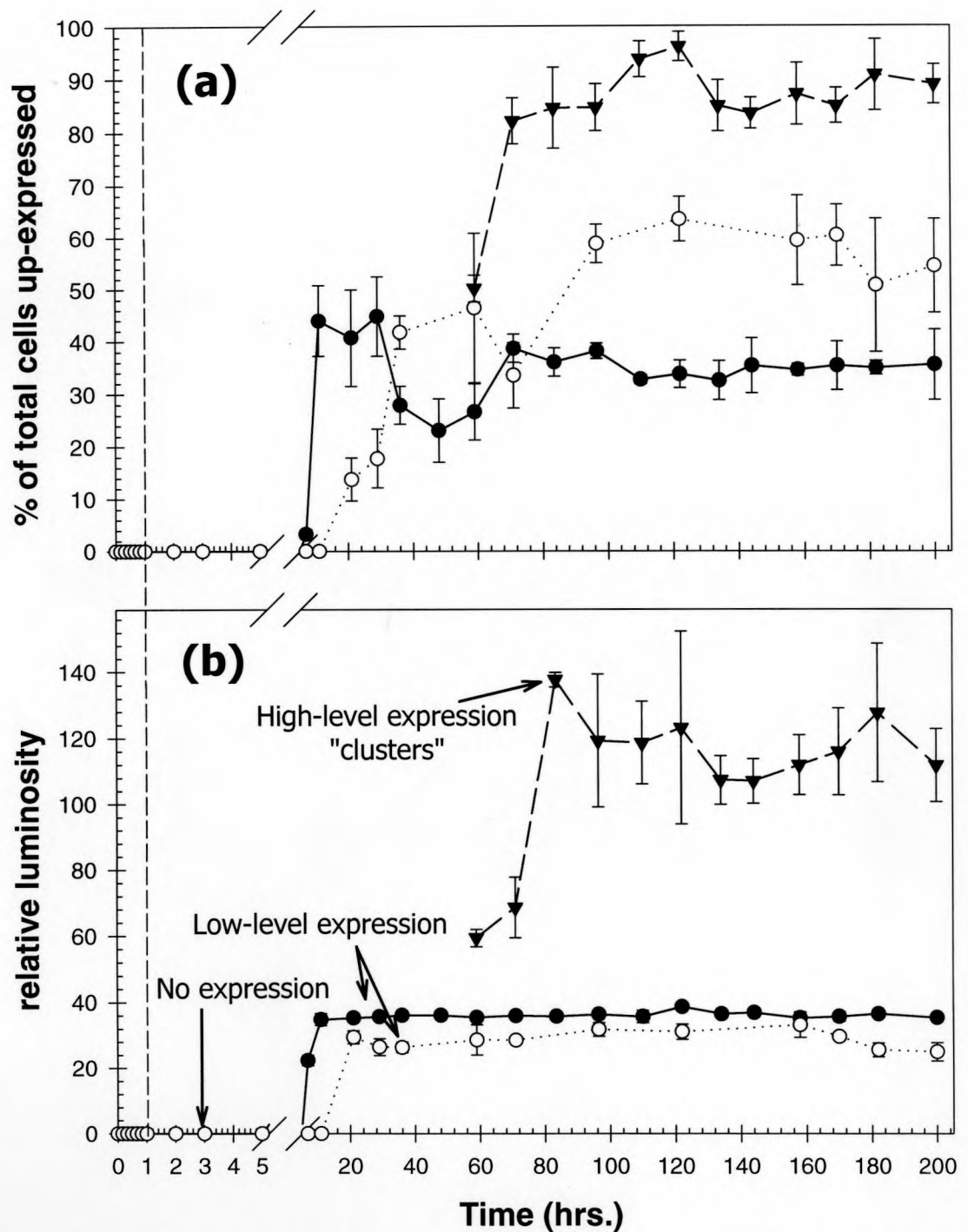
#### Chitinase Gene Expression of Surface Associated Bacteria

Upon initial attachment to either chitin or silicon surfaces, cells displayed no GFP fluorescence, indicating that their *chiA/chiB* genes remained down-expressed. However, some cells on the chitin and silicon surfaces displayed GFP fluorescence after 7 and 21 hrs post-inoculation, respectively (Fig 18b; Fig 18c). Thirty seven percent of the total cells on the chitin surface and 60% of the total cells on the silicon surface were *chiA/chiB* up-expressed at this time (Fig 19a). These *chiA/chiB* up-expressed cells displayed relative luminosities of 35.9 ± 0.8 on the chitin and 28.6 ± 2.6 on the silicon surfaces for the remainder of the experiment (Fig 19b).

On the pure chitin surface intense fluorescence appeared in discrete clusters of attached cells after 59 hrs (Fig 19b). In contrast to the fluorescence intensities of cells



**Figure 18.** DIC (left-panel) and epifluorescence (right-panel) micrographs of (a) the sterile chitin surface, (b) the silicon and (c) the chitin surfaces colonized with S91 at 96.5 hrs post-inoculation, showing low-levels of *chiA/chiB* expression on both surfaces and (d) an area of the chitin surface exhibiting two clusters of *chiA/chiB* expression within the surface associated population.



**Figure 19.** (a) The percentage of the total population that is up-expressed for *chiA/chiB* activity during growth of S91 on the silicon surface (○), the chitin surface (●), and in *chiA/chiB* up-expressed clusters on the chitin surface (▼). (b) Relative luminosity of *chiA/chiB* up-expressed cells. The vertical dashed line represents the time point at which the 1 hr inoculation ended and the sterile seawater flow was initiated.

observed prior to this period, luminosities of cells in these clusters averaged  $117.5 \pm 9.5$  between 80 and 200 hrs. Thus, cells in these clusters displayed higher levels of *chiA/chiB* expression than those cells that were up-expressed outside the clusters (Fig 18d). Ninety three percent of the cells in these clusters displayed these higher relative luminosities (Fig. 19b). These clusters of up-expressed cells varied in size from  $308 \text{ um}^2$  to  $49,125 \text{ um}^2$  with the mean size being  $4845 \text{ um}^2$ , but contained the same cell densities as the cell densities outside the clusters (Fig 17). At the end of the 200 hr experiment, clusters containing cells displaying these high relative luminosities covered approximately 20% of the total surface area of the chitin coupons. In a replicate experiment, clusters of high *chiA/chiB* gene expression covered approximately 16% of the total surface area of the chitin coupons. Therefore, at least three subpopulations of cells were defined on the chitin surface based on *chiA/chiB* gene expression; one subpopulation with no expression, a second with a low level of expression and a third with a high level of expression. In contrast, only two subpopulations were defined on the silicon surface based on *chiA/chiB* gene expression; one subpopulation with no expression and a second with a low level of expression.

### Detachment

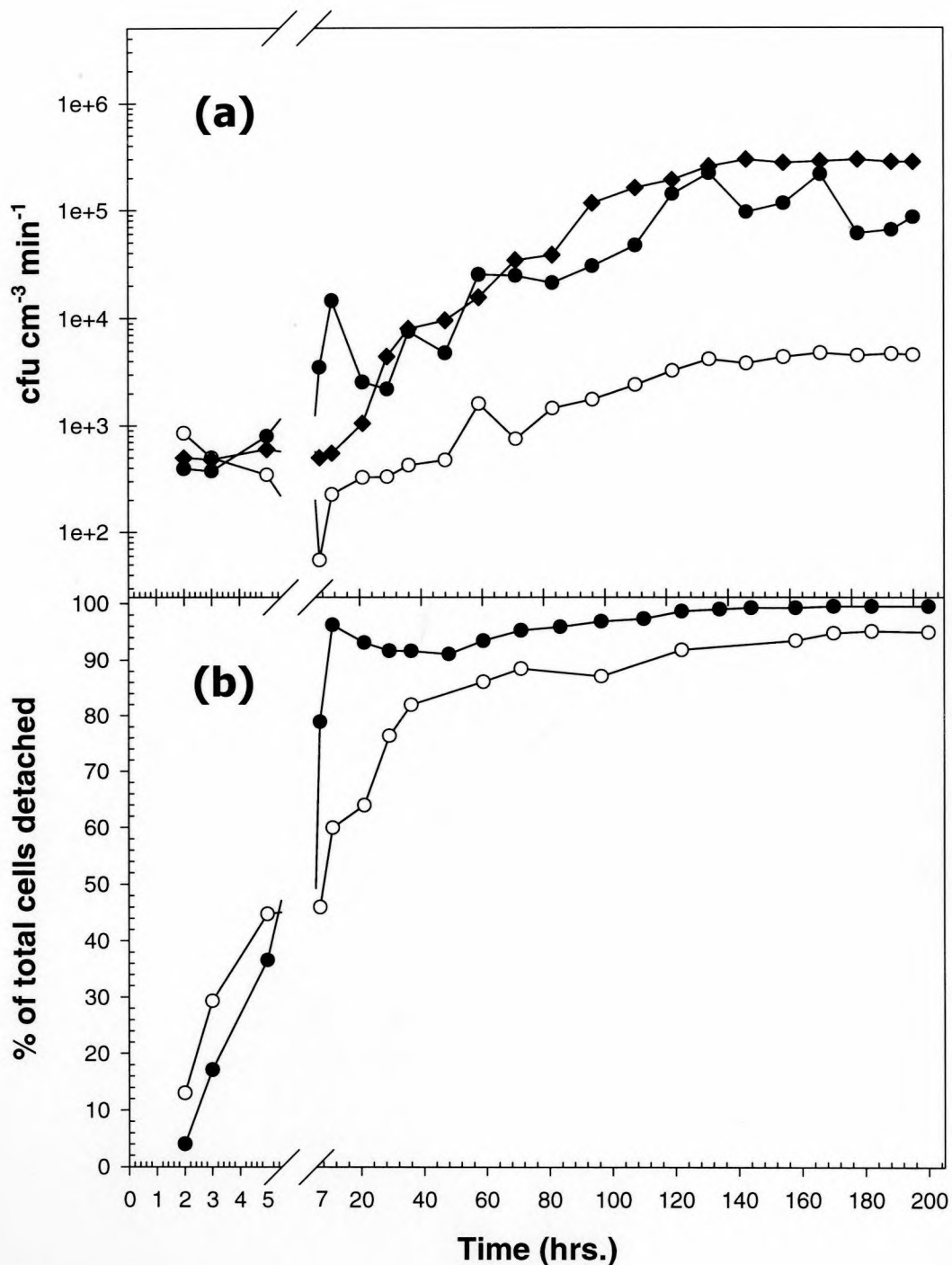
The cell detachment rates were similar in magnitude for both the pure chitin and squid pen chitin surfaces (Fig 20a). Detachment rates were lower on the silicon surface than on the chitin surfaces. Detachment rates from all the surfaces varied during surface colonization and attached cell growth. Detachment rates on the pure chitin surface

displayed the greatest increase during the time at which the cells accumulated most rapidly on that surface. On the squid pen chitin and silicon surfaces detachment rates remained at their maximum values after 130 hrs. Detachment rates continued to fluctuate throughout the experiment on the pure chitin surface.

As early as 7 hrs post-inoculation of the chitin and silicon LFCs, cells which had accumulated in the bulk aqueous phase as a result of detachment, exceeded in number those remaining on the surface (Fig 20b). The percentage of the total cells detaching from the natural squid pen chitin surface could not be calculated, since cells could not be enumerated on the surface. At 130 hrs post-inoculation, when the cell densities on the chitin and silicon surfaces had reached a plateau, 97.3% and 87.1% of the total cells generated on the pure chitin and silicon surfaces, respectively, had detached into the bulk aqueous phase (Fig 20b). Thus, cells displaced from the surface into the bulk aqueous phase represent the majority of the total cells in the system, regardless of the nutritional value of the surface.

### Bacterial Production

Total BP in the LFCs was calculated by combining the integrated surface associated BP from figure 17 with the integrated BP displaced into the effluent from figure 20a for the entire 200 hr time course of the LFC experiment. The average calculated volume and carbon biomass per cell did not differ between the chitin and



**Figure 20.** (a) Culturable cells of S91 that detached into the effluent from the chitin (●), silicon (○) and natural squid pen chitin (◆) surfaces. (b) Percentage of the total cells, that detached from the chitin (●) and silicon (○) surfaces, into the bulk aqueous phase over the 200 hr time course of the LFC experiments.

silicon surfaces. However, cell volume and, hence, carbon biomass per cell in detaching cells from the two surfaces were different. At 200 hrs post-inoculation the mean volume and carbon biomass of attached cells were  $2.07 \text{ } \mu\text{m}^3$  and  $2.03 \times 10^{-10} \text{ mgC cell}^{-1}$ , respectively. In the effluent of both the chitin and silicon LFCs detached cells had a mean volume and carbon biomass of  $1.01 \text{ } \mu\text{m}^3$  and  $1.21 \times 10^{-10} \text{ mgC cell}^{-1}$ , respectively. Total BP in the LFCs containing the silicon and chitin surfaces were 0.09 and  $2.13 \text{ mg C m}^{-3} \text{ d}^{-1}$ , respectively (Table 3).

**Table 3.** Total, surface associated, and surface displaced BP generated on the silicon, chitin, and squid pen chitin surfaces.

Surface	Surface associated BP* ( $\text{mg C m}^{-3} \text{ d}^{-1}$ )	BP displaced from the surface ( $\text{mg C m}^{-3} \text{ d}^{-1}$ )	Total surface derived BP ( $\text{mg C m}^{-3} \text{ d}^{-1}$ )	% BP displaced
Silicon	0.024	0.072	0.096	75
Chitin	0.068	2.060	2.128	96.8
Squid Pen Chitin	ND	4.17	ND	ND

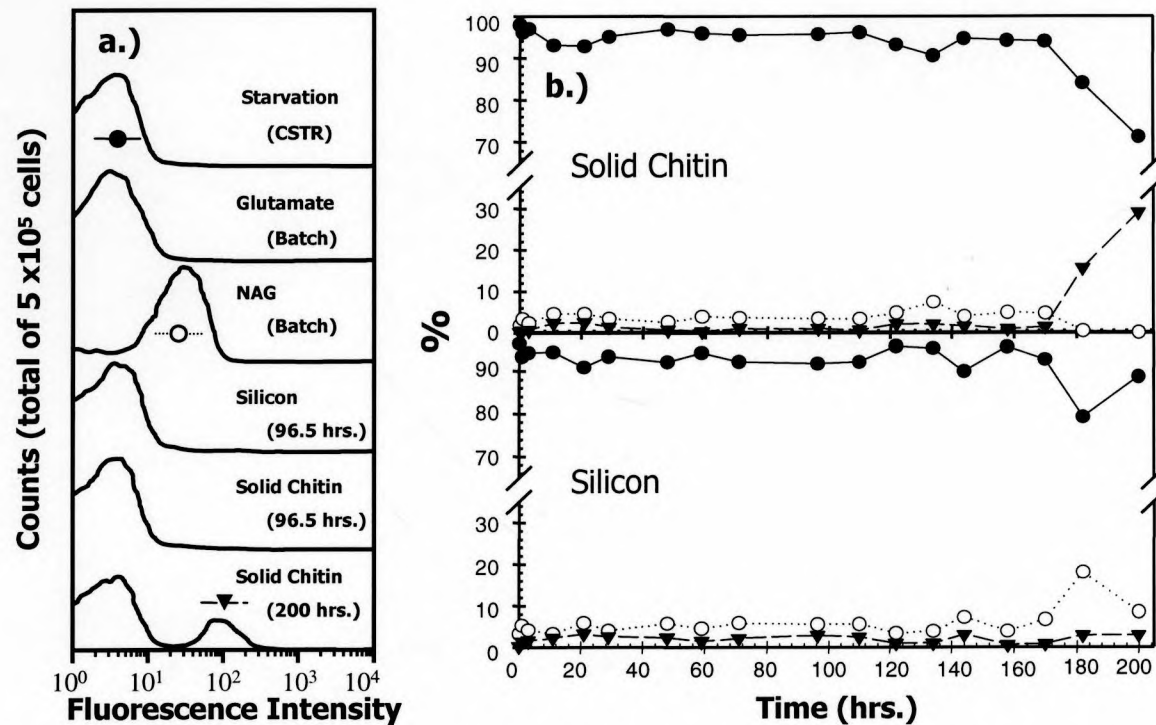
\*based on total cells attached to  $2.0 \text{ cm}^2$  of surface and the volume of seawater that passed through the LFCs at 200 hrs post inoculation.

Because the residence time of the seawater in the LFCs was less than the specific growth rate of the bacterial cells in the seawater, no significant cell growth occurred in the bulk aqueous phase within the LFCs. Therefore, almost all of the BP in the system occurred on the LFC surfaces. Only 3.2% of the total surface derived BP remained associated with the chitin surface, while 96.8% had detached as free living bacteria. Correspondingly, 25% of the total surface derived BP remained associated with the silicon surface, while 75% had detached as free living bacteria. Thus, irrespective of the nutritional value of the surface, the majority of the BP produced on the surfaces was displaced into the bulk aqueous phase. The total BP could not be evaluated from the LFC containing the squid

pen chitin surface but that portion of BP that was displaced into the bulk aqueous phase was twice that displaced from the pure chitin (Table 3).

#### Chitinase Gene Expression of Detached Bacteria

Flow cytometric analysis of the effluent samples from both the silicon and chitin surfaces revealed the majority of detached cells displayed no detectable GFP fluorescence, indicating that this population of cells was not expressing *chiA/chiB* (Fig 21b, top and bottom). This level of *chiA/chiB* expression was approximately the same as that observed for starved cells and glutamate grown cells (Fig 21a). The remainder of the cells (3-4%) that detached from both pure chitin and silicon surfaces during the first 170 h of LFC operation displayed fluorescence intensities equivalent to cells grown in batch cultures containing N-acetyl- $\beta$ -D-glucosamine (Fig 21a). Some cells displaced from the chitin surface after 170 hrs of LFC operation displayed a higher level of fluorescence than those cells displaced from the surface at earlier times, suggesting a higher level of *chiA/chiB* gene expression (Fig 21a). The appearance of cells displaying the higher level of fluorescence in the effluent of the chitin LFC at the two end time points of the experiment, was a result of excessive deterioration of the pure chitin thin film near the end of the experiment, resulting in the release of the highly up-expressed cells associated with the chitin surface.



**Figure 21.** Fluorescence intensity histograms (a) of *chiA/chiB* activity of starved cells, a glutamate grown batch culture, an N-acetyl- $\beta$ -D-glucosamine batch culture, an effluent sample from the silicon surface at 96.5 hrs, and effluent samples from the chitin surface at 96.5 hrs, and at 200 hrs. Detached cells were partitioned on the basis of their fluorescence intensity into one of three levels of *chiA/chiB* expression; no expression (●: 1-10), intermediate expression (○: 10-100), and high-level expression (▼: 30-300). The percentage of the total cells that detached from the chitin (b, top) and silicon (b, bottom) into the bulk aqueous phase with these fluorescence intensities are shown.

### Hydrodynamic Conditions

The flow dynamics in the LFCs were characterized by injecting a slug dose of crystal violet dye. The flow of the crystal violet moved in a plug flow fashion and correlated with a Re of 1.30, suggesting laminar flow of the aqueous phase across the coupons. The flow rate of defined seawater through each LFC was  $0.5 \text{ cm}^3 \text{ min}^{-1}$ , corresponding to a fluid flow velocity of  $75 \text{ m d}^{-1}$ . The shear stress at the surface under these conditions was equivalent to  $0.004 \text{ pN } \mu\text{m}^{-2}$  of force. The flow velocities and Re numbers in the LFC were equivalent to values published for natural sinking aggregates (Alldredge and Gotschalk 1988). The residence time of the bulk aqueous phase flowing through the LFCs was 55.3 s. This residence time was much shorter than the generation time of S91. Therefore, all of the free living bacteria in the bulk aqueous phase were cells derived from an actively replicating, surface associated subpopulation and not cells that had replicated in the bulk aqueous phase.

### Discussion

A starved population of S91 was able to colonize and proliferate on the silicon surface despite the lack of any added carbon, nitrogen or energy sources. However, the population attached to the silicon surface possessed lower specific growth rates when compared to the chitin surface. These specific growth rates, observed in association with a nonnutritional surface, were most likely due to the utilization of trace contaminants, in the form of DOM, in the artificial seawater flowing across the surface. When cells of

S91 were suspended in this artificial seawater no growth occurred and the population immediately entered a mode of starvation. The attachment of these starved bacteria to a surface exposed to flowing artificial seawater, resulted in the reproduction and detachment of culturable cells from the surface. This demonstrates that surface associated bacteria, irrespective of the nutritional value of the substratum with which they are associated, can contribute to the pool of free living bacteria in this model system. However, attachment to a nutritional surface in contact with a low nutrient flowing bulk aqueous phase results in higher growth rates when compared to that of cells associated with a nonnutritional surface.

In the pelagic marine environment, bacterial degradation of detrital POM is carried out under conditions of constant flow as the aggregates sink through the water column. Measured settling velocities for sinking detrital POM in the open ocean range between 1-150 m d<sup>-1</sup> (Asper 1986; Shanks and Trent 1980; Lampitt 1985). Alldredge and Gotschalk (1988) measured settling velocities and dimensions of sinking aggregates off the coast of southern California. The average settling velocity for particles ranging from 2.4 to 75 mm in diameter was  $74 \pm 39$  m d<sup>-1</sup>. Hydrodynamic forces exerted over the surface of natural detrital POM during settling will vary as a consequence of complex geometry. However, the hydrodynamic forces exerted upon the surface associated bacteria in the in-vitro system used in the present study are relatively constant. The shear stress at the surface within these LFCs was  $0.004$  pN  $\mu\text{m}^{-2}$ . This approximates the shear stress at a point perpendicular to the flow of seawater across a natural aggregate sinking

at a rate of  $75 \text{ m d}^{-1}$ , a value similar to that reported above by Alldredge and Gotschalk for natural particles sinking through the water column.

The effect of shear stress on the adhesion of *Staphylococcus epidermidis* to a variety of materials including, glass, siliconized glass, plasma conditioned glass, titanium, stainless steel and Teflon<sup>®</sup>, has been evaluated by Linton et al. (1999). In those studies shear stresses ranging from  $0.2\text{-}1.0 \text{ pN } \mu\text{m}^{-2}$  had little effect on the detachment rate of colonizing bacteria. Furthermore, chitinolytic bacteria may possess specific mechanisms of binding to chitin that are stronger than nonspecific bacteria surface interactions. Chitinases possess chitin binding domains that aid in binding the enzyme to its substrate (Raikhel et al. 1993). These chitin binding domains may also serve to anchor the cell to the chitin surface (Montgomery and Kirchman 1993). It is difficult to assess the significance of shear stress on the process of detachment. However, a shear stress of  $0.004 \text{ pN } \mu\text{m}^{-2}$  would most likely represent a negligible force on the attached bacteria. This suggests other, yet unrealized, mechanisms may be involved in the active detachment of these surface associated bacteria.

That a low level of *chiA/chiB* expression was observed following attachment of S91 cells to both silicon and chitin surfaces suggests the response was surface induced but independent of surface properties. Even cells attached to the floor of the Teflon LFCs upstream of the coupons displayed low levels of *chiA/chiB* expression (data not shown). Up-expression of genes coding for various cellular processes has been reported for other types of bacteria following surface attachment, but the properties of a solid surface that effect gene expression have not been identified (Silverman et al. 1984; Van Loosdrecht et

al. 1990; Davies and Geesey 1995; Zhang and Normark 1996; O'Toole and Kolter 1998). Since chitin is one of the primary organic carbon and nitrogen sources in the pelagic marine environment, cells possessing chitinase genes may express these genes at a low level following contact with any solid surface to synthesize "sensing levels" of enzymes. When a chitin surface is encountered, this could result in the release of small quantities of N-acetyl- $\beta$ -D-glucosamine or chitin oligomers, which upon uptake by the cells, may enhance *chiA/chiB* up-expression, as was observed in cells on some areas of the chitin surface but not in cells on the silicon or Teflon surfaces.

The variable expression of the *chiA/chiB* genes in different cells of the attached population suggests the existence of subpopulations involved in different surface associated activities. The results presented here suggest that *chiA/chiB* up-expressed cells are most likely involved in chitin degradation via chitinase production, with very limited involvement in the process of surface detachment, and contributing little to total system production. In contrast, the subpopulation of attached cells down-expressed for the *chiA/chiB* genes appeared to be involved in the detachment process and responsible for the bulk of the biomass production in the system. It is unlikely that these subpopulations occurred as a result of the loss of the plasmid since all cells in the system were under constant selection for Km resistance conferred by the plasmid. It is also unlikely that bacterial contaminants contributed to the variable expression of the chitinase genes in this study, since all cells in the system were also under constant selection for Sm resistance and exposed to a seawater medium containing no added sources of carbon, nitrogen or energy. Such physiological heterogeneity may allow two energy demanding processes,

such as ectoenzyme production and cell biomass production, to proceed simultaneously but partitioned among different cells within the population. Other types of bacteria also appear to distribute different physiological activities among different members of the population as a means to enhance survival of the population as a whole (Caldwell et al. 1997; Shapiro 1998).

In order for the "division of labor" among subpopulations to mediate the bacterial production observed in the chitin LFC above that observed in the silicon LFC, the replicating cell subpopulation that is down-expressed for chitinase genes must have access to the chitin degradation products generated by the sub population whose chitinase genes are up-expressed. The elaboration of a matrix of extracellular polymeric substances (EPS) by surface associated cells may impede displacement of chitin degradation products as well as chitinase enzyme molecules from the substratum surface into the bulk aqueous phase and promote their accessibility to nearby chitin associated cells down-expressed for the enzyme. Although the mechanism remains to be resolved, surface associated bacterial production by cells whose *chiA/chiB* genes remain down-expressed is likely linked to chitin degradation by surface associated cells whose *chiA/chiB* genes are up-expressed.

Current methods using [ $^3\text{H}$ ]thymidine (Kirchman et al. 1982; Fuhrman and Azam 1982; Ducklow and Kirchman 1983; Riemann and Bell 1990) to estimate bacterial production do not account for the transfer of bacterial biomass between POM and the bulk aqueous phase through the processes of attachment and detachment. Turley and Mackie (1994) reported that the contribution of attached bacteria to total BP ranged

between 1.8-3.4% from aggregates collected in the NE Atlantic. Alldredge et al. (1986) reported that the contribution of attached bacteria to total BP ranged between 3-26% and averaged 8% from marine snow collected in neretic waters off southern California. Likewise, Griffith et al. (1994) reported that attached bacteria were responsible for 5-10% of the total BP in the Chesapeake Bay. Karner and Herndl (1992) found the contribution of attached bacteria to total BP could equal that of the free living bacterial populations. These estimates of POM associated BP are based on the assumption that detachment or exchange of bacterial biomass between the surface of POM and the bulk aqueous phase, is insignificant.

Jacobsen and Azam (1984) found that as much as 90% of bacteria produced by cells attached to copepod fecal pellets were recovered in the surrounding water. They hypothesized that cells may leave the fecal pellet when the bacteria are separated by septation during cell division. These researchers recognized that detachment of POM associated bacteria may result in major errors when estimating BP and bacterial growth rates for POM associated and free living bacterial populations. Our results confirm this source of error and demonstrate surface associated bacteria can contribute to free living bacterial populations through the process of reproduction and detachment while maintaining steady cell densities on the surface during degradative activity. In the experiments presented here, where detachment could be accounted for, the BP that detached from the pure chitin and silicon surfaces accounted for 96.8% and 75%, respectively, of the total BP generated by the attached bacteria. Furthermore, free living cells resulting from detachment exceeded the number of attached cells early in the

process of surface colonization and chitin utilization. Thus, detachment may contribute to the relatively low measured carbon demand of POM associated bacteria in studies by other investigators (Karl et al. 1988; Simon et al. 1990; Ducklow et al. 1992), as BP that detaches from POM would be credited to free living populations. Since detachment rates associated with the surfaces studied here were not constant over time, but varied depending on the age of the surface associated population, it is necessary to evaluate both detachment and BP at multiple time points during POM degradation.

On the basis of biomass calculations derived from measurements of cell dimensions, total biomass produced in the chitin LFC was 40% greater than the estimated available carbon in the chitin thin films. The factors likely responsible for this discrepancy are the empirically derived factors for converting cell volume to biomass used in this study and the uncertainty of the thickness of the chitin thin films. The carbon content per volume can vary widely in marine bacteria (Kogure and Koike 1987; Lee and Fuhrman 1987; Bjornsen and Kuparinen 1991; Kroer 1994; Fakuda et al. 1998). Since this was not determined in our study the conversion factors may be inaccurate. Film thickness may vary as much as 30-40% when the casting temperature varies by 5°C, due to temperature induced changes in the viscosity of the chitosan solution. Since temperature was not controlled during the casting of the films, estimated carbon content may be off by as much as 30-40%. Theoretically, these uncertainties can be minimized in future experiments, permitting an accurate determination of the efficiency of bacterial conversion of chitin to biomass in this model system.

In summary, surface associated bacteria have been shown to be a significant source of free living bacterial biomass as a result of detachment of cells replicating on a surface. Use of a reporter for expression of chitinase genes at the single cell level demonstrated the existence of both up-expressed and down-expressed cells on chitin and nonchitin surfaces, and cells with different levels of expression on a chitin surface. It is hypothesized that cells whose chitinase genes are up-expressed supply chitin degradation products to nearby cells on the chitin surface whose chitinase genes are down-expressed, allowing the latter subpopulation to replicate and disseminate progeny to the bulk aqueous phase to colonize new particle surfaces. This altruistic behavior likely promotes survival of the population in environments such as the pelagic marine environment where particle surfaces represent regions of nutrient enrichment in a nutrient depleted bulk aqueous phase.

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## CHAPTER 5

DIFFERENTIATION OF CHITINASE ACTIVE AND INACTIVE  
SUBPOPULATIONS OF A MARINE BACTERIUM DURING CHITIN  
DEGRADATIONIntroduction

Detrital particulate organic matter (POM) in the pelagic marine environment harbors dense microbial assemblages (Alldredge and Youngbluth 1985). Bacteria associated with POM synthesize ectohydrolytic enzymes to convert insoluble substrates into bacterial biomass. Release of dissolved organic matter (DOM) during ectohydrolytic enzyme degradation of POM is thought to support bacterial production (BP) of free living bacterial populations in the pelagic marine environment (Hollibaugh and Azam 1983; Smith et al. 1992; Vetter and Deming, 1999). This process has been referred to as "sloppy feeding" by the particle associated bacteria. However, POM associated bacteria have been shown to have a low carbon demand when compared to their free living counterparts and the particles they inhabit are relatively few in number (Ducklow et al. 1982; Karl et al. 1988; Simon et al 1990). Therefore, POM associated bacteria are believed to contribute little to the direct production of bacterial biomass in the open ocean.

Recently, it was demonstrated that a large fraction of reproducing surface associated bacteria contribute to free living bacterial biomass through the process of

detachment (Chapter 4). Heterogeneity in chitinolytic gene expression played an important role in the partitioning of bacterial biomass between the surface and bulk aqueous phase during the degradation of chitin. During bacterial reproduction, >96.8% of the total BP generated on the surface of pure chitin detached to form free living bacterial biomass. Furthermore, the majority of the detached bacteria were completely down-expressed for chitinase gene activity, while the highly up-expressed subpopulation remained associated with the chitin surface.

Due to methodological difficulties, this type of physiological diversity within bacterial populations is rarely reported. As a result, bacterial populations are often viewed as clonal and individual cell behavior is only considered as a fraction of the whole. It was the goal of this research to localize chitinolytic gene expression and chitinase activity at the single cell level during chitin degradation. A new precipitating Enzyme Linked Fluorescent (ELF<sup>®</sup>-97) probe was employed to relate chitinase gene expression to extracellular chitinase activity in individual bacterial cells of a population. The new enzyme substrate, ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide, is soluble in aqueous solutions, membrane impermeable, and nonfluorescent until cleaved by extracellular chitinase. The resulting fluorophore crystallizes at the site of enzyme action and is used in conjunction with a Green Fluorescent Protein (GFP) reporter of chitinase gene expression. The combination of these tools permitted the direct observation of chitinase gene expression and the activity of the chitinase gene product at the single cell level during chitin degradation.

## Materials and Methods

### Bacterial Strains

A green fluorescent protein reporter gene (*gfp*) was used to visualize chitinase gene expression of *Pseudoalteromonas sp.* strain S91, during the degradation of solid chitin. Strain S91 was derived from the wild type strain S9, a chitinolytic marine bacterium isolated from the surface waters of Botany Bay, New South Wales, Australia in 1981 (Humphrey et al. 1983). Strain S91 is streptomycin (Sm) resistant and contains the plasmid, pDSK519 that confers kanamycin (Km) resistance. The gene for *gfp* is under control of the *chiA* promoter on the plasmid and a fully functional *chiA* gene was located on the chromosome (Stretton and Goodman 1998; Techkarnjanaruk 1998; Techkarnjanaruk and Goodman 1999). The expression of this gene is essential for utilization of high molecular weight chitin as a carbon, nitrogen and energy source (Techkarnjanaruk et al. 1997). The *gfp* gene is the GFPmut2 derivative of the wild type gene and confers a 30-fold increase in GFP fluorescence intensity (Cormack et al. 1996).

### Bacterial Cultivation

Stock cultures of S91 were grown to exponential phase in MB2216 (Difco, Detroit, MI). Approximately 5  $\mu$ L of this culture was used to inoculate 50 mL of a defined seawater media. Growth of S91 under conditions of *chiA* down-expression was achieved by supplementing the defined seawater with 0.05M glutamic acid as the sole

carbon, nitrogen and energy source (Techkarnjanaruk et al. 1997). When the induction of the *chiA* gene was desired during the growth of S91, 0.05M N-acetyl- $\beta$ -D-glucosamine was added to the defined seawater as the sole carbon, nitrogen and energy source. For the starvation of S91, cells were first grown on glutamic acid. At a culturable cell density of approximately  $4.2 \times 10^7$  cfu mL<sup>-1</sup> the cells were pelleted in a Sorvall RC-5B centrifuge at 16,000 g for 10 min then washed and repelleted 3x in defined seawater media. The cells were resuspended in 10 mL of this seawater medium and added to 1.0 L of defined seawater medium in an air sparged, continuously stirred tank reactor (CSTR) maintained at 20°C. The defined seawater consisted of 201.1 mM NaCl, 4.8 mM H<sub>3</sub>BO<sub>4</sub>, 27.5 mM Na<sub>2</sub>SO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 88.5 mM KCl, 8.4 mM KBr, 54.1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O and 1.5 mM SrCl<sub>2</sub>·6H<sub>2</sub>O suspended in 0.05M Sigma 7-9 buffer using once distilled Millipore water and adjusted to pH 8.0. Following autoclave sterilization, 0.008 mM FeCl<sub>3</sub>, 0.04 mM K<sub>2</sub>HPO<sub>4</sub>, 2.0 mM CaCl<sub>2</sub> were each added separately by filter sterilization through a 0.2  $\mu$ m pore size Millipore syringe filter. To insure retention of the plasmid by the bacterial cells Km was added to a concentration of 600  $\mu$ g mL<sup>-1</sup>. In an effort to minimize contamination of the culture during long term starvation and flow cell experiments, Sm was added to a concentration of 200  $\mu$ g mL<sup>-1</sup>.

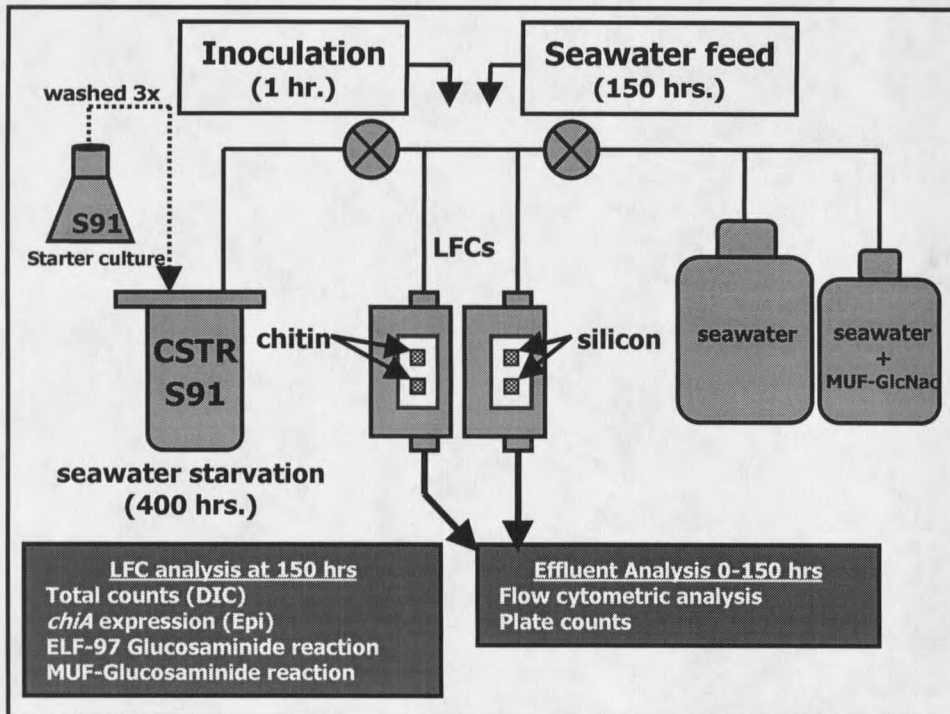
### Substratum Preparation

Thin films of pure chitin served as the only added sources of carbon, nitrogen and energy during chitin degradation. Silicon coupons, 1 cm x 1 cm square and 1.5 mm thick

(Harrick Scientific, Ossining, NY), were used as “nonnutritional” controls. The methods used in the preparation of pure chitin thin films were adapted from previously published methods for spin-casting chitin films from chitosan solutions (Qin 1993; Rathke and Hudson 1994; Bae and Hudson 1997). Chitin thin films were prepared by spin-casting a 1.5% chitosan solution at 4500 rpm, followed by N-acetylation of the chitosan to chitin. Each silicon coupon supported a 100% pure, high molecular weight, homogeneous solid chitin thin film that was approximately 230 nm thick with a dry density of  $2.05 \text{ g cm}^{-3}$ . These chitin thin films were ultra-smooth and nonfluorescent. These physical and chemical properties permitted the observation of attached bacteria at the single cell level during chitin degradation.

#### Flow Cell Operation

Flow cells were used to evaluate bacterial attachment, reproduction and biofilm development on chitin and silicon surfaces under a constant defined seawater flow. Teflon<sup>®</sup> laminar flow cells (LFCs) with glass viewing ports were used to allow direct microscopic examination of the surfaces without disturbing the flowing system. Two separate LFCs were used to monitor surface associated bacterial activity (Fig 22). The first LFC contained two chitin thin films cast onto silicon coupons. The second LFC contained clean silicon coupons and served as nonnutritional control surfaces. The LFCs were designed as described previously (Davies and Geesey 1995; Chapter 4). The LFCs were connected with platinum cured silicon tubing (Cole Palmer, Vernon Hills, IL) to the air sparged CSTR and a 20 L carboy that contained defined seawater. Glass flow breaks



**Figure 22.** Schematic representation of the experimental design showing: laminar flow cells (LFCs), continuously stirred tank reactor (CSTR), peristaltic pumps (⊗). The silicon and chitin LFCs were run in tandem.

placed in line between the media feed and the LFCs prevented back contamination of the sterile seawater feed. All influent lines were fed through a Buchler 12 roller Multistatic® pump (Cole Palmer, Vernon Hills, IL) for inoculation with the starvation culture of S91 and subsequent delivery of the sterile seawater feed.

The chitin and silicon LFCs were simultaneously inoculated with a 400 hr starved population from the CSTR at a cell density of  $4.9 \times 10^5$  cfu  $\text{cm}^{-3}$ . The LFCs were inoculated for a period of 1 hr at a flow rate of  $0.5 \text{ cm}^3 \text{ min}^{-1}$ . After LFC inoculation, the bulk aqueous phase flowing through the LFCs was switched to the sterile defined seawater, without any utilizable carbon, nitrogen or energy sources, but contained Km and Sm to select for plasmid retention and inhibit growth of contaminant bacteria, respectively. The LFCs and the sterile defined seawater were maintained at  $20^\circ\text{C}$  throughout the experiment. The flow rate was maintained at  $0.5 \text{ cm}^3 \text{ min}^{-1}$  for the remaining 150 hrs of the experiment. The residence time of the seawater in the flow cells was 55 s, precluding significant growth of cells in the bulk aqueous phase of this system.

#### Total Chitinase Activity

At 150 hrs post inoculation of the LFCs chitinase activity was determined by using the fluorogenic substrate 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucosaminide dihydrate (MUF-GlcNAc) (Fluka #69585). This chitinase substrate is soluble in water, membrane permeable and reacts with both extracellular and periplasmic  $\beta$ -N-acetylhexosaminidase (chitinase) to yield the fluorophore, 4-methylumbelliferone

(MUF). A stock solution of MUF-GlcNAc was dissolved in dimethyl sulfoxide at a concentration of 100 mM. A 50  $\mu$ M solution of MUF-GlcNAc was prepared by adding 500  $\mu$ L of the stock solution to 1000 mL of seawater. At 150 hrs post inoculation the bulk aqueous phase flowing through the LFC was switched from sterile seawater to sterile seawater containing MUF-GlcNAc. The flow rate was maintained at 0.5 cm<sup>3</sup> min<sup>-1</sup>. After 10 min, nine 2.5 mL effluent samples were collected for determination of chitinase activity contributed by surface associated and bulk aqueous phase enzyme.

The nine effluent samples collected were partitioned into three categories in an effort to locate chitinase activity. The first three effluent samples were immediately frozen at liquid nitrogen temperature after exiting the LFCs with no additional incubation of MUF-GlcNAc. This set of samples captured primarily that activity contributed by surface associated chitinase enzyme as the seawater solution containing MUF-GlcNAc flowed through the LFCs. The second set of three effluent samples was filtered through 0.2  $\mu$ m Millipore filters to remove whole cells and then incubated for 1 hr in the dark at 20°C. After the 1 hr incubation these samples were immediately frozen at liquid nitrogen temperature. This set of samples captured chitinase activity that resulted from surface associated enzyme and any additional activity contributed by free chitinase liberated into the bulk aqueous phase. The final set of three effluent samples was incubated for 1 hr in the dark at 20°C and then frozen at liquid nitrogen temperature. This set of samples captured the combined activity of chitinase associated with the surface, free chitinase associated with the bulk aqueous phase and chitinase activity associated with detached cells. Prior to freezing the samples at liquid nitrogen temperatures, 0.5 mL of

glycine-OH buffer at pH 10.5 was added to the samples to raise the pH to 10, the pH at which maximum fluorescence emission of MUF occurs. The samples were then stored at  $-40^{\circ}\text{C}$  during the four week period prior to measurement of fluorescence.

Immediately prior to measuring sample fluorescence, each sample was thawed and placed in a 3.5 mL optical glass cuvette (Starna Cells Inc., Atascadero, CA) with a wavelength range of 320 to 2500 nm. The cuvette was placed in the analysis chamber of a TD-700 fluorometer (Turner Design Inc., Sunnyvale, CA). A 2.5 mL volume of 50  $\mu\text{M}$  MUF-GlcNAc seawater solution adjusted to pH 10.0 with glycine-OH buffer served as a blank. The fluorescence intensity of the samples was determined at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. MUF concentration was determined by relating fluorescence intensity to MUF concentration using a standard curve over the range of 0.01-10  $\mu\text{M}$ .

#### Surface Localization of Extracellular Chitinase Activity

Spatial distribution of extracellular chitinase activity, on the chitin and silicon surfaces, was identified by using a new precipitating fluorescent probe for  $\beta$ -N-acetylhexosaminidase (chitinase) activity. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide is soluble in water, cell impermeable and reacts with chitinase to yield the fluorophore ELF<sup>®</sup>-97. This fluorophore is insoluble in water and crystallizes at the site of enzyme action. The methods used in the synthesis of this enzyme substrate were adapted from previously published methods for synthesizing ELF<sup>®</sup>-97- $\beta$ -D-glucuronide (Diwu et al.

1997). Briefly, ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was synthesized by oxidatively condensing 4-chloroanthranilamide with 4-chloro-2-formylphenyl-aceto- $\beta$ -D-glucosaminide. The resulting 4-chloro-2-[2'-(6"-chloro-4(3H)-quinazolinonyl)]-phenyl-aceto- $\beta$ -D-glucosaminide was deprotected to yield the enzyme substrate 4-chloro-2-[2'-(6"-chloro-4(3H)-quinazolinonyl)]-phenyl- $\beta$ -D-glucosaminide or ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide. The enzymatic hydrolysis of this substrate with pure *Streptomyces griseus* chitinase (Fluka, #22725) or with whole up-expressed cells of *Pseudoalteromonas sp.* strain S91 yields a bright yellow-green precipitate with the excitation (360 nm) and emission (540 nm) wavelengths of the fluorophore, 2-[2'-hydroxy-5'-chlorophenyl]-6-chloro-4(3H)-quinazolinone) or ELF<sup>®</sup>-97 (Larison et al. 1995).

At 150 hrs post-inoculation, and after the MUF-GlcNAc analysis, the LFCs were incubated with ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide for a period of 1 hr in an effort to spatially resolve chitinase activity with respect to total cells and cells up-expressed for *chiA* activity on both the chitin and silicon surfaces. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was dissolved in dimethyl sulfoxide at a concentration of 10 mM. A 50  $\mu$ M solution of ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide was prepared by adding a 10  $\mu$ l volume of the 10 mM stock solution to 2.0 mL of defined seawater. The mixture was filtered through a 0.2  $\mu$ m PTFE Millipore membrane to remove any residual ELF<sup>®</sup>-97 crystals and injected into the LFCs. The LFCs were allowed to incubate in the dark at 20°C for 1 hr. The ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide-seawater solution was then

rinsed out of the flow cells and the ELF<sup>®</sup>-97 activity was assessed using epifluorescence microscopy and image analysis.

### Microscopy and Image Analysis

At 150 hrs post-inoculation total cells, *chiA* up-expressed cells and sites of ELF<sup>®</sup>-97 activity were directly enumerated and spatially related on both the chitin and silicon surfaces. Images were acquired using an Olympus B-max 60 microscope (Olympus Optical Co., Tokyo, Japan) with both reflected differential interference contrast (DIC) and epifluorescence optics. All images were acquired using a Nikon infinity corrected, 40x, water immersion objective (Nikon Inc., Torrance, CA) and a mercury lamp (Chiu Technical Corporation, Kings Park, NY). Digital images were gathered using a Photometrics Imagepoint<sup>™</sup> cooled CCD camera (Photometrics, Tucson, Az). Fluorescence images of *chiA-gfp* reporter gene expression were acquired using an excitation of 481 nm and an emission of 507 nm. Fluorescence images of ELF<sup>®</sup>-97 were acquired using an excitation of 360 nm and an emission of 540 nm. All images were analyzed with Image-Pro Plus<sup>™</sup> software (Media Cybernetics, Silver Springs, MD). Image manipulation of the DIC images consisted of a background correction, adjustment of the gray scale contrast, and one pass of a 3x3 sharpening filter. The images of *gfp* expression were pseudocolored green and the images of ELF<sup>®</sup>-97 activation were pseudocolored red. Total cells, *chiA* up-expressed cells and sites of ELF<sup>®</sup>-97 activity were counted using triple image overlays. Individual cells were counted as chitinase

active if sites of ELF<sup>®</sup>-97 activity were touching the cell. Four physiological states were established at the single cell level using these image overlays: cells that were *chiA* down-expressed and chitinase inactive (*chiA*<sup>-</sup>/chitinase<sup>-</sup>), cells that were *chiA* up-expressed and chitinase inactive (*chiA*<sup>+</sup>/chitinase<sup>-</sup>), cells that were *chiA* up-expressed and chitinase active (*chiA*<sup>+</sup>/chitinase<sup>+</sup>), cells that were *chiA* down-expressed and chitinase active (*chiA*<sup>-</sup>/chitinase<sup>+</sup>). Chitinase active sites not associated with cells (cell<sup>-</sup>/chitinase<sup>+</sup>) were also located and enumerated. The software was used to count individual cells and individual sites of ELF<sup>®</sup>-97 activity in all images using manually adjusted threshold values for each individual image. Four random fields were counted in each of ten images on the chitin surface and three random field in each of eight images were counted on the silicon surface.

### Flow Cytometric Analysis

A total of seventeen LFC effluent samples were gathered throughout the time course of the 150 hr experiment and analyzed using a Becton Dickenson FACSCalibur<sup>™</sup> fluorescence activated cell sorter (FACS). *chiA* expression was monitored by measuring GFP fluorescence upon excitation at 481 nm and emission at 507 nm. A maximum of 50,000 counts were acquired for each sample. Batch cultures of S91 grown on glutamic acid and N-acetyl-D-glucosamine were used as controls for *chiA* down- and up-expression, respectively in cells. The *chiA* down-expressed, glutamic acid grown batch culture displayed relative fluorescent intensities ranging from 1-12. The *chiA*

up-expressed, N-acetyl-D-glucosamine grown batch culture displayed relative fluorescent intensities ranging from 12-1000. Detached cells were partitioned on the basis of their fluorescence intensity using ranges defined by the batch culture controls.

### Culturability of Bacterial Cells in the Bulk Aqueous Phase

The extent to which dissolved organic matter (DOM), released by the surface associated bacterial population, supported culturability of bacterial cells in the bulk aqueous phase after their detachment from the chitin surface was determined after increasing periods of chitin degradation. Effluent from the chitin LFC was sampled at 50, 65, 78, 90, 102, 114, 126, 138 and 150 hrs. Each sample was incubated at 20°C. Densities of culturable cells were determined by serial dilution and plating of subsamples after 0, 24, 48 and 72 hr incubations.

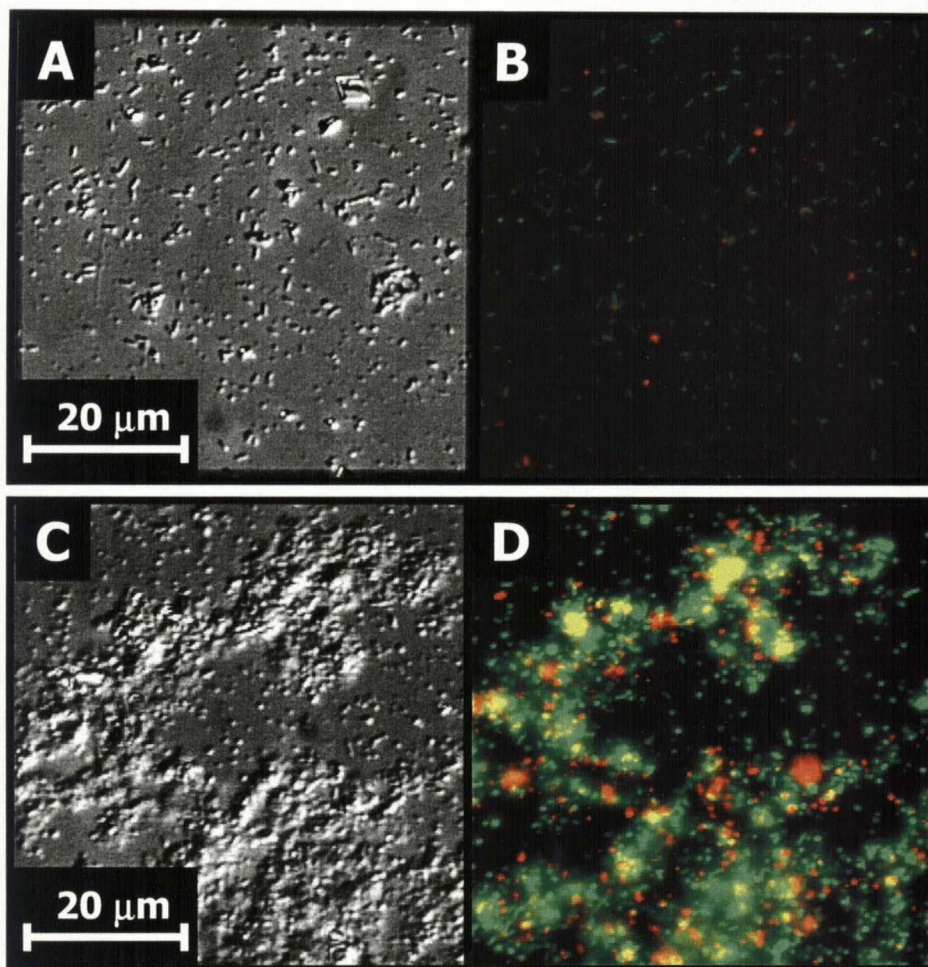
## Results

### Chitinolytic Activity of the Surface Associated Population

Silicon and chitin surfaces were rapidly colonized by cells that had been starved for 400 hrs. Following the switch to sterile seawater, attached cells increased in size and proliferated across each surface. The results of the experiments described here closely paralleled those of previous studies throughout the 150 hr time course of the experiment (Chapter 4). DIC images of the chitin and silicon surfaces, at 150 hrs post-inoculation,

reveal higher cell densities on the chitin surface when compared to the silicon surface (Fig 23A; Fig 23C). The mean cell densities  $\pm 1$  standard error on the chitin and silicon surfaces were  $7.6 \times 10^6 \pm 1.5 \times 10^5$  and  $3.4 \times 10^6 \pm 6.4 \times 10^4$  cells  $\text{cm}^{-2}$ , respectively. On the chitin surface  $3.2 \times 10^6 \pm 1.5 \times 10^5$  cells  $\text{cm}^{-2}$  or 41.5% of the total cell population was up-expressed for *chiA*. On the silicon surface  $1.8 \times 10^6 \pm 4.9 \times 10^4$  cells  $\text{cm}^{-2}$  or 53.8% of the total cell population was up-expressed for *chiA*. Thus, a significant fraction of attached cells become up-expressed for *chiA* regardless of the presence of chitin.

Image overlays revealed *chiA* up-expressed cells were associated with chitinase active sites on the chitin and silicon surfaces (Fig 23B; Fig 23D). Evaluation of the area displaying ELF<sup>®</sup>-97 fluorescence showed that  $11.6 \pm 1.5\%$  of the total surface area on the chitin surface displayed chitinase activity, whereas, only  $2.9 \pm 0.3\%$  of the silicon surface displayed chitinase activity. However, it was difficult to evaluate the relationship between *chiA* up-expression and ELF<sup>®</sup>-97 activity at the single cell level for many areas on the chitin surface as high cell densities and enzyme activities made it difficult to resolve individual cells in the image overlays. Other areas of the surface with lower cell densities, however, permitted the evaluation of the relationship between *chiA* up-expression and ELF<sup>®</sup>-97 activity by individual cells. These areas were used to evaluate phenotypic differences among cells associated with the chitin and silicon surfaces. In most cases where chitinase activity was observed, it was either directly associated with *chiA* up-expressed cells (Fig 24A) or directly adjacent to *chiA* up-expressed cells (Fig 24B). Many cells on both the chitin and silicon surfaces exhibited no *chiA* activity.



**Figure 23.** DIC (left panel) and epifluorescence micrographs (right panel) of silicon (A and B) and chitin (C and D) surfaces at 150 hrs post inoculation. *chiA* up-expression is depicted in green and sites of active enzyme activity, as reported by ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide enzyme substrate are depicted in red. Overlap of *chiA* gene activity (green) and chitinase activity (red) appears as yellow. DIC and epifluorescence image overlays (B and D) show the tight association between *chiA* up-expressed cells and *chiA* activity at the single cell level.

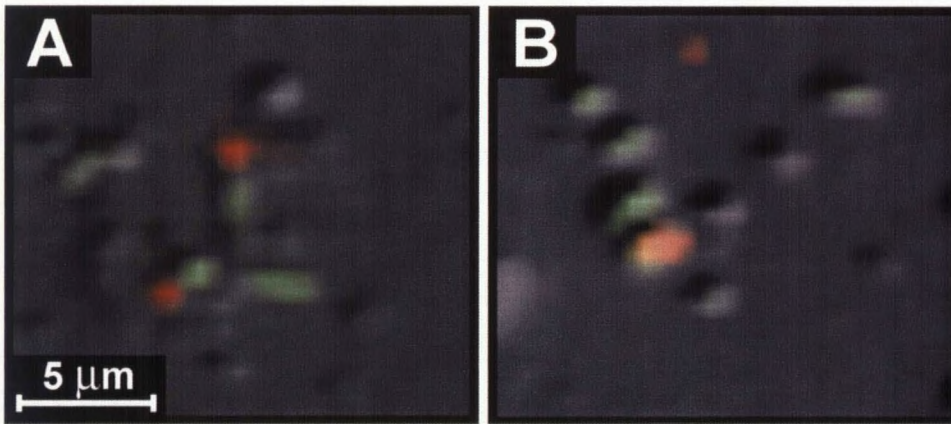
Four physiological states were resolved for the surface associated cell population based on *chiA* gene expression and chitinase activity (Table 4).

**Table 4.** Contribution of subpopulations to total cell population associated with chitin and silicon surfaces.

Surface	Sub-populations				
	<i>chiA</i> <sup>+</sup> /chitinase <sup>+</sup>	<i>chiA</i> <sup>+</sup> /chitinase <sup>-</sup>	<i>chiA</i> <sup>-</sup> /chitinase <sup>-</sup>	<i>chiA</i> <sup>-</sup> /chitinase <sup>+</sup>	<i>cell</i> <sup>-</sup> /chitinase <sup>+</sup>
Chitin	25.2 ±7.1%	31.8 ±8.7%	39.0 ±12.4%	1.33 ±0.19%	1.61 ±0.29%
Silicon	20.8 ±4.6%	44.2 ±8.2%	32.7 ±6.1%	1.25 ±0.27%	0.88 ±0.26%

A total of 1780 and 1149 cells were counted on the chitin and silicon surfaces, respectively.

The data show, 25.2 ±7.1% and 20.8 ±4.6% of the total population is *chiA*<sup>+</sup>/chitinase<sup>+</sup> on the chitin and silicon surfaces, respectively. Thus, some cells of S91 become *chiA* up-expressed and excrete active chitinase enzyme upon attachment to a surface regardless of whether the surface contains chitin. In addition, 39.0 ±12.4% and 32.7 ±6.1% of the total population is *chiA*<sup>-</sup>/chitinase<sup>-</sup> on the chitin and silicon surfaces, respectively. Therefore, not all cells become up-expressed for *chiA*, even in the presence of chitin. A large fraction of *chiA* up-expressed cells displayed no detectable chitinase activity (*chiA*<sup>+</sup>/chitinase<sup>-</sup>). The ELF<sup>®</sup>-97 enzyme substrate also identified a very small percentage of free enzyme activity not associated with cells (*cell*<sup>-</sup>/chitinase<sup>+</sup>), and enzyme active sites containing cells down-expressed for *chiA* (*chiA*<sup>-</sup>/chitinase<sup>+</sup>). These results suggest that chitinase activity, when present, closely correlated with *chiA* up-expression.



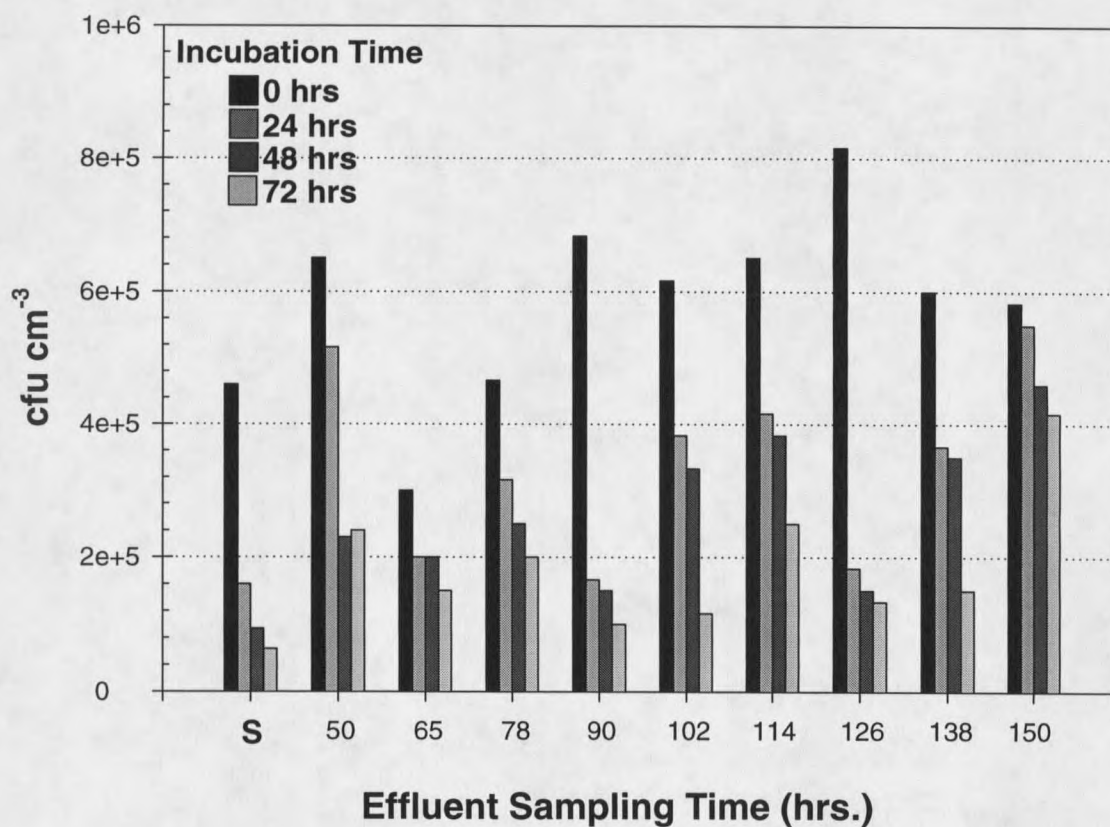
**Figure 24.** DIC, epifluorescence image overlays of *chiA* up-expression and ELF<sup>®</sup>-97 activity at the single cell level. *chiA* up-expression are depicted in green and sites of active enzyme activity, as reported by the ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide enzyme substrate are depicted in red.

### Physiological State of Detached Cells

Similar to results reported previously, the majority of the total biomass generated in the system was free living cells that had detached from either chitin or silicon surfaces (Chapter 4). At 50 hrs post-inoculation of the chitin surface, when the surface associated population reached a semi-steady cell density, an average of  $2.98 \times 10^5$  cfu  $\text{cm}^{-3}$   $\text{min}^{-1}$  were displaced from the chitin surface (Fig 25). When integrated over the 50-150 hrs period following reactor inoculation,  $1.78 \times 10^9$  cfu, representing 99.8% of the total bacteria generated from the surface associated population, detached as free living bacteria from the chitin surface. After detaching from the surface, the free living cell population displayed no reproductive activity (Fig 25). In fact, cell culturability decreased during a 72 hr incubation period, similar to the decrease in cell culturability displayed by a culture of S91 subjected to starvation conditions after growth on glutamic acid. Therefore, cells that detach from the surface, in this system, appear to resemble cells entering a starvation mode of existence.

### Chitinolytic Activity of Detached Bacteria

Flow cytometric analysis of the effluent samples from LFCs containing the chitin and silicon surfaces revealed that very few detached cells were *chiA* up-expressed (Fig 26). Throughout the 150 hr time course of the experiment, only  $0.2 \pm 0.03\%$  ( $n = 17$ ) and  $0.1 \pm 0.02\%$  ( $n = 17$ ) of the cells that detached from both the chitin and silicon surfaces respectively, to form the free living bacterial population, were *chiA* up-expressed. The

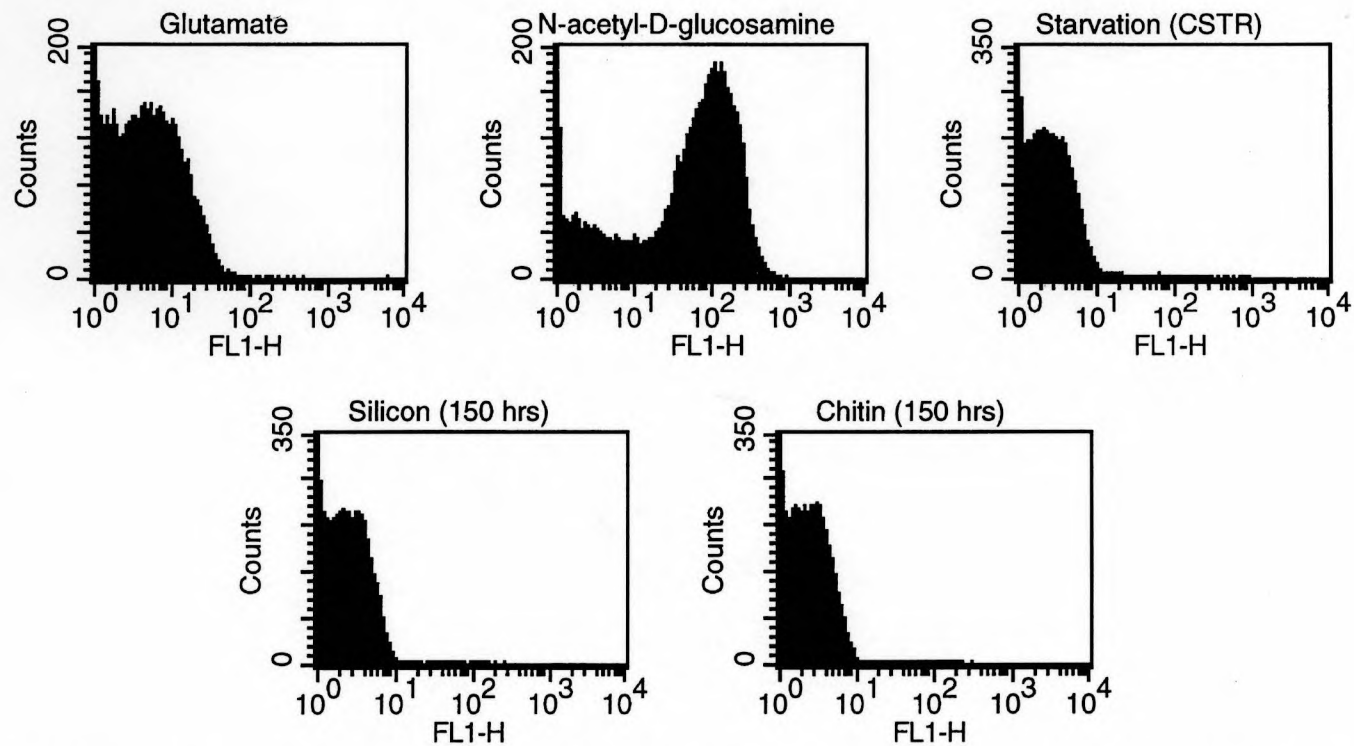


**Figure 25.** Culturable cells of S91 in the effluent collected over the 150 hr period following inoculation of the chitin LFC and of a 400 hr starved batch culture (S) after further incubation for 0, 24, 48 and 72 hrs.

percentage of the total free living bacterial population in the reactor effluent, that was up-expressed for *chiA* was lower than the percentage of total free living cells up-expressed for *chiA* ( $3.3 \pm 0.8\%$ ) in a batch culture grown on glutamate as the sole carbon, nitrogen, and energy source and lower than the total free living population that displayed *chiA* up-expression ( $0.8 \pm 0.05\%$ ) after 400 hrs of starvation. In contrast,  $71.5 \pm 3.4\%$  of the total cells were *chiA* up-expressed in a batch culture with monomeric N-acetyl- $\beta$ -D-glucosamine as the sole carbon, nitrogen and energy source. Therefore, the population that detached from the chitin surface has a smaller fraction of cells up-expressed for *chiA* than the starved population used to inoculate the surface.

#### Localization of Chitinase Activity

To assess the distribution of chitinase activity between the surface and bulk aqueous phase, chitinase activity was determined with MUF-N-acetyl- $\beta$ -D-glucosaminide, a soluble, membrane permeable analog to the ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide substrate (Table 5). Using the same molar concentration for MUF-N-acetyl- $\beta$ -D-glucosaminide as ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide, enzyme activity in the effluent from the LFCs containing chitin and silicon were  $0.47 \pm 0.05$  and  $0.24 \pm 0.03 \mu\text{mol L}^{-1} \text{hr}^{-1}$ , respectively, when no further incubation was carried out. When the effluent samples were incubated



**Figure 26.** Fluorescence intensity histograms of *chiA* activity in a population of cells in an N-acetyl-D-glucosamine batch culture, a glutamate grown batch culture, starved cells, and effluent samples from the silicon and chitin LFCs at 150 hrs post-inoculation. Detached cells in the effluent were partitioned on the basis of their fluorescence intensity using ranges defined by the batch culture controls. Fluorescent intensities between 1-12 defined *chiA* down-expressed cells, while up-expressed cells displayed fluorescent intensities ranging between 12-1000.

**Table 5.** Chitinase activity of S91 associated with chitin and silicon surfaces, free-living cells released into the bulk aqueous phase, and free enzyme released into the bulk aqueous phase at 150 hrs post-inoculation.

Surface	Rate of MUF-GlcNAc hydrolysis ( $\mu\text{mol L}^{-1} \text{hr}^{-1}$ )		
	Surface Associated Cells	Surface Associated Cells + Free Enzyme + Free Cells	Surface Associated + Free Enzyme (< 0.2 $\mu\text{m}$ Filtrate)
Chitin	$0.47 \pm 0.05$	$0.50 \pm 0.04$	$0.46 \pm 0.05$
Silicon	$0.24 \pm 0.03$	$0.21 \pm 0.02$	$0.22 \pm 0.02$

for an additional 60 min, no additional chitinase activity was detected, suggesting there was no significant activity contributed by free enzyme or enzyme associated with detached, free living cells. Therefore, the majority of chitinase activity in this system was surface associated. The lack of chitinase activity among detached free living bacteria indicate this subpopulation maintains the *chiA*<sup>-</sup>/chitinase<sup>-</sup> phenotype. Furthermore, the subpopulation on either the chitin or silicon surface that was *chiA*<sup>+</sup>/chitinase<sup>+</sup> was not part of the subpopulation of cells detaching from the surface or releasing free enzyme into the bulk aqueous phase.

### Discussion

Previous studies revealed two subpopulations of surface associated cells of S91; one up-expressed for *chiA/chiB* and presumably producing and excreting chitinase to degrade chitin present in the system, and another subpopulation down-expressed for *chiA/chiB* which was involved in the production of cells that were released from the

surface into the bulk aqueous phase (Chapter 4). In this study, a new method for localizing extracellular chitinase activity confirmed the presence of both subpopulations: one actively synthesizing and excreting extracellular chitinase enzyme ( $chiA^+$ /chitinase<sup>+</sup> and  $chiA^-$ /chitinase<sup>+</sup>), and the other not producing extracellular chitinase enzyme ( $chiA^-$ /chitinase<sup>-</sup> and  $chiA^+$ /chitinase<sup>-</sup>).

The detection of  $chiA^+$ /chitinase<sup>-</sup> and  $chiA^-$ /chitinase<sup>+</sup> subpopulations was unexpected. Cells displaying the  $chiA^+$ /chitinase<sup>-</sup> phenotype may have recently initiated chitinase synthesis, with not enough active enzyme excreted to convert a threshold amount of enzyme substrate to a detectable fluorescent product. Detection of the  $chiA^-$ /chitinase<sup>+</sup> phenotype, on the other hand, suggests cells are capable of liberating an enzyme, which can hydrolyze ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide that is encoded by a gene that operates independently of the *chiA* promoter. Many chitinolytic bacteria possess multiple chitinase genes controlled by different promoters (Watanabe et al. 1990; Miyashita et al. 1991; Svitil et al. 1997) as does S91 also Tachkarnjanaruk and Goodman (1999). The small contribution made by this phenotype to the total population, however, suggests that the *chiA* gene product accurately reflected chitinase activity, as reported by ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide under the conditions of this study.

We cannot exclude the possibility that the  $chiA^+$ /chitinase<sup>-</sup> subpopulation produced an endochitinase that may not have reacted with ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide. It is possible using the current synthesis method for ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide to synthesize the corresponding

ELF<sup>®</sup>-97-N,N'-diacetyl- $\beta$ -D-chitobioside and -N,N',N''-triacetyl- $\beta$ -D-chitotrioside enzyme substrates to distinguish different chitinase enzymes. In combination with the 4-methylumbelliferone analogs, these substrates should enable the distinction of different chitinase activities at the single cell level.

That chitinase activity was also observed in association with cells attached to the silicon surface in the absence of chitin demonstrates that induction of *chiA* was a surface induced phenomenon, independent of surface chemistry. While it is recognized that numerous genes are regulated by cell attachment to a surface (Belas et al. 1986), we know little about those properties of the surface that influence or control gene expression. In this study, surface attachment promoted the synthesis and excretion of "sensing levels" of extracellular chitinase, regardless of surface properties. On a silicon surface, this sensing level of chitinase activity reflected a low level of *chiA* expression as reported previously (Chapter 4), and a small area of ELF<sup>®</sup>-97 fluorescence as reported here. However, when the surface consisted of chitin, the *chiA* gene became up-regulated in some of the attached cells causing them to display a high level of GFP fluorescence as reported previously (Chapter 4) and more enzyme was synthesized, causing a larger area of ELF<sup>®</sup>-97 fluorescence than on the silicon surface as described in this work. Thus, surface properties appear to influence the level of gene expression, and not just whether a gene will or will not be expressed.

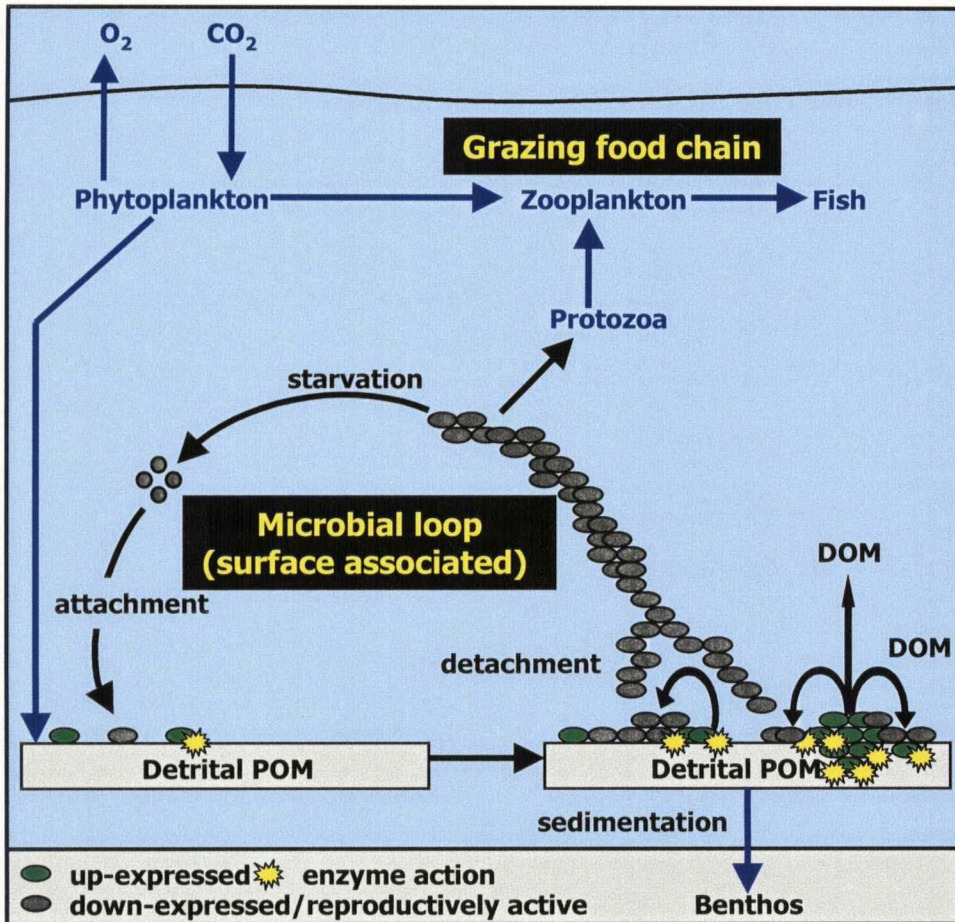
It has been hypothesized that POM associated bacteria generate more DOM from the hydrolysis of POM than they can utilize, resulting in the release of DOM into the bulk aqueous phase for utilization by free living bacteria (Azam and Cho 1987; Cho and Azam

1988; Azam 1998). In our model system, the *chiA*<sup>+</sup>/chitinase<sup>+</sup> subpopulation was likely responsible for the generation of the excess chitin hydrolysate. This hydrolysate was likely utilized by the *chiA*<sup>-</sup>/chitinase<sup>-</sup> subpopulation on the chitin surface to produce bacterial biomass that was displaced into the bulk aqueous phase. Since the residence time of the bulk liquid in the system is less than the generation time of the bacteria, the biomass originating from the *chiA*<sup>-</sup>/chitinase<sup>-</sup> free living cell population must have originated from the surface associated population of the same phenotype. Free living cells do not appear to have access to chitin hydrolysate for growth and replication in the bulk aqueous phase. Rather, the free living cell population displayed the starvation mode of existence, with a decline in cell culturability over time.

The apparent dependence of the surface associated *chiA*<sup>-</sup>/chitinase<sup>-</sup> subpopulation on chitin degradation products produced by the *chiA*<sup>+</sup>/chitinase<sup>+</sup> subpopulation suggests cell-cell interaction. Intra-species cooperation may result in more efficient utilization of the carbon, nitrogen and energy available in chitin by the surface associated population as a whole, and allow for two energy intensive cellular activities (chitinase production and cell replication) to occur simultaneously. Phenotypic variation within a bacterial population is generally recognized when individual traits manifest themselves through distinguishable morphological features (Ben-Jacob et al. 1998). In one of the best known examples, vegetative cells of *Myxococcus xanthus* aggregate and undergo morphogenesis to form fruiting bodies that differentiate into myxospores (Shimkets 1987; Munoz-Durado and Arias 1995). Likewise, as *Streptomyces* colonies age, cells differentiate into

aerial filaments called sporophores that give rise to spores called conidia that germinate to form new vegetative cells (Chater 1993). *Caulobacter crescentus* is known to attach to surfaces, form a stalk that serves as an adhesive hold fast and undergo cell division that results in a flagellated swarmer cell (Newton 1987; Gober et al. 1991). In all of these examples, morphological differentiation distinguishes members of a population with different life histories that work cooperatively to enhance the survival of the population. However, unlike the above examples, differentiation among members of S91 was not manifested as a gross change in morphology. Rather, the differentiation occurred at the level of gene expression and extracellular enzyme production.

In summary, evaluation of microbial processes at the single cell level may be necessary in order to resolve pathways of carbon flux in the marine environment. We have demonstrated that synthesis of extracellular chitinase enzyme, like the up-regulation of chitinase genes in surface associated bacteria varies among individual cells within a population exposed to apparently homogeneous environmental conditions (Fig 27). The ability of a population to coordinate chitinase activity, cell reproduction and surface detachment among different cells on a surface should enhance their ability to locate new sources of nutrients in the pelagic marine environment.



**Figure 27.** Cartoon, depicting the events that govern chitin degradation and biomass production within this in-vitro system. Starved *Pseudoalteromonas sp.* strain S91 attaches to a surface. On the chitin surface, S91 differentiates into two distinct sub-populations: one whose *chiA* gene becomes up-expressed resulting in synthesis and excretion of active chitinase, and another whose *chiA* gene is down-expressed and whose cells are involved in bacterial replication and dissemination of progeny into the bulk aqueous phase.

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## CHAPTER 6

## SUMMARY

The synthesis of extracellular hydrolytic enzymes in an effort to solubilize detrital POM for the production of bacterial biomass directly impacts carbon cycling in the pelagic marine environment. Defining the range of physiological diversity within a single bacterial population as well as within the community colonizing detrital POM will expand our knowledge of the mechanistic details that regulate bacteria organic matter interactions. The main objective of this research was to localize chitinase gene expression and chitinase activity at the single cell level during chitin degradation, and to quantify (if it exists) the effect this physiological diversity has on the production of bacterial biomass on the surface and in the corresponding bulk aqueous phase. There were three main goals of the study. The first was to quantify BSP and growth rates of the marine bacterium *Pseudoalteromonas sp.* strain S91 during the degradation of solid chitin. With respect to this goal the results were summarized as follows:

- 1.) Thin films of the natural biopolymer, chitin, were developed to enable the observation and quantification of microbial activities at the cellular level. These thin films were ultra-smooth, non-fluorescent and 100% pure. These qualities permitted the evaluation of single cell behavior in response to chitin in a flowing seawater environment.

- 2.) Following initial attachment cells proliferated across the chitin surface until a cell density was reached that was representative of a monolayer of cells.
- 3.) During bacterial reproduction, <95% of the total BSP generated on the surface of pure chitin detached to form free living bacterial biomass.
- 4.) Following detachment from the chitin surface, cells appeared to enter a starvation survival mode of existence.

The second goal was to quantify chitinolytic gene expression of S91 at the single cell level during the processes of bacterial attachment, reproduction and detachment from the surface of chitin. With respect to this goal the results were summarized as follows:

- 1.) Not all the bacteria that attached to the chitin surface became up-expressed for *chiA* or *chiA/chiB* genes.
- 2.) Almost all of the bacterial biomass that detached to form the free living population was completely down expressed for *chiA* and *chiA/chiB* gene activity.
- 3.) The highly up-expressed subpopulation remained associated with the chitin surface.

The third goal was to spatially localize chitinolytic gene expression with extracellular chitinase activity at the single cell level during chitin degradation. With respect to this goal the results are summarized as follows:

- 1.) A new precipitating enzyme substrate was developed to identify extracellular chitinase activity associated with individual bacterial cells of a population. ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide is soluble in water, cell impermeable and reacts with  $\beta$ -N-acetylhexosaminidase and exochitinases to yield the insoluble fluorophore ELF<sup>®</sup>-97.
- 2.) The combination of a *gfp* reporter for chitinase gene expression with the ELF<sup>®</sup>-97-N-acetyl- $\beta$ -D-glucosaminide enzyme substrate enabled the differentiation of at least four physiologically distinct subpopulations within a single species of chitinolytic marine bacteria on the surface of chitin.
- 3.) Among the subpopulations identified, a chitinase active subpopulation was directly contributing to chitin degradation through the production of extracellular chitinase, while a chitinase inactive subpopulation was responsible for the bulk of the biomass production associated with the surface and the corresponding bulk aqueous phase.
- 4.) Chitinase activity as revealed by the chitinase enzyme, MUF-N-acetyl- $\beta$ -D-glucosamine, showed that all of the enzyme activity was associated with the surface associated bacterial population. No enzyme activity was detected in cells that detached to form the free living population.
- 5.) Active extracellular chitinase enzyme was also observed in association with cells attached to the silicon surface in the absence of chitin, demonstrating the initial induction of *chiA* is a surface induced response independent of surface chemistry. The initial induction of the *chiA* gene by a surface may serve to generate "sensing levels" of extracellular chitinase enzyme.

This study demonstrates that gene expression of surface associated bacteria is highly variable even within a population exposed to homogeneous environmental conditions. The combined activity of chitinase active and chitinase inactive cells during the degradation of chitin resulted in a high rate of BSP on the surface by the chitinase inactive subpopulation that was subsequently displaced into the bulk aqueous phase through the process of detachment during chitin degradation. This type of physiological heterogeneity may serve as a mechanism to support both the energy intensive activity of ectohydrolytic enzyme production and the energy intensive activity of cellular reproduction, in an environment where resources are limited.

The data presented here suggests POM associated bacteria may not only function in particle hydrolysis but also in the production of free living bacterial biomass through the process of reproduction and detachment. If detachment rates in the pelagic marine environment are equivalent to those observed here a significant under estimation of bacterial growth rates would result using current methodologies. Currently, no detachment rates are known for POM associated bacteria in the pelagic marine environment. Thus, the "particle decomposition paradox" might be at least partly explained by detachment rates of POM associated bacteria.

This work provides a glimpse into the physiological diversity that is present within a population of marine bacteria during the degradation of chitin. The micron scale processes observed here might have profound influences on the total microbial community in the pelagic marine environment. In light of these results, resolution of

rates of organic matter transformation will require new methodologies to assess activities at the single cell level. However, the need to demonstrate these processes in the natural environment will be essential in an effort to gain an understanding of the complex association of bacteria-POM coupling that will ultimately enable the incorporation of bacterial activities into mechanistic models.

MONTANA STATE UNIVERSITY - BOZEMAN



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