EFFECTS OF LOW TEMPERATURE, COLD SHOCK AND VARIOUS CARBON SOURCES ON THE PHYSIOLOGY OF A PSYCHROTROPHIC *ACINETOBACTER* SP.

BY

SUSAN E. BARBARO

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Abstract

Growth of psychrotrophic *Acinetobacter* sp. HH1-1 at low temperatures was investigated by monitoring cell membrane properties, enzyme activity, and protein synthesis during growth at 25°C and 5°C, and following a 25°C to 5°C decrease in growth temperature (cold shock). The effect of different carbon sources on the ability of HH1-1 to grow at low temperatures and respond to cold shock was also investigated. Cells were grown in batch cultures with acetate, Tween 80, or olive oil as the sole source of carbon. Membrane permeability was monitored after cold shock by measuring K^+ concentrations in culture supernatant. At various time points during growth at 25°C, 5°C, and after cold shock, cell membrane fluidity was examined by measuring the fluorescence polarization of the membrane-probe parinaric acid. The fatty acid composition of cells was determined using gas chromatography. Activities of the enzymes isocitrate lyase, esterase, and lipase were also monitored using spectrophotometric assays, and protein synthesis was assessed using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Cell-membrane changes were observed after cold shock for all carbon sources. Cells became leaky and membranes less fluid. Acetate-grown cells responded more quickly to cold shock than did cells grown with either Tween 80 or olive oil by restoring membrane fluidity and by taking K^+ back into cells. Concentrations of the fatty acid palmitoleic acid (*cis*-Δ9-hexadecenoic, 16:1) increased 2 h after cold shock in acetate-grown cells and 24 h after cold shock in Tween 80- and olive oil-grown cells. In addition, an increased content of C16:1 fatty acid was observed in olive oil-grown cells during growth at 5°C relative to cells grown at 25°C. These data indicated that this fatty acid may be important for maintaining membrane fluidity at low temperatures.

Growth at low temperatures and cold shock had varying effects on isocitrate lyase, esterase, and lipase activities. During growth at 25°C, isocitrate lyase activity was measured in cell sonicate, but at 5°C and after cold shock, activity was measured primarily in cell culture supernatant. This response supported the conclusion that a loss in membrane permeability occurred at low temperatures. HH1-1 produced two cell-associated esterases and an extracellular esterase and lipase. Activities of the extracellular esterase and lipase were reduced at 5°C and after cold shock. These results indicated that the reduction in extracellular esterase and lipase activities may be related to inefficient transport of the enzymes across the cell membranes. In contrast, an increased synthesis of a 53-kDa cell-associated esterase observed 50 h after cold shock suggested a requirement for this enzyme at low temperatures.

HH1-1 responded to cold shock by synthesizing both cold shock proteins (csps) and cold acclimation proteins (caps). The synthesis of 3 csps (csps 89a, 36a, and 18) was increased 2 h after cold shock by all cells. An additional csp (csp12), with an estimated molecular mass of 12-kDa, was observed in olive oil-grown cells only. Csp12 was also induced when cells were grown at 30°C, this strain's maximal growth temperature, suggesting that csp12 may be a general stress protein rather than one required solely for cold-temperature growth. In addition to csps, fifteen cold acclimation proteins (caps) were observed at 72 h (acetate-grown cells) and at 140 h (Tween 80- and olive oil-grown cells) post cold shock. Two caps were common for all substrates whereas the other 13 proteins were unique to only one or two of the substrates tested.

The data collected in this study demonstrated that cells utilizing olive oil as the sole carbon source are affected by rapid decreases in temperature and growth at low temperatures

to a greater extent than either acetate- or Tween 80-grown cells. Substrate effects on the physiology of *Acinetobacter* sp. HH1-1 would need to be considered if the low-temperature growth of this bacterium is to be used in industry, biotechnology, or for bioremediation schemes.

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Dedication

I would like to dedicate this thesis to my parents Gordon Albert and Eleanor Dale Hipkin for their continued love and encouragement.

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Introduction

Microorganisms that can survive and grow in environments that are frequently exposed to low temperatures or permanently cold are common in nature (Russell, 1990). Psychrophilic microorganisms were first recognized over 100 years ago (Gounot, 1991). Literally meaning cold loving, microorganisms capable of growth at 0°C are called "psychrophiles". Psychrophilic microorganisms are further subdivided into two groups based on minimum, optimal and maximum growth temperatures. The most widely used terms distinguishing the two groups were first proposed by Morita (1975). Bacteria capable of growing at 0°C, having optimal growth temperatures below 16°C and an upper growth temperature limit of 20°C are termed "psychrophiles", while cold tolerant bacteria, those capable of growing at 0°C but having optimal growth temperatures above 20°C are termed "psychrotrophs". As a consequence of the potential uses of psychrophilic microorganisms and their enzymes in industry and biotechnology, an increased interest in microorganisms inhabiting extreme environments (extremophiles) has recently emerged. Studies evaluating potential uses for extremophiles have focused primarily on thermophiles, while relatively few studies have examined microorganisms capable of growing at low temperatures (Margesin & Schinner, 1994).

Potential Uses of Psychrophilic Microorganisms

Increased growth rates and enzymatic activities of psychrophilic microorganisms at low temperatures could provide competitive advantages to industries that rely on microbially-derived products and enzymes. For instance, proteolytic and lipolytic enzymes from cold-adapted microorganisms could be used in the production of fermented foods and

cheeses at low temperatures, thus avoiding the risk of contamination by mesophilic microorganisms (Gounot, 1991). The increased use of enzymatic components in detergents and lower washing temperatures have resulted in increased research into the potential benefits of psychrophilic proteases, lipases, amylases and cellulases (Margesin & Schinner, 1994). Cold-adapted bacteria with low proteolytic activity could also be useful in producing medically important compounds, particularly ones that are vulnerable to proteolysis (Bio/Tech Commentary, 1992). Psychrophilic microorganisms may also be useful in the treatment of wastewater and in the degradation of environmental contaminants at ambient temperatures (Margesin and Schinner, 1997). Presently, the mechanisms that permit microorganisms to grow at low temperatures are not completely understood. Further investigation will likely extend the uses of psychrophilic bacteria in industry, biotechnology, and bioremediation.

Bacterial Growth at Low Temperatures

Membrane Associated Changes

One response to decreasing temperatures is the change in the membrane lipid composition of cells. Bacteria respond to a variety of environmental stimuli by changing the fatty acid composition of the phospholipids as a means to maintain a constant degree of fluidity. This mechanism is called "homeoviscosic" adaptation (Keweloh & Heipieper, 1996). For instance, Cronan & Vagelos (1972) reported an increase in the content of cardiolipin and cyclopropane fatty acids concurrent with a decline in the content of phosphatidylglycerol and unsaturated fatty acids for *Escherichia coli* following the transition from exponential to stationary phase during batch growth. Membrane fluidity is determined in part by the fatty acyl chains making up phospholipids. Lipids containing primarily long

chain unbranched or saturated fatty acids will pack more tightly than lipids containing shorter chained, branched or unsaturated fatty acids. Tightly-packed lipids in phospholipids will therefore decrease the fluidity of membranes (Cronan & Gelman, 1975).

When subjected to decreases in growth temperature bacteria respond by either increasing the amount of lipid in the membrane or by increasing the proportion of unsaturated fatty acyl residues in phospholipids (Gounot, 1991). For example, following a drop in temperature (30°C to 3°C), an increase in phosphatidylethanolamine content was observed in *Pseudomonas fluorescens* (Gill, 1975). When the growth temperature was decreased from 35°C to 10°C, the content of palmitic and methylene hexadecanoic fatty acids decreased in the mesophilic bacterium *E. coli*, while the percentage of octadecenoic fatty acids increased (Marr & Ingraham, 1962). Gill (1978) also noted increases in the unsaturated fatty acid composition of phospholipids isolated from *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Lactobacillus casei* in response to decreasing temperatures.

Bacteria can alter membrane fatty acids by a variety of mechanisms. The psychrophilic bacteria *Vibrio* spp. and *Micrococcus cryophilus* alter membrane fatty acids by decreasing fatty acyl chain length, through the action of a membrane-bound elongase, or by increasing branched-chain or cyclopropane fatty acids, through the actions of transaminases, ACP transacylases and fatty acid synthetases (Russell, 1990). In addition, membrane fatty acids can be altered in response to decreasing growth temperatures as a result of *de novo* synthesis of unsaturated fatty acids (Russell, 1984), or by the induction of desaturase activity (Wada et al., 1989; Russell, 1984).

De novo synthesis of unsaturated fatty acids occurs in response to temperature and does not involve lipid acyl chain turn over. Fatty acid synthesis can produce both saturated and unsaturated fatty acids. At a branch point in the pathway, a 10-carbon fatty acid

intermediate is elongated through the action of β-ketoacyl-ACP synthetase. Two forms of this enzyme exist. β-Ketoacyl-ACP synthetase I is active at high temperatures and catalyzes the elongation reaction while dehydrating the fatty acid to a *trans*-2 unsaturated isomer which eventually gives rise to a saturated fatty acid. In contrast, β-ketoacyl-ACP synthetase II is heat-labile, and at low temperatures will elongate the fatty acid while dehydrating it to a *cis*-3 unsaturated intermediate, eventually giving rise to an unsaturated fatty acid (Russell, 1984).

The alternative strategy involves the presence of membrane-bound fatty acid desaturase enzymes. Bacteria can possess either an oxygen-dependent membrane bound fatty acid desaturase and/or an oxygen-independent membrane bound fatty acid desaturase. Both enzymes convert existing saturated fatty acyl chains to unsaturated fatty acyl chains (Wada et al. 1989).

Precise lipid composition at low temperatures is necessary for the normal functioning of membrane-associated processes (De Kruyff et al., 1973). Increasing unsaturation in lipids in response to low temperatures causes a lipid phase transition from a crystalline to a liquid-crystalline state. Studies on the membrane-phase properties of a psychrotrophic and a mesophilic *Bacillus* using wide-angle X-ray diffraction indicated that the phase transition temperature (Tm), the temperature at which lipids will change from a fluid to gel state, was not a determinant of the minimum growth temperature (Inniss et al., 1979). However, increased unsaturation of fatty acids in lipids in response to low temperatures does suggest a requirement for a liquid-crystalline rather than a crystalline lipid environment in membranes (Rose, 1989).

Published information on outer-membrane adaptations to low-temperature growth is limited. Ray et al. (1994a) have proposed that changes to the outer membrane in response to

low temperatures may also help in cold-temperature adaptation. These investigators discovered a lipopolysaccharide kinase, isolated from a psychrotrophic Antarctic *Pseudomonas syringae* strain that phosphorylated more lipopolysaccharide at high temperatures compared to low temperatures. *In vitro* studies using ^{32}P-labelled P_i showed that the major part of the radioactivity (>85%) was associated with the core oligosaccharide region. Phosphate groups in the core oligosaccharide region bind divalent cations such as Mg^{2+}. These bonds help anchor the outer membrane creating an effective permeability barrier (Mäkelä et al., 1984). The role that a reduced number of phosphate groups in the core oligosaccharide region plays in the adaptation for growth at low temperatures is presently not clear.

Ray et al. (1994b) have also described temperature-dependent phosphorylation and dephosphorylation of membrane proteins. Three proteins with molecular masses of 30 kDa, 65 kDa, and 85 kDa were detected in *P. syringae*. The 30-kDa and 85-kDa proteins were phosphorylated only at relatively high temperatures (20°C-30°C), whereas the 65-kDa protein was phosphorylated only at low temperatures (0°C-15°C). Proteins of similar mass that are differentially phosphorylated in response to different temperatures have also been found in other genera including *Micrococcus*, *Planococcus* and *Arthrobacter* (Ray et al., 1994b). The differential dephosphorylation and phosphorylation of proteins have been found to play essential roles in the signal transduction process for nitrogen regulation, chemotaxis and sporulation in bacteria (Bourret et al., 1991). Ray et al. (1994b) propose that the differential dephosphorylation and phosphorylation of membrane proteins may constitute a mechanism that psychrophilic bacteria use to detect changes in temperature so that the necessary adjustments required for low temperature growth can be made.

Properties of Psychrophilic Enzymes

Enzymes isolated from psychrophilic microorganisms have higher catalytic efficiencies and greater stability at low temperatures relative to enzymes isolated from mesophilic microorganisms (Feller et al., 1996). High catalytic efficiencies at low temperatures are believed to be due to greater flexibility of the enzyme rather than changes in the catalytic site. A number of hypotheses have been put forth to explain what properties are required to give more flexibility to enzymes. These include adjustments to the number of ion pairs, hydrophobic interactions, and changes in the amino acid sequences (Brenchley, 1996). The protease enzyme, subtilisin, was isolated from the psychrophilic bacterium *Bacillus* TA41, and the amino acid sequence was determined (Davail et al., 1994). From the predicted model, several features were identified that differed from the mesophilic subtilisins previously characterized. For instance, salt bridges and aromatic-aromatic interactions, highly conserved in mesophilic subtilisins, were missing in the psychrophilic subtilisin. In addition, the external shell of the protein was generally more hydrophilic due to the presence of additional aspartate residues. Similarly, investigations with a lipase gene (*lip1*) isolated, cloned, and sequenced from *Psychrobacter immobilis* B10 indicated that the molar ratio of basic amino acid residues arginine/(arginine+lysine) (0.28) was lower than the mean value obtained for mesophilic (0.40) and thermophilic (0.48) proteins (Arpigny et al., 1993). Studies have shown that arginine has a greater stabilizing effect than does lysine due to the formation of intramolecular bonds; therefore, a reduction in the number of arginine residues in the cold-adapted lipase may produce a more flexible structure (Arpigny et al., 1993).

Inactivation of psychrophilic enzymes at only moderately high temperatures is another common characteristic that distinguishes psychrophilic enzymes from mesophilic and thermophilic enzymes. This characteristic is believed to be due to a less rigid enzyme

structure. Kobori et al. (1984) found that 26 of 139 psychrophilic bacteria, previously isolated from the Antarctic, produced heat-labile alkaline phosphatases. In each case, the enzymes were inactivated when incubated at 60°C for only 10 min. Additional heat-sensitivity studies were conducted using a purified and well-characterized alkaline phosphatase isolated from one of these Antarctic bacterial strains. Results showed that the optimum temperature for this enzyme occurred at 25°C, and while only 17% activity remained at 0°C, the enzyme was completely inactivated at 50°C (Kobori et al., 1984). Similar results were obtained with an alkaline phosphatase isolated from the psychrophilic *Arthrobacter* sp. D10A. It was found to be heat-labile, losing over 60% of its activity within a 15-min incubation at 55°C (De Prada et al., 1996). β-Lactamase, secreted by the psychrophile *Psychrobacter immobilis* A8, is another example of an enzyme displaying a relatively low optimum temperature of activity (35°C) and a low level of thermal stability (less than 50% activity remaining after a 4-min incubation at 50°C) (Feller et al., 1995).

Enhanced excretion of extracellular enzymes at low temperatures is also observed in psychrophilic bacteria. Lipase excretion by four different *Moraxella* strains isolated from Antarctic sea water was maximal at 3°C, which was 22°C lower than the cells optimal growth temperature (Feller et al., 1990). Similarly, Gügi et al. (1991) studied the effect of growth temperature on the export of proteins by *P. fluorescens*. Both extracellular protease and periplasmic phosphatase activity was greatest at 17.5°C, while the optimal growth temperature was 25°C-30°C. In chemostat studies, with cells incubated at either 25°C or 30°C and several dilution rates, Gügi et al. (1991) demonstrated that the observed variations of enzyme activity were not related to growth rate, as observed for mesophilic bacteria, but were regulated by growth temperature.

Protein Synthesis

The ability to synthesize proteins at low temperatures is another important characteristic that distinguishes psychrophilic bacteria from mesophilic bacteria. Inhibition of protein synthesis at low temperatures was first demonstrated with *E. coli* after noting that growth ceased at temperatures below 8°C. Broeze et al. (1978) demonstrated that protein synthesis was severely inhibited when mesophilies were subjected to low temperatures (5°C), but in the same experiment, psychrophilic bacteria continued to synthesize proteins at a rate corresponding to their growth rates at 5°C. Similar results reported by Bobier et al. (1972) showed that proteins synthesized by whole cells of two psychrotrophic bacteria *Bacillus psychrophilus* and *Bacillus insolitus*, corresponded directly with the cell's growth at 5°C, 20°C, and 30°C. The inability of *E. coli* to synthesize proteins at 5°C was attributed to a block in the initiation of translation resulting in polysomal runoff and a subsequent accumulation of ribosomal subunits (Broeze et al., 1978). Other studies have indicated that the capacity of psychrophilic bacteria to synthesize proteins at low temperatures resides in the ribosomes. Szer (1970) described a unique protein (factor P) found in extracts of ribosomal washings from a psychrophilic *Pseudomonas* sp. strain 412 that activated *E. coli* ribosomes at low temperatures. Factor P added to the ribosomes of a cold sensitive *Pseudomonas* sp. 412 mutant that exhibited limited growth at 0°C and did not yield factor P also initiated poly U-directed incorporation of phenylalanine at 0°C.

Cold Shock Response

To further elucidate the effects of low growth temperature on protein synthesis, additional studies were performed in which cells were subjected to down-shifts in

temperature (cold shock), and the protein content examined. These studies led to the discovery of the cold shock response, which describes a pattern of an induction of proteins, designated cold shock proteins (csps), and the continued synthesis of transcriptional and translational proteins during the lag period following cold shock (Jones & Inouye, 1994). These proteins are thought to allow the survival of cells during the stress of a cold shock and also to prepare cells for sustained growth at low temperatures (Whyte & Inniss, 1992). The most extensive cold shock studies have been conducted with *E. coli*. When subjected to a 37°C to 10°C cold shock, growth ceased for up to 4 h to 5 h. During this time, the synthesis of most proteins was repressed but the induction of 13 proteins was observed (Jones et al., 1987). Cold shock proteins identified in *E. coli* include NusA (involved in termination and antitermination), RecA (involved with recombination and the SOS response), H-NS (a nucleoid-associated DNA-binding protein), GyrA (the α subunit of topoisomerase DNA gyrase), initiation factor 2 (responsible for mediating binding of charged *f*met-*t*RNA to the 30s ribosomal subunit for the initiation of translation), polynucleotide phosphorylase (responsible for degradation of mRNA), and cspA (Berger et al., 1996).

CspA is the most prominently induced protein in *E. coli* following a down-shift in temperature. CspA is a 7.4-kDa protein that reaches levels equivalent to 13% of the total protein synthesized within 1-1.5 h after cold shock (Goldstein et al., 1990). As of yet, the exact function of this protein is not known, but based on its three-dimensional structure, Jones et al. (1994) proposed that cspA may function as a RNA chaperone, facilitating translation at low temperatures. Characterization of the *cspA* gene has shown that cspA has high similarity with the eukaryotic DNA binding proteins DbpA, DbpG and YB-1 (Tanabe et al. 1992). YB-1 has demonstrated binding to the CCAAT motif within the conserved regulatory sequence or Y box of the histocompatibility complex class II genes (Wistow,

1990). The CCAAT motif was also revealed on the leading edge of the RNA polymerase binding site for H-NS isolated from *E. coli*. In addition, a protein, similar in size to cspA, acted as a transcriptional activator in response to cold shock (La Teana et al., 1991). These findings led to the conclusion that cspA may serve to enhance the activity of mRNAs for other csps. Recently, it has been discovered that *cspA* belongs to a familiy of genes consisting of at least six others genes (*cspB*, *cspC*, *cspD*, *cspE*, *cspF* and most recently *cspG*) in addition to the *cspA* gene (Nakashima et al., 1996). However, only *cspA*, *cspB* and *cspG* are cold shock inducible.

More recently, cold shock experiments have been conducted with psychrophilic microorganisms. Psychrophilic microorganisms in their natural environment are often subjected to relatively rapid changes in temperature. Therefore it was of interest to investigate the changes in protein synthesis that occurred in these cold-adapted microorganisms in response to a temperature decrease. One of the first studies to examine the response of a psychrophilic microorganism to a down-shift in growth temperature was performed by Julseth & Inniss (1990). A two-dimensional electrophoresis analysis was used to assess protein synthesis by the psychrotrophic yeast, *Trichosporon pullulans*, after being subjected to varying degrees of cold shock. After a 21°C to 5°C cold shock, 26 csps were maximally induced 12 h following cold shock, and after a 15°C to 5°C cold shock, 6 proteins were induced (Julseth & Inniss, 1990). The influence of the range in temperature of cold shock on the induction of proteins has been observed for other psychrotrophic bacteria. Cold shocks of 20°C to 0°C, 5°C or 10°C resulted in the induction of nine, seven and five cold shock proteins respectively, by the psychrotrophic bacterium *B. psychrophilus* (Whyte & Inniss, 1992). Berger et al. (1996) subjected the psychrotrophic bacterium *Arthrobacter globiformis* SI55 to varying magnitudes of cold shock (25°C to 15°C, 10°C, 8°C, 6°C and

4°C), analyzed protein content by two-dimensional electrophoresis, and used a statistical approach (correspondence analysis of protein appearance) to assess protein induction. Based on the induction of proteins, data indicated that the temperature shifts fell into three categories. The first category, represented by a 25°C to 15°C shock, was characterized by a reduced number of over-expressed proteins compared to proteins expressed at 25°C and a reduced lag phase, but did not induce a cold shock response. The second category, represented by 25°C to 10°C, 8°C and 6°C cold shocks, was characterized by a lag phase and the over-expression of several proteins that increased with the magnitude of the cold shock, suggesting a mild shock. The last category, represented by a 25°C to 4°C cold shock, was characterized by a longer lag phase and the appearance of cold shock proteins in addition to those observed in categories 1 & 2, and was considered a severe stress. Similar responses to these have been observed with other psychrophilic bacteria (Araki, 1991a; Roberts & Inniss, 1992).

Increasingly, csps with a high degree of homology to cspA have been identified in psychrophilic microorganisms. Ray et al. (1994c) detected *cspA* homologous genes in various psychrotrophic Antarctic bacteria representing both gram-negative and gram-positive bacteria. Two-dimensional gel electrophoresis revealed the presence of a 7.5-kDa protein after a psychrotrophic *Bacillus cereus* strain was subjected to a 30°C to 7°C cold shock (Mayr et al., 1996). Sequencing of the gene encoding this protein revealed 63% homology to *E. coli cspA* gene.

In addition to the induction of csps, psychrophilic and psychrotrophic bacteria also produce a distinct set of proteins called cold acclimation proteins (caps). Caps are proteins synthesized at a greater level during continuous growth at low temperatures. *B.*

psychrophilus, while growing at 0°C, 5°C or 10°C, synthesized 11, 10 and 4 caps, respectively (Whyte & Inniss, 1992). When growing at 5°C, a psychrotrophic *Pseudomonas fluorescens* produced 5 caps (Colucci & Inniss, 1996), and Araki (1991b) noted an increased synthesis of 28 proteins at 0°C compared to growth at 13°C by a psychrophilic *Vibrio* sp. strain ANT-300. Presently, the functions of caps in microorganisms are unknown. Recently, Berger et al. (1997) described an acclimation protein (CapA) isolated from *A. globiformis* SI55 that had a high level of identity with the sequences of CspA, CspE and related proteins from various sources. CapA was not only expressed during prolonged growth at low temperatures, but was also induced immediately after cold shock. These results indicated that the synthesis of this protein is regulated at the translational level, and suggested that the functions of cold acclimation proteins may be similar to those of csps.

Physiological and Metabolic Characteristics of *Acinetobacter* spp.

Acinetobacter spp. are gram-negative rods (0.9-1.6 μm in diameter and 1.5-2.5 μm in length) that become spherical in the stationary phase. During exponential growth, cells are plump rods that occur in pairs or sometimes in chains. At maximum stationary phase, cells may become coccoid while other cells retain their diplobacillus shape (Baumann et al., 1968). They are oxidase-negative, strictly aerobic bacteria, and although they have the ability to utilize NO_3^- and NO_2^- as the sole source of nitrogen, they are unable to use these compounds as electron acceptors (Juni, 1978). Prior to the creation of the genus *Acinetobacter*, previously isolated gram-negative, oxidase-negative diplobacilli were placed into many different genera based solely on phenotypic characteristics (Juni, 1972). Many of the first *Acinetobacter* spp. isolated were found to have growth temperature optima around

33-35°C and were occasionally associated with humans and other animals, not as primary pathogens of animals, but associated with disease in debilitated or immunologically-compromised individuals (Juni, 1978). It was only later that investigators discovered that *Acinetobacter* spp. are widely distributed in nature and that in addition to human sources, can be readily isolated from soil, water and sewage (Juni, 1978; Baumann et al., 1968).

Strains isolated from water, soil, and waste water, are predominately psychrotrophic; they are capable of growing at temperatures ranging between 4-30°C (Breuil et al., 1975). Recently, *Acinetobacter* spp. have been gaining more attention due to their diverse metabolic capabilities and their potential uses in industry and biotechnology. Various physiological characteristics of *Acinetobacter* spp. of industrial and technological importance are presently being investigated. Such characteristics include exopolymer and lipolytic enzyme productions. However, the adaptations that allow these bacteria to grow at low temperatures and the effects of low temperatures on specific physiological features have not been extensively examined. Many processes that currently utilize *Acinetobacter* spp. could be enhanced and other applications could be discovered if more information was available regarding their ability to grow at low temperatures.

Cell Envelope Characteristics

Studies that have examined the phospholipid composition of *Acinetobacter* membranes have indicated that the major phospholipid in both the cytoplasmic and the outer membrane is phosphatidylethanolamine (Scott et al., 1976; Borneleit et al., 1988; Borneleit & Kluber, 1991). Scott et al. (1976) investigated the phospholipid composition of membranes from a hydrocarbon-oxidizing *Acinetobacter* sp. HO1-N. Phosphatidyl-ethanolamine was present in the greatest amounts in both membranes regardless of whether

cells were grown with hexadecane or in nutrient-broth yeast-extract medium. Similar results were also obtained by Borneleit et al. (1988) when membrane lipids from *Acinetobacter calcoaceticus* 69V grown with hexadecane were compared to membrane lipids from cells grown with acetate. Both studies reported the presence of small amounts of cardiolipin, lysocardiolipin, phosphatidylglycerol, and lysophosphatidylethanolamine in both membranes in addition to phosphatidylethanolamine. Slightly larger amounts of cardiolipin and lysophosphatidylethanolamine were present in the outer membrane. Although the fatty acid composition of phospholipids is subject to various environmental factors such as temperature, nutrient availability, and growth phase, the major fatty acids found in membrane phospholipids of *Acinetobacter* 69V included dodecanoic acid, hexadecanoic acid, septadecanoic acid, octadecanoic acid, *cis*-Δ9-hexadecenoic acid, *cis*-Δ9-septadecenoic acid, and *cis*-9Δ-octadecenoic acid (Loffhagen et al., 1995; Hartig et al., 1999).

The overall composition of *Acinetobacter* outer membranes is similar to the outer membranes in the Enterobacteriaceae, with proteins, phospholipids and lipopolysaccharides (LPS) making up the main components. However, the structure and composition of *Acinetobacter* LPS is unique. For instance, *Acinetobacter* LPS is primarily R-type rather than O-type LPS (Borneleit et al., 1990), the lipid A component is linked to the core oligosaccharide by 2-octulosonic acid instead of 3-deoxy-D-manno-2-octulosonic acid (Kawahara et al., 1987), and 12-carbon fatty acids instead of 14-carbon fatty acids are associated with the lipid A. In addition to the structural differences in the LPS between enteric bacteria and *Acinetobacter* spp., *Acinetobacter* releases the LPS from the cell surface, particularly when cells are grown in the presence of hydrophobic compounds. One function of the outer membrane is to serve as a selective barrier to the external environment. Although some small solutes can cross the outer membrane, it typically prevents the passage

of hydrophobic compounds. The structural differences in the outer membrane structure of *Acinetobacter* may allow passage of certain hydrophobic compounds which require transport across the outer membrane, so that cells may use them for growth substrates (Borneleit & Kleber, 1991).

Carbohydrate Metabolism

Acinetobacter resemble saprophytic pseudomonads in terms of the compounds that are utlilized as growth substrates, and the pathways used to metabolize these substrates. All strains examined to date have the ability to grow in minimal media containing a single carbon and energy source; growth factors are not required. Utilization of carbohydrates is extremely restricted among *Acinetobacter*. Disaccharides, sugar alcohols (including glycerol), and hexoses are utilized by very few members of this genus (Baumann et al. 1968; Juni, 1978). The most commonly utilized sugars include D-glucose, D-xylose, D-ribose, and L-arabinose. The few strains that are capable of utilizing glucose do so exclusively via the Entner-Doudoroff pathway. *Acinetobacter* strains unable to grow on glucose may possess an aldose dehydrogenase that results in the acidification of the medium due to production of gluconic acid (Juni, 1978). This also occurs when other sugars such as D-xylose, L-arabinose, D-galactose, D-mannose, maltose, and lactose are supplied in the growth medium. Pentose utilization is similar to that observed in pseudomonads. *Acinetobacter* possess a non-specific aldose dehydrogenase that converts L-arabinose, D-xylose, and D-ribose to the appropriate pentanoic acids which are then converted to α-ketoglutarate involving a series of hydration and dehydration steps (Baumann et al., 1968; Juni, 1978).

In *Acinetobacter*, enzymes of the tricarboxylic acid cycle function during growth. However, when cells are grown on ethanol or acetate as sole source of carbon, isocitrate

lyase and malate synthase activity have been observed, indicating that the glyoxylate bypass pathway is active (Baumann et al., 1968). Not all microorganisms are capable of growing on acetate as the sole source of carbon. The glyoxylate bypass allows carbon, from acetate, to be rerouted and incorporated into cellular structures, avoiding complete loss of carbon to the tricarboxylic acid cycle (Hoyt et al., 1994). In *E. coli*, growth on acetate causes a reduction in $NADP^+$-isocitrate dehydrogenase activity. This is a tricarboxylic acid cycle enzyme responsible for the conversion of isocitrate to α-ketoglutarate, that is inactivated due to a reversible phosphorylation by an ATP-dependent phosphatase/kinase (Hoyt et al. 1991). At the same time, isocitrate lyase, a glyoxylate bypass pathway enzyme that converts isocitrate to glyoxylate, is activated as a result of its phosphorylation. Inactivation of isocitrate dehydrogenase and activation of isocitrate lyase allows isocitrate to enter the glyoxylate pathway.

The regulation of this pathway in *Acinetobacter* is more complex. *Acinetobacter* has two forms of isocitrate dehydrogenase (I and II). When growing on acetate, isocitrate dehydrogenase I is inactivated while isocitrate dehydrogenase II and isocitrate lyase activities are dramatically increased (Hoyt et al., 1994). Isocitrate dehydrogenase II is an allosteric form believed to regulate isocitrate flow between the glyoxylate bypass pathway and the tricarboxylic acid cycle (Reeves et al., 1986). However, the exact mechanism is presently not well understood.

Lipolytic Enzymes

Ester linkages between carboxylic acid groups and hydroxyl groups are the basic chemical structures of carboxyesters and lipids (McKay, 1993). Lipases and esterases are enzymes that hydrolyze the ester linkages, allowing microorganisms to metabolize these

compounds as substrates for growth. Esterases and lipases are carboxylic ester hydrolases (Kok et al. 1993). Esterases (carboxyesterases) (EC 3.1.1.1.) hydrolyze water-soluble or emulsified esters with relatively short fatty acid chains, while lipases (triacylglycerol acyl hydrolases) (EC 3.1.1.3.) preferentially attack emulsified water-insoluble substrates with long-chain fatty acids (Gilbert et al., 1991). *Acinetobacter* spp. are prolific lipase and esterase producers and as a consequence can utilize lipid compounds as sole sources of carbon (Kok et al., 1995). Lipases and esterases can be produced separately or concurrently under the same growth conditions (Kouker & Jaeger, 1987) and during growth on complex media, production of lipases and esterases is generally growth-phase-dependent (Kok et al., 1993).

Acinetobacter BD413 was grown in batch culture and incubated at 30°C with esterase and lipase activity monitored at intervals over 24 h (Kok et al., 1993). Cell-bound esterase activity was initially reported after 90 min of incubation. Maximal activity was reached between exponential and stationary phases and remained high after an additional 19 h of incubation. In contrast, lipase activity increased fivefold within 60 min after exponential growth ended, and decreased 90 min after the stationary phase had been reached (Kok et al., 1993). Growth-phase-dependent expression of lipolytic enzymes has been reported for other *Acinetobacter* isolates (Breuil & Kushner, 1975a; Shabtai & Gutnick, 1985). Breuil & Kushner (1975b) isolated a lipolytic, psychrophilic *Acinetobacter*, strain O_{16}, from sediment taken from a heavily polluted stream. Unlike BD413, O_{16} esterase was cell-bound only during early growth and then was released into the extracellular medium late in growth. This trend was also observed with *A. calcoaceticus* RAG-1. Like *Acinetobacter* BD413, both O_{16} and RAG-1 also produce lipases that are secreted into the medium during exponential growth (Breuil & Kushner, 1975b).

In addition to differences in the localization of enzyme activity, the temperatures at which lipolytic enzyme production is optimal varies among *Acinetobacter* strains. Chappe et al. (1994) compared lipolytic activities of four *Acinetobacter* isolates: SY1, IB2 and BO2 isolated from activated sludge, and SYD isolated from a commercial bioadditive. Based on the ability to hydrolyze olive oil, optimal lipolytic activity for isolates SY1 and SYD occurred at 25°C compared to 40°C-45°C for isolates IB2 and BO2. Thermostability studies showed lipolytic activity measured in SY1 and SYD diminished quickly at temperatures greater than 25°C, with complete inactivation occurring at 45°C, while lipolytic enzymes produced by IB2 and BO2 were inactivated at 55°C and 60°C, respectively (Chappe et al., 1994). Similarly, little difference in the production of cell-associated esterase was found between a mesophilic *Acinetobacter* strain O_{14} and a psychrophilic strain O_{16} when cells were incubated at 15°C, 20°C and 30°C (Breuil & Kushner, 1975a). However, optimal lipase production occurred at 15-20°C for O_{16} compared to 20-30°C for O_{14}.

Bacterial lipases and esterases are presently used in a number of industrial processes. These include the production of flavour compounds in foods and beverages, the conversion of low-value triglycerides into more confectionary fats, use in detergents, the removal of fat and oils from processed foods, and more recently the synthesis of drugs (Harwood, 1989; McKay, 1993; Margolin, 1993). In addition, esterase and lipase enzymes have possible applications in bioremediation schemes since the production of these enzymes has been linked to the ability of certain cells to degrade hydrocarbons.

Storage Compounds

In the absence of growth, energy is still required for the maintenance of intracellular pH, osmotic regulation, and turnover of proteins and nucleic acids (Dawes and Senoir, 1973). In response to nutrient-limited conditions, microorganisms will either metabolize existing protein and RNA for an energy and carbon source, or they will synthesize special energy reserve compounds. Energy reserves accumulate when growth is limited by a nutrient in the presence of excess carbon and energy source and are utilized when there is insufficient exogenous carbon and energy for growth (Fixter & Sherwani, 1991). Examples of energy storage compounds include glycogen, glycogen-like polymers, poly-β-hydroxybutyrate and other poly-β-hydroxyalkanoates. Three main energy storage compounds have been identified in *Acinetobacter*. These include two common storage sources, poly-β-hydroxybutyrate and polyphosphate, and a novel energy storage source, wax esters (Fixter & Sherwani, 1991).

Formation of wax esters during alkane oxidation by *Acinetobacter* has been observed by a number of researchers. Wax esters produced during alkane oxidation were generally thought of as overflow metabolites because they accumulated primarily extracellularly rather than within the bacterium (Fixter & Sherwani, 1991). However, a number of studies have demonstrated that wax esters do accumulate inside the cell. Wax esters have also been observed in *E. coli*, *Serratia marcescens*, *Nocardia* sp. and *Thiobacillus thioparus* but only in trace amounts (Fixter et al., 1986). In contrast, *Acinetobacter* store significant amounts of wax esters when grown on succinate, acetate, and other carbon sources in addition to alkanes, in nutrient-limited batch cultures (Fixter & Fewson, 1984).

Wax ester composition has been determined for only a few strains. Fixter et al. (1986) studied the production of wax esters in stationary phase cells of two strains of

Acinetobacter (NCIB 8250 and NCIB 10487) when grown under nitrogen-limited conditions on either succinate or acetate as the sole source of carbon. Stored wax esters were comprised primarily of C_{14} to C_{18} saturated and mono-unsaturated alkane-1-ols esterified with C_{14} and C_{18} saturated and mono-unsaturated fatty acids. Gallagher (1971) characterized wax esters in three *Acinetobacter* strains (G37, G39 and G249) during growth in nutrient broth containing Tween 80. These wax esters were comprised of a variety of saturated and mono-unsaturated alcohols ranging from C3 to C5 and from C8 to C20.

Gallagher (1971) also investigated the effect of changing the growth temperature on the composition of the wax esters. In response to a decrease in temperature, the degree of unsaturation in the wax ester increased dramatically for a mesophilic *Acinetobacter* strain G37. In contrast, small increases in unsaturation were observed for a psychrotrophic strain G39, while decreases in the proportion of long-chain alcohols were observed for the other psychrotrophic strain G249. The differences in wax ester composition observed between these two psychrotrophic strains after a decrease in temperature were believed to be related to a difference in growth rate at 1°C. However, an increase in the degree of unsaturation of wax esters, in response to low-growth temperature, has been observed for other *Acinetobacter* sp. (Fixter & Sherwani, 1991).

The biosynthesis of wax esters is not well understood. However, there is evidence that activity of an NADP-dependent alcohol dehydrogenase may be responsible for the production of these compounds (Fixter & Nagi, 1984). The breakdown of wax esters is initiated by the hydrolysis of the ester link. Two different enzymes, alcohol dehydrogenase and fatty aldehyde dehydrogenase, have been identified in *Acinetobacter* sp. strain HO1-N and are believed to be responsible for wax ester catabolism (Singer & Finnerty ,1985a; 1985b).

Poly-β-hydroxybutyrate (PHB) is a more common carbon and energy reserve polymer produced by a variety of microorganisms. Initially, investigators concluded that *Acinetobacter* spp. did not accumulate PHB even under nutrient-limited growth conditions. The most common means to detect PHB granules in cells was to treat the cells with Sudan Black and then observe the cells microscopically. The method is limited by a low degree of sensitivity, particularly when PHB reserves are present in low concentrations. With the development of more sensitive methods for detecting PHB, it became apparent that members of this group do produce and store PHB. Rees et al. (1993) looked at PHB production by *Acinetobacter* spp., strains RA3757 and RA3117, under nutrient-limited concentrations of ammonia, phosphate, or sulphate with acetate as the sole source of carbon. Strain RA3757 produced PHB under all limiting conditions. Production of PHB commenced only when the limiting nutrient was exhausted. Levels of PHB varied between 2.0-11.5% per cell dry weight. In contrast, PHB was accumulated by RA3117 only under phosphate-limiting conditions, with PHB concentrations never exceeding 0.9% per cell dry weight. Differences in the accumulation of PHB have been observed between *Acinetobacter* strains when different carbon sources (butyrate, propionate, succinate, valerate, caproate, methanol, and ethanol) were supplied in the growth medium. Variations in the time of maximal PHB production and conditions affecting PHB utilization have also been observed (Vierkant et al., 1990).

Typically, polyphosphate, which is stored in cells, is not used as an energy source, but rather as a source of phosphate under phosphate-limited conditions. However, there are bacteria that will use polyphosphate stores as an energy reserve compound. Polyphosphate accumulation is common among *Acinetobacter*, and for most strains, polyphosphate is used as an energy source. Deinema et al. (1980) isolated several strains of *Acinetobacter* from

activated sludge. Three of the 7 isolates were used for phosphate removal studies. The isolates were grown in batch cultures on butyrate-minimal medium containing 0.15 g/L KH_2PO_4, and incubated at 28°C. During incubation, phosphate removal, pH, and cell dry matter were monitored. Results showed that dividing cells took up more phosphate, between 15-30 mg phosphate per 100 mg cell dry weight, than was necessary for normal cell components.

Several different types of activated sludge treatment plants have been designed for phosphate removal. The anaerobic-aerobic system is one treatment that has been extensively studied. The model for this process involves the breakdown of polyphosphate and the production of PHB during the anaerobic stage, and the breakdown of PHB and the synthesis of polyphosphate in the subsequent aerobic stage (Rees et al., 1993). Close examination of the aerobic stage of the treatment process and studies evaluating orthophosphate uptake in batch cultures indicated that *Acinetobacter* were the primary microorganisms responsible for phosphate removal in anaerobic-aerobic sewage treatment systems (Deinema et al., 1980). During anaerobic growth, short chain fatty acids, alcohols, and lactic acid are produced by facultative anaerobic bacteria. These compounds are preferred substrates for *Acinetobacter* and will be metabolized during the aerobic stage with simultaneous phosphate uptake (Deinema et al. 1980).

Exopolysaccharide Production

Most *Acinetobacter* spp. produce capsules under most growth conditions. Composition is the same regardless of the carbon source used for growth. The capsular polysaccharide is composed of D-glucose and L-rhamnose units. No amino sugars, sugar alcohols, uronic acid or pentose components have been detected (Taylor & Juni, 1961).

In addition to capsules, *Acinetobacter* produce exopolysaccharides distinguished from capsules by their amphipathic nature. Originally, extracellular compounds were thought to be capsular material, but some studies have shown that emulsifying polysaccharides are produced in addition to capsular polysaccharides (Kaplan and Rosenberg, 1982). For example, *Acinetobacter* strain BD4 produced a capsule and an extracellular polysaccharide composed of rhamnose and glucose. Strain BD413 was derived from BD4 through transformation (Juni, 1972). The single gene mutation in BD413 caused a reduction in capsular formation concurrent with an increased production of the extracellular polysaccharide. BD4 and BD413 extracellular rhamnose polysaccharides were detected starting from mid-exponential phase growth and accumulated to the onset of the stationary phase. The molar ratio of rhamnose to glucose (3:1) was found to be similar for the BD4 capsule and the BD413 emulsifier, but when the BD4 capsule was released from the cell surface by artificial shearing and tested for emulsifying properties, none was detected (Kaplan and Rosenberg, 1982).

Rosenberg et al. (1979a) isolated and characterized an exopolysaccharide from *Acinetobacter* RAG-1, called emulsan, that consisted of an N-acetyl-D-galactosamine and N-acetyl hexosamine uronic acid backbone combined with approximately 15% fatty acids and approximately 15-20% non-covalently bound proteins. Optimal conditions for emulsifying activity were obtained when the pH of the environment was adjusted in the range of 6.5-7.5 in the presence of 2-5 mM Mg^{2+} (Kaplan and Rosenberg, 1982). The production of emulsan by RAG-1 was monitored using an immunochemical enzyme-linked immunosorbent assay to assess cell-bound exopolysaccharide activity, while extracellular activity was monitored based on the ability of supernatant to form an emulsion of hexadecane and 2-methyl naphthalene. Emulsan was found to accumulate on the cell surface during exponential

growth and was released from the cell surface by early stationary phase (Shabtai and Gutnick, 1985). The release of emulsan depended on a suitable carbon and nitrogen source as well as the production and release of an esterase from the cell surface (Alon and Gutnick, 1993).

Acinetobacter RAG-1 is a hydrocarbon-degrading bacterium (Rosenberg et. al., 1979b). It can utilize a wide variety of hydrophobic growth substrates such as C_{10}-C_{20} chain length alkanes, crude and fuel oils, and triglycerides and waxes (Alon and Gutnick, 1993). The hydrocarbon-degrading ability has been linked to the production of emulsan. BD413 and RAG-1 emulsifiers are chemically different and exhibit different hydrocarbon specificities. BD413 emulsifier is unable to emulsify straight crude oil but is able to emulsify a mixture of aliphatic and aromatic hydrocarbons while emulsan will emulsify straight crude oil. All microbial surfactants isolated so far lower the interfacial tension between oil and water or stabilize hydrocarbon-water emulsions (Rosenberg et al.,1988a). As a result, these compounds have been used to enhance the biodegradation of environmental contaminants such as crude oils and gasoline (Rosenberg et al., 1988a; Elkeles et al., 1994).

Since the discovery of emulsan, extracellular polysaccharides possessing different bioemulsifying properties have been isolated from many *Acinetobacter* strains. Rosenberg et al. (1988b) screened a number of *Acinetobacter* spp. for their ability to produce emulsifying polysaccharides. A unique extracellular dispersing agent, now known as biodispersan, was isolated from *A. calcoaceticus* strain A2. The chemical structure and activity of biodispersan was compared to emulsan. Both compounds are heteropolysaccharides and are anionic due to the presence of uronic acid residues. Emulsan has a much higher molecular weight, approximately 100,000, compared to biodispersan which has a molecular weight of

approximately 51,400 (Rosenberg et al. 1988b). Unlike emulsan, biodispersan is incapable of emulsifying hydrocarbons. Instead, this compound is used to adhere to inorganic surfaces.

Biodispersan is presently used in mining operations and in the manufacturing of paper products (Rosenberg et al.,1988b). Some paper products will contain up to 20% limestone fillers (Elkeles et al.,1994). A grinding and dispersing agent is required to process limestone for use as filler. Biodispersan has increased grinding efficiency resulting in its use by paper, paint, and ceramics industries (Elkeles et al., 1994). The surface properties of limestone are changed in the presence of biodispersan, enough to increase its dispersability in water solutions (Elkeles et al., 1994). The dispersion properties of biodispersan are due to at least two ionizable groups: uronic acid (pK_a =3.1) and an amino sugar (pK_a=8.0) (Rosenberg et al., 1988b). When the pH of a solution is above the pK_a of the amino group, biodispersan will bind to the surface of limestone particles creating a strong negative charge on the surface (Rosenberg et al., 1988b). Because limestone naturally carries a negative charge, an increase in repulsion between limestone particles will occur, permitting the limestone to remain dispersed in water.

Objectives

Despite their psychrotrophic nature, *Acinetobacter* spp. presently under investigation are studied primarily for lipolytic enzymes, biosurfactant production and their role in the treatment of wastewater. Although studies have examined temperature optima for enzyme activity, the physiological adaptations utilized by these bacteria for low-temperature growth have not been extensively investigated. Recently, Yamashita et al. (1998) reported the production of antifreeze-like proteins produced by an *Acinetobacter* sp., KINI-1, during growth at low temperatures indicating one possible mechanism used by *Acinetobacter* for growing at low temperatures. In this study, the effects of low temperatures on different physiological characteristics of an *Acinetobacter* sp. HH1-1 were investigated. In addition, the effects of various carbon sources on the ability of this strain to grow at low temperatures and to respond to a rapid decrease in temperature were also investigated. This research will add to the current understanding of bacterial growth at low temperatures in addition to elucidating the mechanisms used by *Acinetobacter* spp. for low-temperature growth. Exploiting *Acinetobacter* abilities to grow at low temperatures could lead to additional applications in industry, biotechnology and bioremediation.

The objective of this study was to identify the possible adaptations that allow *Acinetobacter* HH1-1 to grow at low temperatures. Three microbial physiological characteristics were investigated. Cell membrane properties were examined by monitoring membrane permeability, membrane fluidity, and fatty acid composition. Second, the effects of low temperatures on the activity and site of action of isocitrate lyase, esterase, and lipase were examined. In addition, because esterase activity has been linked with biosurfactant activity, the effects of low temperatures on biosurfactant production were also examined. Third, protein synthesis and the cold shock response were also characterized using two-

dimensional polyacrylamide gel electrophoresis (2-D PAGE), and a search for a *cspA* homolog was conducted. Finally, preliminary results in the present study revealed that carbon source greatly affected the ability of these cells to grow at low temperatures. Consequently, all experiments were repeated using acetate, Tween 80, or olive oil as sole sources of carbon, and effects of these on the different physiological characteristics were investigated.

Bacterium:

HH1-1 was isolated from a sediment sample removed from Hamilton Harbour, Hamilton, Ontario. Results from fatty acid analysis (Microbial I.D. Inc., Newark, DE, USA) identified HH1-1 as belonging to the genus *Acinetobacter*. This result was confirmed using the Biolog System (Biolog Inc., Hayward CA, USA) for bacterial identification.

Maintenance of Culture:

HH1-1 was grown at 25°C in Basal Salts Medium (BSM) (4.3 g K_2HPO_4, 3.4 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.34 g $MgCl_2 \cdot 6H_2O$, 0.6 mg $FeSO_4.7H_2O$, 26 mg $CaCl_2.H_2O$, 2 mg $NaMoO_4 \cdot 2H_2O$, 1.0 L H_2O, pH 7.0) (Furukawa et al., 1983) supplemented with 0.1% (w/v) Na-acetate (J. T. Baker Chemical Co., Phillipsburg, N.J., USA) as the sole source of carbon. At mid-exponential phase, sterile glycerol (20% v/v final concentration) was added. One-ml aliquots were dispensed into sterile cryogenic tubes, which were stored at -80°C. To ensure purity of the culture throughout the study, a frozen culture was thawed at room temperature prior to its use in the different experiments and whenever the purity of the culture was believed to be compromised.

Media and Culture Conditions:

HH1-1 was grown in 250-ml Erlenmeyer flasks containing 50 ml BSM with acetate (0.1% w/v), Tween 80 (0.2% v/v) (BDH Inc., Toronto, Canada) or refined olive oil (0.2% v/v) (Sigma Chemical Co., St. Louis, MO, USA) as the sole source of carbon. Acetate and Tween 80 were added to BSM prior to sterilization. Olive oil was steam treated for 30 min

on 3 consecutive days and aseptically added to sterile BSM. BSM containing acetate (0.1% w/v) plus vitamin supplementation (biotin 0.5 μg/ml, niacin 1.0 μg/ml, calcium pantothenate 1.0 μg/ml, pyridoxine HCl 1.0 μg/ml, thiamine HCL 1.0 μg/ml-final concentrations) (Provence & Curtiss III, 1994) was used for growth temperature range study only. Flasks were incubated while shaking at approximately 150 revs/min.

Growth was determined spectrophotometrically by monitoring turbidity at O.D=650nm. The generation time (g) was determined from the linear portion of a semi-logarithmic plot of the growth data constructed using turbidity measurements (Appendix I). Growth data was collected for triplicate samples.

Agar plates were prepared by adding 1.5% (w/v) agar to BSM. Carbon sources were added to their appropriate final concentrations prior to autoclaving.

Cold Shock Conditions:

Cultures were grown at 25°C until mid-exponential phase was reached. At this point, 25-ml aliquots of the cultures were aseptically transferred to empty sterile 250-ml Erlenmeyer flasks cooled to 5°C. The cultures were maintained at the new lower growth temperature. Samples were removed at various times following the decrease in growth temperature, and different parameters were measured and compared to 25°C mid-exponential control samples and to cultures grown at 25°C and 5°C. For all cold shock experiments, the data points at 0 h represent the data observed for the 25°C mid-exponential control samples.

Growth Temperature Range Studies:

HH1-1 was grown in BSM, with vitamin supplementation and acetate (0.1% w/v) as the sole source of carbon. For growth temperatures ranging between 0°C-10°C, incubation took place in water bath shakers (New Brunswick Scientific Co. Inc., Edison, NJ, USA). At higher growth temperatures (15°C-37°C), incubations took place in Isotemp Incubators (Fisher Scientific, Nepean, ON., Canada).

Additional growth temperature studies were conducted at 25°C, 5°C, 0°C and during a 25°C to 5°C cold shock. HH1-1 was grown in BSM containing acetate (0.1%), Tween 80 (0.2%) or olive oil (0.2%) as the sole source of carbon. An Isotemp Incubator (Fisher Scientific Co. Inc.) was used for growth at 25°C, while waterbath shakers (New Brunswick Scientific) were used for 5°C and 0°C growth temperatures.

Cell Membrane Permeability Studies:

Samples removed for analyses during growth at 25°C and 5°C were taken from culture vessels at times corresponding to early-exponential, mid-exponential, late-exponential and early-maximum stationary phases. For cold shock conditions, samples were removed from the culture vessels at mid-exponential growth at 25°C prior to transferring culture to 5°C and at 2 h, 4 h, 6 h, 24 h post cold shock and at mid-exponential growth phase post cold shock.

Extracellular Potassium Concentrations:

Potassium (K^+) concentrations were monitored in cell culture supernatant following a 25°C to 5°C decrease in growth temperature. HH1-1 was grown in BSM containing acetate,

Tween 80 or olive oil. One-ml samples were removed from the culture vessel at 2 h, 4 h, 6 h, 24 h (acetate-, Tween 80- and olive oil-grown cells), 50 h (acetate-grown cells) and 72 h (Tween 80- and olive oil-grown cells) post shock. Samples were centrifuged at 14,000 x g for 10 min in a microcentrifuge. The supernatant was removed and analyzed for K^+ using a K^+ specific ion meter probe (Horiba Cardy Compact Ion Meter, Horiba Instruments Inc., Irvine CA, USA). Potassium concentrations for cold shocked samples were compared to the 25°C mid-exponential control samples processed as described above. All experiments were conducted in triplicate.

Fluorescence Polarization Studies:

Membrane fluidity was monitored during growth at 25°C, 5°C, and after a 25°C to 5°C cold shock by measuring the fluorescence polarization of *trans* and *cis* parinaric acid. HH1-1 was grown in BSM containing acetate, Tween 80, or olive oil as the sole source of carbon. During growth at 25°C, two1.0-ml aliquots of culture were removed at 3 h, 5 h, 6 h, and 7 h (acetate-grown cells), at 2 h, 4 h, 6 h, 8 h, and 10 h (Tween 80-grown cells), and at 3 h, 5 h, 7 h, 9 h, and 11 h (olive oil-grown cells). During growth at 5°C, two1.0-ml aliquots of culture were removed at 30 h, 48 h, 54 h, 72 h, and 78 h (acetate-grown cells), and at 11 h, 24 h, 48 h, 72 h, 100 h, and 140 h (Tween 80- and olive oil-grown cells). Cold shocked samples were removed at 2 h, 4 h, 6 h, 24 h, and 50 h (acetate-grown cells) or 72 h (Tween 80- and olive oil-grown cells) after cold shock and compared to 25°C mid-exponential samples. Cell suspensions were washed by centrifuging the suspensions at 14,000 x g for 10 min, removing supernatant and resuspending cell pellet in 20 mM HEPES (N-[2-hydroxyethyl piperazine]-N'-[2-ethanesulfonic acid]) (pH 7.0) buffer. This procedure was performed twice. Ten µl of

either *trans* or *cis* parinaric acid (4 mM) were added to washed cell suspensions that had been resuspended in a final volume (1 ml) of HEPES buffer. Samples were incubated at either 25°C or 5°C for 30 min to allow the probe to partition into the cell membrane. To inhibit *de novo* synthesis of fatty acids during the 30-min incubation, cerulenin (5 μg/ml final concentration) was added to the suspensions and incubated for 10 min prior to the addition of probe.

Fluorescence was monitored using a polarizing spectrofluorometer (model SLM Smart 8000, Urbana-Champaign, IL, USA). Excitation and emission monochrometers were set at 325 nm and 410 nm, respectively. The excitation beam was vertically polarized using a 10-mm Glan-Thompson calcite prism. Fluorescence intensity (I) was measured with the emission polarizer parallel (II) and perpendicular (⊥) to the excitation polarizer. All fluorescence measurements were taken at room temperature. The polarization ratio (II/⊥) was plotted against time. All experiments were conducted in triplicate.

Fatty Acid Analysis:

Changes in the fatty acid composition of cells were monitored during growth at 25°C, and at 5°C and after a 25°C to 5°C cold shock. During constant growth at 25°C, 1.0 ml of cell suspension was removed from the culture flask at 3 h, 5 h, 6 h, and 7 h (acetate-grown cells), at 2 h, 4 h, 6 h, and 9 h (Tween 80-grown cells), and at 3 h, 5 h, 9 h, and 11 h (olive oil-grown cells). During constant growth at 5°C, 1.0 ml of cell suspension was removed at 24 h, 48 h, 58 h and 72 h (acetate-grown cells) and at 10 h, 24 h, 72 h and 140 h (Tween 80- and olive oil-grown cells). Cold shocked cell suspensions were removed at 2 h, 4 h, 6 h , 24 h and 50 h (acetate-grown cells) or 72 h (Tween 80- and olive oil-grown cells) and compared

to 25°C mid-exponential control samples. Cell suspensions were washed twice and resuspended in 20 mM HEPES as described for the fluorescence polarization study. Total lipids were extracted from cells by the method described by Bligh and Dyer (1959). Lipids were subjected to methanolysis for 1 h at 90°C in boron trifluoride methanol (1.0 ml). The resultant fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography (Hewlett-Parkard Co., Palo Alto, CA, USA) using a flame-ionization detector and fused silica capillary column (15 m × 0.25 mm I.D., 0.20-μm film thickness) (Supelco, Inc., Bellefonte, PA, USA). Flow rate was set at 1.0 ml/min, the column temperature was 180°C and the injector and detector temperatures were set at 250°C (Fobel et al., 1987). Five μl of the internal standard, fatty acid 19:0 (10 mg/ml) was added prior to analysis. Fatty acids were identified by comparison of the retention times with those of standards. Relative proportions of fatty acids were calculated based on percent total peak area. All experiments were conducted in triplicate.

Statistical Analysis:

Statistical analysis was performed on raw data collected for K^+ concentrations, fluorescence polarization of parinaric acid and fatty acid analysis to determine significant changes in extracellular K^+ polarization ratios and fatty acid composition in carbon and incubation treatments. The repeated measures design analysis of variance (ANOVA) was used for all data. Data for 25°C and 5°C data was analyzed together while cold shock data was analyzed separately. Separate analysis of the incubation treatments was required because cold shock data were collected at additional time points. The normality assumption

and homogeneity of variance was checked by plotting residuals versus expected values and plotting residuals versus treatments, respectively.

Once the ANOVA had been carried out and significant effects determined, paired comparisons between means (protected least significant difference) were conducted for comparisons of interest between the independent variables (carbon and incubation treatment) and the dependent repeated measure factors (time). The p-value associated with an F-ratio was used as an indicator of significance. Because a repeated measures design was implemented, in which repeated measures were not randomly allocated, the Greenhouse-Geisser (G-G) adjusted p-value was used when the G-G epsilon was estimated to be less than 0.8000 (Winer, 1971). The calculated *t*-statistic (t_{calc}) was determined and compared to the critical t' value (t'_{crit}) determined at $\alpha(2)$ and p=0.10, 0.05 and 0.01 (Appendix II) (Steel and Torrie, 1980).

All statistical analysis was conducted using the statistical software SYSTAT Version 5.03 (Wilkinson, 1989).

Esterase, Lipase and Isocitrate Lyase Activity Measurements:

Esterase, lipase and isocitrate lyase activity were measured during constant growth at 25°C and 5°C and during a 25°C to 5°C cold shock. HH1-1 was grown in 50 ml of BSM containing acetate (esterase and isocitrate lyase), Tween 80 (esterase and lipase) or olive oil (esterase and lipase) as sole sources of carbon. During growth at 25°C, 5 ml of culture were removed at 0 h, 3 h, 5 h, 7 h and 10 h (acetate-grown cells), at 0 h, 3 h, 5 h, 9 h and 11 h (Tween 80-grown cells) and at 0 h, 3 h, 6 h, 9 h and 12 h (olive oil-grown cells). During constant growth at 5°C, 5 ml of culture were removed at 0 h, 24 h, 50 h, 72 h and 80

h (acetate-grown cells), at 0 h, 11 h, 30 h, 74 h, and 144 h (Tween 80-grown cells) and at 0 h, 11 h, 53 h, 77 h, 171 h (olive oil-grown cells). Cold shocked samples were removed at 2 h, 8 h, 20 h, 26 h and 44 h (acetate-grown cells), and at 2 h, 20 h, 44 h, 140 h and 308 h (Tween 80- and olive oil-grown cells) and compared to 25°C mid-exponential control samples. Extracellular enzyme activity was monitored in the culture supernatant which was removed following centrifugation of the culture at 12,000 x g for 15 min at 5°C. The resulting cell pellet was washed twice and resuspended in 20 mM-HEPES buffer (pH 7.0). The cell suspension was sonicated for 6 min on ice (1-min intervals) using an ultra tip sonicator (Sonic Systems, Inc., Newtown, PA). This suspension was centrifuged at 12,000 x g for 5 min to remove unbroken cells and cellular debris. Cell-associated enzyme activities were monitored in the resulting supernatant. All experiments were conducted in triplicate.

Esterase Activity

Esterase and lipase are distinguished from each other based on their ability to hydrolyze different fatty acids. Often, these enzymes have overlapping substrate specificities that can cause difficulty in an accurate identification of the enzyme. For the purposes of this study, esterases were operationally distinguished from lipases based on the ability of cell culture supernatant and cell sonicate to hydrolyze either *p*-nitrophenylacetate (esterase) or *p*-nitrophenylpalmitate (lipase).

Esterase activity was measured as described by Kok et al. (1993). A substrate assay solution was prepared by adding 100 µl of *p*-nitrophenyl acetate (*p*NPA) stock solution (36 mg dissolved in 1 ml methanol) to 20 ml of 20 mM-HEPES buffer (pH 7.0) yielding a final substrate concentration of 1 mM. The reaction was started by adding 200 µl of culture

supernatant or cell sonicate to 1.8 ml of substrate assay solution (final volume of 2 ml). The reaction was monitored spectrophotometrically (Pharmacia-Ultra Spec Plus, Pharmacia Biotech, Ste-Anne-De Bellvue, Que., Canada) by measuring the change of absorbance at 410 nm over a 20-min interval. Enzyme activity was calculated from the rate of *p*-nitrophenol formation. An increase in asorbance of 0.100/min=1 Unit of enzyme activity.

Lipase Activity

Lipase activity was measured as described by Kok et al. (1993). The substrate assay solution was prepared by adding 2.0 ml of *p*-nitrophenol palmitate (pNPP) stock solution (37.5 mg in 2.0 ml propanol) to 50 ml of buffer containing 50-mM Tris/HCl (pH 8.0) and 0.1% Triton X-100, yielding a final substrate concentration of 2 mM. The reaction was started by adding 200 µl of cell culture supernatant or cell sonicate to 1.8 ml of substrate assay solution (final volume of 2.0 ml). Activity was calculated as outlined for esterase activity.

Isocitrate Lyase Activity

Isocitrate lyase activity was measured by the phenylhydrazine method described by Kleman and Strohl (1994). The substrate assay solution contained 300 µl $MgCl_2$, 10 µmol; 300 µl cysteine-HCl, 5 µmol; 300 µl phenylhydrazine-HCl, 10 µmol; 800 µl KH_2PO_4-K_2HPO_4 buffer, 64.5 µmol (pH 7.4); and 300 µl isocitrate, 10 µmol. The reaction was started by adding 1 ml of cell culture supernatant or cell sonicate to 2.0 ml of substrate assay solution (final volume of 3.0 ml). The reaction was monitored spectrophotometrically by measuring the change in absorbance at 324 nm over a 20-min interval. Activity was

calculated from the rate of glyoxylate phenylhydrazone formation produced from isocitrate and phenylhydrazine. Activity was calculated as outlined for esterase activity.

Protein Content:

Protein concentration was measured in cell culture supernatant and cell sonicate from cells grown at 25°C, 5°C and after a 25°C to 5°cold shock. HH1-1 was grown in 50 ml of BSM containing acetate, Tween 80 or olive oil. During growth at 25°C, 5 ml of culture were removed at 0 h, 3 h, 5 h, 7 h and 10 h (acetate-grown cells), at 0 h, 3 h, 5 h, 9 h, and 11 h (Tween 80-grown cells) or at 0 h, 3 h, 6 h, 9 h, and 12 h (olive oil-grown cells). During constant growth at 5°C, 5 ml of culture were removed at 0 h, 24 h, 72 h and 80 h (acetate-grown cells), at 0 h, 11h, 30 h, 74 h, and 144 h (Tween 80-grown cells) or at 0 h, 11 h, 53 h, 77 h and 171 h (olive oil-grown cells). Cold shocked samples were removed at 2 h, 8 h, 20 h, 26 h and 44 h (acetate-grown cells) or at 2 h, 20 h, 44 h, 140 h and 308 h (Tween 80- and olive oil-grown cells) and compared to 25 °C mid-exponential controls. Cell suspensions were centrifuged for 10 min at 12,000 x g. Supernatant was removed and assayed for protein content. The resulting cell pellet was resuspended in 20 mM HEPES buffer (pH 7.0) and sonicated for 6 min in 1.0 min intervals at 35 watts. To remove unbroken cells and cellular debris sonicated samples were centrifuged at 14,000 x g. The amount of protein was measured in the supernatant. Protein content was quantified spectrophotmetrically using the Bio-Rad Protein Assay (Bio-Rad Laboratories Mississauga, Ont., Canada) based on the method of Bradford (1976). Protein content was determined by monitoring absorbance at 510 nm. Absorbance units were converted to μg/ml protein with the aid of a standard curve prepared using bovine serum albumin. All experiments were conducted in triplicate.

Esterase Substrate Specifity:

Esterase substrate specificity was assessed in cell culture supernatant and cell sonicate samples. HH1-1 was grown in 50 ml of BSM containing Tween 80 (*p*-nitrophenyl ester substrates) and acetate, Tween 80 and olive oil (naphthyl ester substrates). Cultures were grown at 25°C until mid-exponential growth was reached. Twenty-five ml of culture were aseptically transferred to empty 250-ml sterile flasks cooled to 5°C (cold shocked). Five ml of the 25°C mid-exponential control culture were removed, processed and assayed for substrate specificity. After 50 h at 5°C, 5 ml of the cold-shocked culture were removed, processed and assayed for substrate specificity. The 5-ml samples were centrifuged at 12,000 x g for 15 min at 5°C. Extracellular esterase was monitored in the supernatant. The cell pellets were washed twice and resuspended in 5 ml of 20-mM HEPES buffer (pH 7) and sonicated as previously described. The sonicated suspension was centrifuged at 12,000 x g for 5 min at 5°C to remove unbroken cells and cellular debris. Cell-associated esterase was monitored in the resulting supernatant.

p-Nitrophenyl Ester Substrates

Specificity assays were conducted by measuring the formation of nitrophenol from different *p*-nitrophenyl ester substrates (Kok et al., 1993). Substrates included *p*-nitrophenyl acetate, palmitate, butyrate, caprylate, N-carboxybenzyloxy (CBZ) lysine and N-CBZ phenylalanine. Substrate stock solutions were prepared by dissolving 40 mg of substrate in methanol (1 ml). Assay mixture was prepared by adding 100 µl of substrate stock solution to 20 ml of 20 mM-HEPES buffer (pH 7). The reactions were initiated by adding 200 µl of supernatant or cell sonicate suspension to 1.8 ml of assay mixture. The reaction was

followed monitoring the change in absorbance at 410 nm over a 20-min interval. Activity measurements were determined as previously described for esterase activity. Experiments were conducted in triplicate.

Naphthyl Ester Substrates

Before assaying cell culture supernatant and cell sonicate samples for esterase substrate specificity, fractions were concentrated using NanoSpin Plus centrifugal ultrafiltration with a molecular mass cut off of 10 kDa (GelmanSciences Inc., Montreal, Que., Canada). After centrifugation at 13,000 x g for 30 min at 5°C, concentrated sample was removed from the tube and used for gel electrophoresis. Specificity assays were conducted using a modified method described by Rosenberg et al. (1975). Proteins were separated by non-denaturing gel electrophoresis using a mini-PROTEAN II electrophoresis apparatus (Bio-Rad Laboratories Ltd., Mississauga, Ont. Cananda). A 7% acrylamide separation gel (5 ml lower gel buffer (1.5 M Tris, pH 8.8), 4.6 ml 30% acrylamide, 10 ml 75% glycerol, 9.2 ml H_2O) was prepared, poured into glass plates using 0.75-mm spacers and allowed to polymerize for 45-60 min after adding 30 µl of 10% ammonium persulphate and 10 µl N, N, N', N' tetramethylethyldiamine (TEMED). After polymerization, a 3% stacking gel (1.25 ml upper gel buffer (0.5 M Tris pH 6.75), 0.5 ml 30% acrylamide, 3.25 ml H_2O, 15 µl of 10% ammonium persulphate, 10 µl TEMED) was prepared and poured onto the separation gel. A 0.75-mm 10-well comb was inserted into the stacking gel. The gel was allowed to polymerize for 20 min, the comb was gently removed and the wells washed with tank buffer (10x stock solution: 288.4 g glycine, 60.3 g Tris, and 2 L H_2O). Thirty µl of sample (either concentrated cell culture supernatant or concentrated cell sonicate) were

mixed with 5 µl of tracking dye (20% glycerol, 0.125 M Tris (pH 6.8), and 0.01% bromophenol blue). Twenty-five µl of sample (triplicate samples of cell culture supernatant and cell sonicate) were loaded onto the gel. Tank buffer was added to the apparatus. The gels were run at 50 V for 5 h at 5°C.

A gel was prepared for each substrate tested. The gels for esterase staining were prepared as follows. After electrophoresis, the gels were removed from the glass plates and placed in 0.05 M KH_2PO_4-K_2HPO_4 buffer for 15 min at room temperature. The gels were then transferred to the substrate-coupler staining solution and incubated for 1 h at room temperature. The reaction was stopped by removing the staining solution and replacing it with 2% acetic acid. The substrates used in this study included α-naphthyl acetate, butyrate and laurate, and β-naphthyl acetate, butyrate and laurate. The substrate-coupler solutions were prepared by adding 4 ml of substrate stock solution (20 mg substrate dissolved in 4 ml N,N-dimethylformamide) to 36 ml of phosphate buffer containing 20 mg of Fast Blue D Salt (ICN Biomedicals Inc. Aurora, OH USA).

Additional gels were prepared and stained for protein. After electrophoresis, gels were placed into Coomassie Blue protein stain (0.15% Coomassie Blue, 10% acetic acid, 40% methanol) and incubated at 60°C for 10 min. The gels were then transferred to destaining solution (10% acetic acid, 40% methanol) and incubated at 60°C for 30 min. Colour photographs were taken of protein and esterase gels using Kodak-Gold colour film 100 speed with the aid of a light box.

Surface Tension Measurements:

Surface tension of culture supernatant, removed from acetate-, Tween 80- or olive oil-grown cultures, was measured during growth at 25°C, 5°C and after a 25°C to 5°C cold shock. During constant growth at 25°C, 10 ml of cell suspension were removed from flasks at 2 h, 3 h, 5 h and 9 h (acetate-grown cells) and at 2 h, 4 h, 7 h, 10 h (Tween 80- and olive oil-grown cells). During constant growth at 5°C, 10 ml of cell suspension were removed at 24 h, 48 h and 72 h (acetate-grown cells) and at 8 h, 24 h, 72 h and 140 h (Tween 80- and olive oil-grown). Cold shocked cell suspensions were removed at 2 h, 4 h, 6 h, 24 h, 50 h (acetate-grown cells) or 72 h (Tween 80- and olive oil-grown cells) and compared with 25°C mid-exponential control samples. Suspensions were centrifuged at 12,000 x g for 10 min at 5°C. Supernatant was decanted into 50-ml beakers and surface tension was measured using a Fisher Surface Tensiomat Model 21 (FisherScientific, Nepean, ON., Canada). The beaker was placed on the sample table positioned beneath the palladium-titanium ring that was attached to the torsion arm. The ring was immersed in the supernatant 4 cm below the surface by lowering the torsion arm and the scale reading adjusted to zero. After the index arm and image were aligned with the reference, the vernier was adjusted to zero. The sample table was then lowered, keeping the index and image in alignment, until the film at the surface broke. The scale reading, measuring apparent surface tension, was recorded then converted to absolute surface tension after multiplying the scale reading by the correction factor (F) calculated as follows:

$$F = 0.7250 + \sqrt{\frac{0.01452(P)}{C^2(D-d)} + 0.04534 - \frac{1.679r}{R}}$$

where: (F) is the apparent surface tension reading, (R) is the radius of the ring, (r) is the radius of the wire of the ring, (C) is the circumference of the ring, (D) is the density of the lower phase and (d) is the density of the upper phase (FisherScientific, Nepean, ON, Canada).

All experiments were conducted in triplicate.

Partial Characterization of HH1-1 Emulsifier:

HH1-1 was grown in BSM with .2% (v/v) olive oil as sole source of carbon. Cultures were incubated for 48 h at 25°C while shaking at 150 revs/min. Fifteen-ml of culture were removed and cells were separated from supernatant by centrifugation at 12,000 x g for 15 min at 5°C. Supernatant was collected and concentrated using a NanoSpin Plus centrifugal ultrafiltration device as previously described. The pH of the concentrated supernatant was adjusted to 2.0 with 1 N HCl prior to extracting the supernatant with an equal volume of chloroform:methanol (2:1). The solvent layer was removed and resulting aqueous phase extracted once again with chloroform:methanol. The solvent layer was removed and combined with the previous extract. The extract was concentrated under vacuum using a rotoevaporator (Evapotec Rotary Evaporato, Haake Buchler Product, Berlin, Germany). Concentrated extract was spotted onto 3 separate Baker Si250F Thin Layer Chromatography (TLC) plates (JT Baker, Phillipsburg, NJ, USA). Pure rhamnolipid, isolated from *Pseudomonas aeruginosa* UW-1 (Sim et al., 1997) and sterile olive oil were used for comparison. Plates were developed for 10 min in a chloroform:methanol:water solvent

system (65:15:2) and visualized with TLC reagents. One plate dipped into α-naphthol-H_2SO_4 acid (Molish Reagent) and then hot air-dried was used to detect sugars, a second plate was exposed to iodine vapour used to visualize lipids, and a third plate was sprayed with orcinol (20 mg orcinol in 100 ml 75% H_2SO_4) to detect amino groups.

One-Dimensional and Two-Dimensional Polyacrylamide Gel Electrophoresis:

Radiolabelling of Proteins

Whole cells

Cells subjected to a 25°C to 5°C cold shock were grown in BSM containing acetate, Tween 80 or olive oil. Cells incubated at 30°C, 25°C, 20°C, 15°C, 10°C, and 5°C were grown in BSM with vitamin supplementation and acetate as the sole source of carbon. When mid-exponential growth was reached, 200 µl of cell suspension were removed and aseptically transferred to sterile 1.5-ml Eppendorf tubes. For cold shock experiments, 200 µl of cell suspension were removed from the 25°C mid-exponential phase control cultures, prior to aseptically transferring 25 ml of culture to empty sterile 250-ml Erlenmeyer flasks preacclimated to 5°C. At 2 h and 72 h (acetate-grown cells) and 140 h (Tween 80- and olive oil-grown cells) post cold shock, 200 µl of culture were transferred to sterile 1.5-ml Eppendorf tubes. To label proteins, mid-exponential samples and cold shocked samples were incubated at either 25°C or 5°C for 1 h with 150 µCi/ml of ^{35}S-L-methionine (ICN, Biomedical Canada Ltd. Montreal, Que. Canada). Radiolabelling was stopped by centrifuging cells at 14,000 x g for 5 min in a microcentrifuge at 4°C. Supernatant was discarded and pellets stored at –20°C or lysed for either 1- or 2-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Cell Fractions

Two-ml samples were removed from 25°C mid-exponential controls, 2 h and 72 h (acetate-grown cells) and at 140 h (Tween 80- and olive oil-grown cells) after cold shock and transferred to sterile 15-ml centrifuge tubes. Proteins were radiolabelled as described above. After 1 h at either 25°C or 5°C, radiolabelling was stopped by centrifugation at 1,100 x g for 10 min. The supernatant was removed, transferred to a sterile 1.5-ml Eppendorf tube, stored at –20°C and later subjected to 2-D SDS-PAGE to evaluate the presence of radiolabelled extracellular proteins.

Radiolabelled periplasmic proteins were released using the method described by Ames et al. (1984). After a brief vortexing to resuspend the cell pellet in residual medium, 20 µl of chloroform ($CHCl_3$) were added. After a 15-min incubation at room temperature, 1.0 ml of 0.01 M Tris-HCl (pH 8) was added to each sample. Cells were separated from supernatant by centrifugation at 6,000 x g for 20 min. The supernatant fraction, containing radiolabelled periplasmic proteins, was removed and transferred to a sterile 1.5-ml Eppendorf tube, stored at –20°C and later used for 2-D SDS-PAGE.

Using the remaining cell pellet, radiolabelled proteins in crude cell envelope were separated from radiolabelled intracellular proteins. The pellet was resuspended in 5 ml of 20 mM HEPES buffer (pH 7) and sonicated on ice for 6.0 min in 1.0-min intervals at 35 watts. To remove unbroken cells, samples were subjected to low speed centrifugation (1,100 x g for 5 min). Supernatant containing crude cell envelope and intracellular proteins was transferred to a 3.5-ml ultracentrifuge tube and centrifuged at 45,000 x g for 20 min. Intracellular proteins contained in the supernatant were separated from the cell envelope pellet and stored at –20°C.

Cell Lysis

One-D SDS PAGE

One-D SDS PAGE was used to monitor the degree of protein labelling following cold shock in order to determine when the induction of csps occurred. Cells were labelled for 1 h as previously described after cells reached 25°C mid-exponential growth and at 1 h, 2 h, 5 h and 6 h following cold shock. Ten µl of 1.0% lysozyme (in 0.25 M Tris-HCl, pH 8.0) and 10 µl of nuclease solution (20 mM $CaCl_2$, 50 mM $MgCl_2$, 1.0 mg/ml Rnase, 1.0 mg/ml Dnase in 0.5 M Tris-HCl pH 7.0) were added to the cell pellet. The suspension was vortexed, then incubated at room temperature for 5 min prior to adding 50 µl of Electrophoretic Sample Buffer (ESB) (0.757 g Tris, 10 ml 20% SDS, 10 ml glycerol, 5 ml β-mercaptoethanol, and H_20 to 100 ml). The suspension was vortexed and incubated for an additional 20 min at room temperature to allow for the digestion of nucleic acids. Suspensions were frozen in dry ice and thawed at room temperature twice, centrifuged at 14,000 x g at 4°C for 10 s and either stored at –20°C or used directly to determine acid insoluble counts.

Two-D SDS PAGE

Cell lysis for 2-D SDS PAGE was performed as described above, with the following exceptions. Thirty µl of O'Farrell's (1975) Lysis Buffer (9.5 M urea, 2% (w/v) Nonidet-P-40, 2% 3-10 ampholytes (Bio-Rad Laboratories, Mississauga, Ontario) and 5% β-mercaptoethanol) were added to the suspension after the 5-min incubation instead of ESB. Urea (11.4 mg) was added to all samples to return the urea concentration to 9.5 M.

Acid Insoluble Radioactivity Counts and Equilibration of Samples

Counts were determined based on duplicate samples. Two µl of sample (cell extract or extracts of cell fractions) were dispensed onto glass fiber filters (Whatman GF/C, VWR Canlab Mississauga, Ont. Canada). To precipitate protein onto the filter, 3 ml cold 25% tricholoroacetic acid (TCA) containing 1 mg/ml L-methionine, then 10 ml cold 8% TCA containing 1 mg/ml L-methionine, and finally 5 ml cold 95% ethanol were passed through the filters. The filters were placed into scintillation vials (Fisher Scientific Nepean, Ont. Canada) followed by the addition of 10 ml of scintillation fluid (Beckman Ready Safe, Liquid Scintillation Cocktail, Beckman Instruments, Inc. Fullerton, CA USA). Precipitate radioactivity counts on each filter were determined using a Beckman LS7000 liquid scintillation counter (Beckman Instruments, Inc.). The duplicate samples were used to calculate the volume of sample to be added to either ESB (1-D SDS PAGE) or O'Farrell's lysis buffer (2-D SDS PAGE) to obtain 100,000 cpm for 1-D SDS PAGE, 200,000 cpm (whole cell extracts), 1,000 cpm (extracellular proteins), 2,000 cpm (periplasmic proteins), 200,000 cpm (cell envelope proteins), and 200,000 cpm (intracellular proteins) for 2-D SDS PAGE in a final volume of 20 µl. Two µl of 0.001% bromothymol blue was also added, as a tracking dye, to the samples used in 1-D SDS PAGE, and these samples were boiled for 5 min to denature the proteins. Samples were either stored overnight at –20°C or loaded onto 1-D or 2-D gels.

One-D SDS PAGE

One-D SDS PAGE was run using a Mini-PROTEAN II electrophoresis apparatus (Bio-Rad) and 10% separation gels. These were prepared with 5 ml of lower gel buffer (1.5 M Tris, pH 8.8), 7 ml of 30% acrylamide (29.2 g acrylamide, 0.8 g piperazine diacrylamide

(PDA), H_2O to 100 ml), 2.5 ml 75% glycerol, 200 µl 10% SDS, and 5.5 ml H_2O. After degassing for 15 min, 30 µl of freshly made 30% ammonium persulphate and 10 µl of N, N, N', N' tetramethylethylenediamine (TEMED) were added. The mixture was poured into glass plates set up with 0.75-mm spacers. When multiple gels were required, a mini-Protean II multi-casting apparatus (Bio-Rad) was used. The gel was poured to approximately 1 cm below the bottom of the comb wells, over-laid with 300 µl of H_2O saturated isobutanol and allowed to polymerize for 45-60 min. After polymerization, a 5% stacking gel solution (2.5 ml upper gel buffer (0.5 M Tris, pH 6.75), 1.5 ml 30% acrylamide, 6 ml H_2O, 100 µl 10% SDS) was prepared and degassed for 15 min. Thirty µl of 10% ammonium persulphate and 10 µl of TEMED were added to polymerize the stacking gel mixture. This mixture was poured onto the separation gel after the isobutanol overlay had been removed and the surface of the separation gel washed with water. A 0.75-mm, 10-well comb was inserted into the stacking gel. After polymerization (requires 25 min), the comb was removed, and the wells were washed with water. Twenty µl of the appropriate radiolabelled sample were loaded into each well using a Hamilton syringe. Tank buffer (200 ml 10x stock tank buffer (288.4 g glycine, 60.6 g Tris, 2 L H2O), 10 ml 20% SDS, H_2O up to 2 L) was added to the upper and lower chambers of the electrophoresis apparatus. The gels were run at 200 V until the dye front reached the bottom of the gels. Gels were removed from between the glass plates, the stacking gel was discarded, and the separation gel washed in water prior to suspending it in enlightening fluid (50 ml Enlightening Rapid Autoradiography Enhancer (Du Pont Boston, MA USA), 20 ml 95% ethanol, 30 ml H_2O). After a 20-min incubation, the enlightening mixture was poured off and replaced with 5% glycerol for an additional 20-min incubation. The gel was dried between filter paper (Whatman, 3mm Chromatography Paper, VWR

Canlab) and cellophane (BioRad) using a gel-slab drier (BioRad Model 583) for 50 min at 80°C. After drying, gels were placed on film (Biomax MR, Kodak, Toronto, Ont., Canada) using autoradiography cassettes (Fisher Scientific) for 2 days at –80°C. After 2 days exposure, the autoradiography cassettes were equilibrated to room temperature (25°C), then the film was developed in the dark room by exposing it for 4 min to developer (Kodak), 20 s with agitation to stop bath (5% acetic acid), and finally to fixer (Kodak) for 4 min with agitation every 30 s. The fluorograms were rinsed with H_2O and air dried.

Two-D SDS PAGE

Iso-Electric Focusing

Iso-electric focusing (IEF) was performed using the mini-PROTEAN II 2-D electrophoresis aparatus (BioRad). A 10% acrylamide solution containing 9.5 M of urea, 1.33 ml 30% IEF acrylamide (28.4 g acrylamide, 1.62 g PDA, H_2O up to 100 ml), 2.0 ml 10% Nonident-P-40, 250 µl 3-10 ampholytes (BioRad), 250 µl 5-7 ampholytes (BioRad), and 1.97 ml sterile H_2O was prepared. The gel mixture was degassed for 20 min, then 10 µl of ammonium persulphate and 10 µl TEMED were added, and the mixture was added to a multi-casting tube containing capillary tubes of 1.0-mm internal diameter. The gel was allowed to polymerize for 2 h, then the capillary tubes were removed from the multi-caster and rinsed with H_2O. Sample reservoirs were attached to the capillary tubes and filled with degassed upper tank buffer (0.1 M NaOH). The tubes were fitted into the mini-Protean II apparatus. Twenty µl of sample were added to each reservoir using a Hamilton syringe and overlayed with 25 µl of overlay buffer (3.0 g urea, 2.0 ml Nonident-P-40, 250 µl 3-10 ampholytes, 250 µl 5-7 ampholytes, 10 ml sterile H_2O). The upper chamber of the apparatus

was filled with degassed upper tank buffer to just above the level of the reservoirs. The lower chamber was filled with 800 ml of freshly made, degassed, lower tank buffer (0.001 M H_3PO_4 made from a 1 M stock solution). Gels were run at 500 V for 10 min then at 750 V for 3.5 h. The tubes were removed and stored at –20°C.

Second Dimension Separation Gel

The second dimension was run as previously described for 1-D SDS PAGE. Separation acrylamide gels (10% for cold shock experiments, 12% for temperature experiments) were prepared and poured between glass plates set up in a mini-PROTEAN multi-casting unit using 1.0-mm spacers. After polymerization (45-60 min), a 5% stacking gel was prepared and poured onto the separation gel. One-mm 2-D casting combs were inserted into the stacking gels. The separation gel was loaded by extracting the thawed tube gel, using a tube gel ejector, onto the surface with the basic top end of the tube gel closest to the molecular mass marker well. Fifty ml of ESB with 0.001% bromophenol blue tracking dye was overlayed on the tube gel. Five µl of ^{14}C-molecular mass markers (200-kDa myosin, 97.4-kDa phosphorylase, 69-kDa serum albumin, 53-kDa globulins, 22.5-kDa globulins, 12.3-kDa cytochrome C) were added to the single well. After the second dimension gel was electrophoresed at 200 V, dried between filter paper and cellophane, it was exposed to X-ray film (BioMax MR, Kodak) for 4 days at –80°C. This film was developed as described above.

Molecular Mass Determination of Proteins

The molecular mass of individual proteins was determined from 2-D SDS PAGE using the ^{14}C molecular markers. Linear regression was used to determine a best-fit line

based on distance traveled versus log molecular mass of ^{14}C markers. Molecular masses of selected proteins were then determined based on their distance traveled and the determined best-fit line.

Computing Scanning Laser Densitometry

ImageQuant software (Molecular Dynamics Version 3) and computing scanning laser densitometry were used to quantify relative protein synthesis from fluorograms. Fluorograms were scanned using the densitometer and stored in ImageQuant for quantification. Key proteins were identified on the fluorograms. Under 3x magnification and colour palette mode, changes in the intensity of the key proteins were measured as follows. First, the total intensity (intensity of all proteins on the gel except for molecular markers) was determined. The area of the entire gel was outlined using the rectangle mode. A line was drawn in the rectangle best representing the background density. Volume integration (number of pixels within the object) was performed on the entire gel to obtain the density or sum above background. Individual intensities of each key protein were determined essentially the same way except that each protein was encircled using the ellipse mode. The perimeter of the ellipse represented the background for that protein. Volume integration was performed to determine sum above background. For each key protein, percent of total protein (% of total intensity) was calculated by dividing the sum above background of that protein by that of the total protein on the appropriate gel and multipling by 100. The percent of total protein values for the same proteins under the different incubation conditions were compared. Proteins showing a 1.5-fold or greater increase in intensity compared to control conditions, based on replicate samples, were considered to

have been induced under that specific incubation condition, and were designated as either a csp or cap.

Polymerase Chain Reaction (PCR):

DNA Extraction

Inoculated LB agar plates were incubated at 25°C until well-isolated colonies were visible (24 h). Four colonies were aseptically removed from the agar plate, added to 500 µl of sterile demineralized H_2O contained in a 1.5-ml Eppendorf tube, vortexed, then boiled for 10 min. To remove cellular debris, suspensions were cooled on ice then centrifuged for 2 min at 14,000 x g. The supernatant containing DNA was aseptically transferred to a sterile 0.5-ml Eppendorf tube, stored at –20 °C or immediately used as the template for PCR. Duplicate samples of HH1-1 DNA were prepared for PCR analysis.

PCR

Five µl of extracted HH1-1 DNA were added to a sterile 500-µl microcentrifuge tube containing 45.5 µl of PCR reaction mixture (25.5 µl H_2O, 5.0 µl PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl) (Boehringer Mannheim, Laval, Que. Canada), 2.0 µl of 20.0 mM $MgCl_2$, 8.0 µl of 1.25 µM dNTP's (dATP, dCTP, dTTP, dGTP obtained from Pharmacia Biotech, Baie d'Urfe, Que. Canada), 2.0 µl of each 20 µM primer F1 and R1, and 0.5 µl of 2 U Taq polymerase (Boehringer Mannheim)). The primers were constructed from the known sequence of the *Escherichia coli csp*A gene (Goldstein et al., 1990). Primers were kindly supplied by Dr. Lyle Whyte (BRI, NRC, Montreal Quebec). Positive and negative control tubes were prepared similarly. Five µl of *csp*A DNA encoded on the pUC9 plasmid

(provided by Dr. M. Inouye, Department of Biochemistry, Robert Wood Johnson Medical School, Rutgers University of Medicine and Dentistry, NJ, USA) was added to the positive control tube and 5.0 µl of sterile distilled H_2O was added to the negative control tube.

The contents of each tube were overlayed with 50 µl of sterile mineral oil to prevent evaporation during DNA amplification. The tubes were placed into a DNA Thermal Cycler (Perkin Elmer Cetus) with PCR parameters set as follows: 1 cycle at 94°C for 5 min to denature DNA; 30 cycles at 94°C for 1 min, 57°C for 1 min to anneal primers, and 72°C for 1 min for polymerase amplification. After completion of the 30 cycles, all samples were held at 72°C for 3 min for a final extension. After the reaction was completed, sample and controls tubes were kept on ice prior to agarose electrophoresis.

Agarose Electrophoresis

Agarose electrophoresis was used to visualize amplified PCR product. Three µl of bromophenol blue (0.25% bromophenol blue, 40% (w/v) sucrose in H_2O) was added to 20 µl of PCR product (HH1-1 DNA sample, positive and negative control). Five µl of each PCR product were then loaded onto a 2.0% agarose gel (0.6 g agarose, 30 ml 0.5x Tris-Borate Electrophoresis (TBE) buffer (TBE 5x stock; 54 g Tris Base, 27.5 g boric acid, 20 ml 0.5 M EDTA, and H_2O to a final volume of 1 L, pH 8)). To confirm the molecular size of PCR products, 5 µl of HincII digested ϕX174 DNA (Pharmacia Biotech Products) was also loaded onto the gel. The gel was submerged in 0.5x TBE buffer. DNA was stained by adding ethidium bromide (0.5 µg/ml) to the buffer at the positive terminal end. The gel was electrophoresed at 80 V for 2.5 h or until the dye front had migrated to within 2.5 cm of the bottom of the gel. After electrophoresis, the gel was rinsed 3 times in H_2O to remove any

unbound ethidium bromide. PCR products were then visualized under UV light on a UV transilluminator

Results for Temperature Range and Cell Membrane Experiments

Preliminary growth experiments with HH1-1 showed that this bacterium could grow at 0°C with acetate or Tween 80 as the sole source of carbon, but it was unable to utilize olive oil at this temperature. As a result, the experiments conducted in this study were designed to compare results obtained for acetate- or Tween 80-grown cells to findings obtained for cells grown in the presence of olive oil. A detectable difference in the physiological response by olive oil-grown cells to a temperature downshift, for example, could suggest a characteristic critical to enabling this psychrotroph to grow in the cold.

Extracellular K^+ concentrations were monitored in cell culture supernatant following a 25°C to 5°C cold shock. This experiment suggested that cell membranes became leaky following a decrease in temperature. To investigate causes for the loss in membrane permeability during growth at low temperatures and after a rapid decrease in temperature, membrane fluidity and membrane fatty acid composition were monitored and compared to cells grown at their optimal growth temperature.

Growth Temperature Studies

Optimal growth temperature and growth temperature range for HH1-1 were determined using cultures grown in acetate (0.1% w/v) and vitamin supplemented BSM. HH1-1 grew at all incubation temperatures tested with the exception of 37°C, and generation times ranged from 37.2 h at 0°C to 1.2 h at 25°C (Table 1). Therefore, optimal growth for HH1-1 occurred at 25°C. Using the definition first proposed by Morita (1975) HH1-1 is a psychrotrophic bacterium. Growth temperature studies were repeated so that generation

Table 1. Generation times for *Acinetobacter* sp. HH1-1 grown at various incubation temperatures. Cells were grown in Basal Salts Medium (BSM) with vitamin supplementation and acetate (0.1% w/v) as the sole source of carbon. Values are mean ± S.D. of triplicate samples.

Temperature (°C)	Generation Time (hours)
0	37.2 ± 0.7
5	9.0 ± 0.4
10	4.3 ± 0.1
15	1.9 ± 0.1
20	1.3 ± 0.1
25	1.2 ± 0.1
30	1.4 ± 0.2
37	NG

NG=no growth

times for HH1-1 growing on acetate (0.1% w/v), Tween 80 (0.2% v/v) or olive oil (0.2% v/v) could be compared. Table 2 gives the generation times for HH1-1 growing on the three substrates. Generation times for acetate-grown cells in BSM were higher than the generation times reported when cells were grown in acetate-BSM plus vitamin supplementation. However, generation times were lower at all temperatures tested, compared to cells grown with Tween 80 and with olive oil (Table 2). Although HH1-1 was able to grow with olive oil as sole carbon source at 25°C and 5°C, it was unable to grow at 0°C. These findings prompted further investigation into the effects of substrate on the physiology of this bacterium when growing at low temperatures.

Cell Size and Cell Morphology

Size and morphology of cells were compared when HH1-1 was grown with acetate, Tween 80 or olive oil. Cells were incubated at 25°C, removed at early-maximum stationary phase and Gram stained. Figure 1 shows cells observed under bright field microscopy at 100x magnification. Average size of cells grown with acetate was 2.6 μm long by 0.8 μm wide. Cells were typically found in pairs and were rod shaped (Figure 1a). Although Tween 80- or olive oil-grown cells also occurred primarily in pairs, these cells were typically smaller in size than cells grown with acetate, measuring 1.8 μm long by 0.8 μm wide and were more coccoid in shape (Figure 1b,c).

Cold Shock Growth Curves

In this study, cold shock experiments were used as a tool to help interpret differences observed between cells incubated at 25°C and cells incubated at 5°C. Figure 2 shows growth

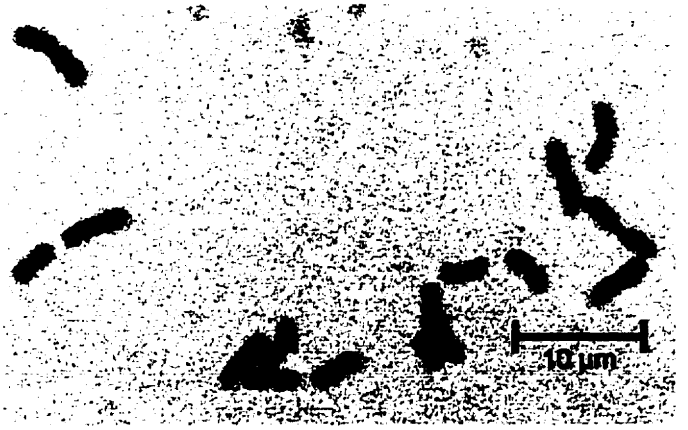
Table 2. Generation times for *Acinetobacter* sp. HH1-1grown in BSM containing acetate, Tween 80 or olive oil as the sole source of carbon. Values are mean ± S.D.

Temperature (°C)	Generation Time (hours)		
	Acetate	Tween 80	Olive Oil
0	51.80 ± 2.8	172.40 ± 6.2	N.G.
5	7.76 ± 0.2	10.52 ± 0.2	16.12 ± 2.5
25	1.05 ± 0.1	2.16 ± 0.1	2.54 ± 0.7

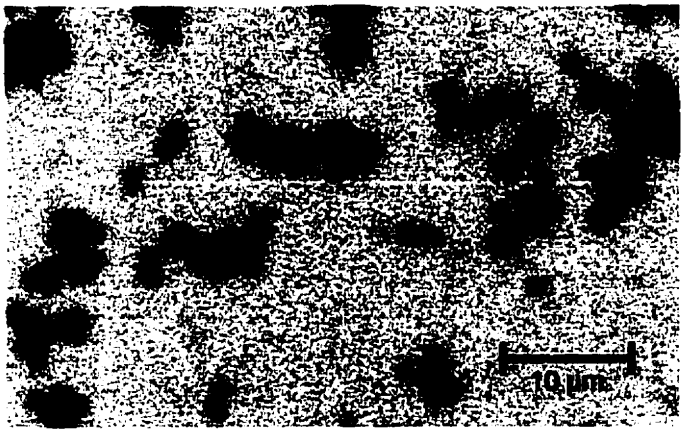
N.G=No growth

Figure 1. HH1-1 was grown in a) acetate b) Tween 80 or c) olive oil. Cells were removed from culture vessels at early stationary phase and Gram stained prior to viewing under Bright Field Microscopy (x1000).

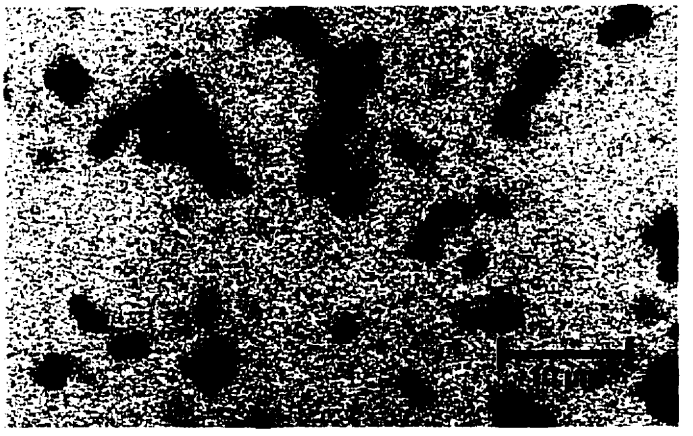
a)



b)



c)



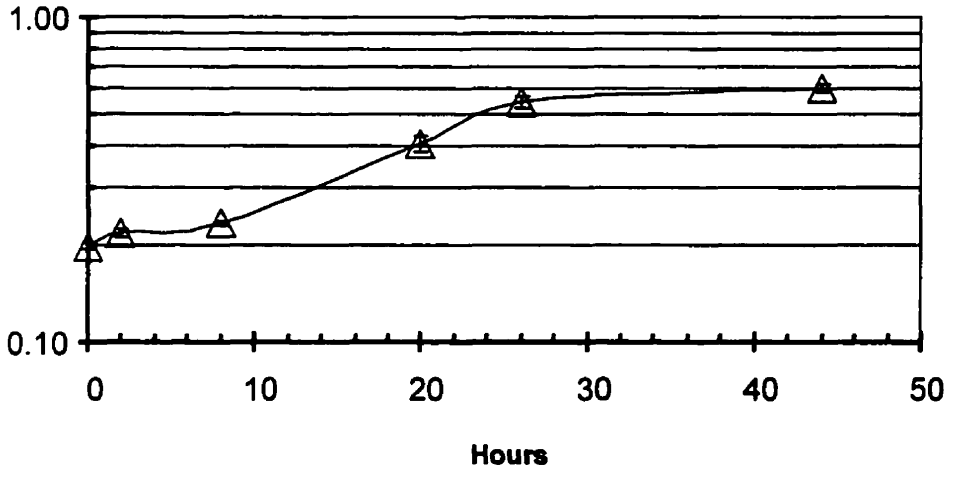
of HH1-1 following a 25°C to 5°C decrease in growth temperature. Data points at 0 h represent the 25°C mid-exponential phase control cultures prior to a decrease in growth temperature. After cold shock, growth slowed for cells supplied either acetate or Tween 80 compared to sustained growth at 25°C. Turbidity measurements remained relatively constant up to 8 h after shock. By 20 h post cold shock, growth increased as indicated by an increase in turbidity (Figure 2 a & b). In contrast, an increase in turbidity from 0.2 to 0.7 was observed over the first 2 h after cold shock for cultures grown with olive oil (Figure 2c). This was the result of the emulsification of olive oil immediately after the drop in temperature, and as a consequence, the exact duration of the lag phase could not be determined. However, cell density had started to increase by 44 h post cold shock. After the lag phase, generation times were similar to those observed for cultures growing at 5°C regardless of sole carbon source.

Extracellular Potassium Concentrations

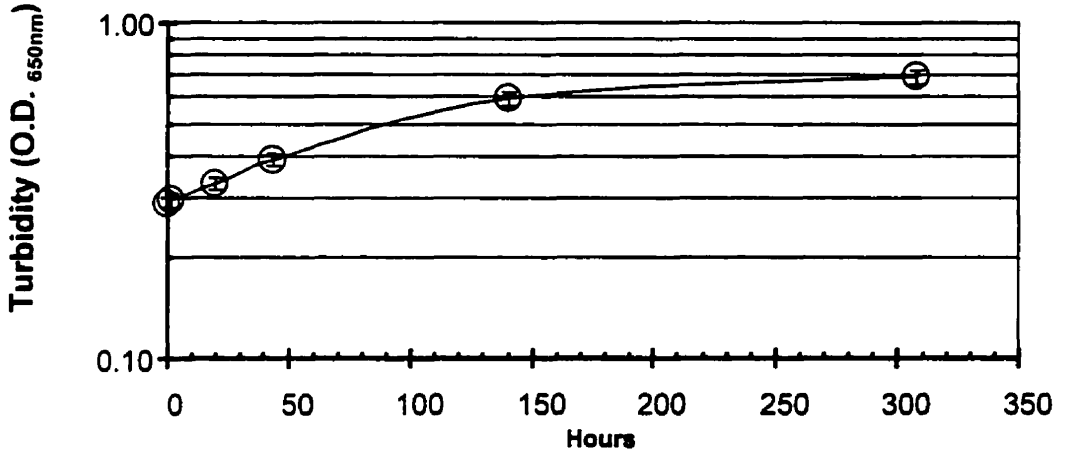
A significant increase in extracellular K^+ concentration was observed 2 h after cold shock for all carbon sources ($0.01<p<0.05$ for acetate- and olive oil-grown cells and $p<0.01$ for Tween 80-grown cells) (Figure 3, insert) (Appendix II). The greatest increase in K^+ (33 μg/ml) at 2 h post cold shock was observed for Tween 80-grown cells. Supernatant K^+ levels continued to signficiantly increase for up to 6 h post cold shock, with greater increases observed for Tween 80- (56 μg/ml) and olive oil-grown cells (46 μg/ml) compared to cells grown with acetate (12 μg/ml). Between 6 h and 24 h post cold shock, K^+ levels significantly declined ($p<0.01$) and by 24 h post cold shock, the K^+ concentration for acetate-grown cells was lower than that observed for the 25°C mid-exponential control cells (Figure

Figure 2. Growth of HH1-1 after a 25°C to 5°C cold shock. Cells were grown in acetate (△), Tween 80 (○), or olive oil (□). Growth was monitored by measuring turbidity at O.D.=650 nm. Each data point is the mean ± S.D. of triplicate samples.

a)


1.00
0.10
0
10
20
30
40
50
Hours

b)


Turbidity (O.D. 650nm)
1.00
0.10
0
50
100
150
200
250
300
350
Hours

c)

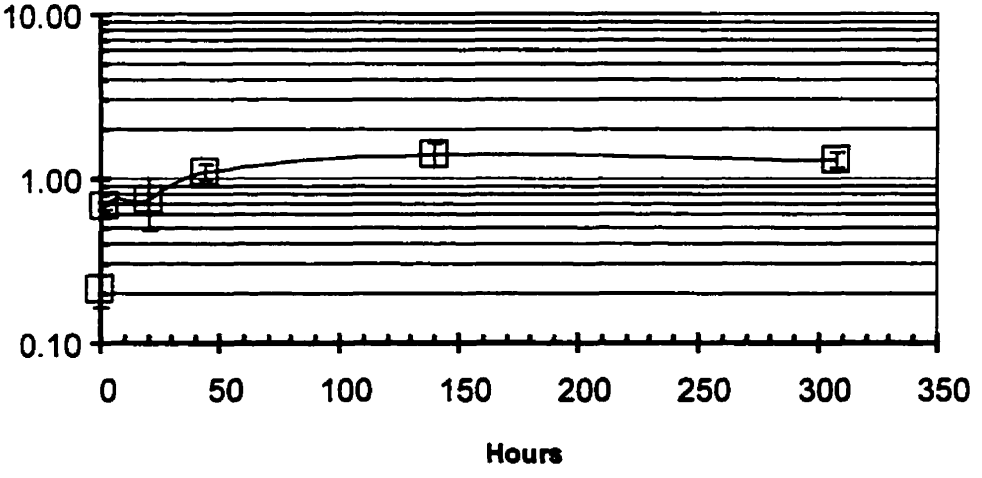
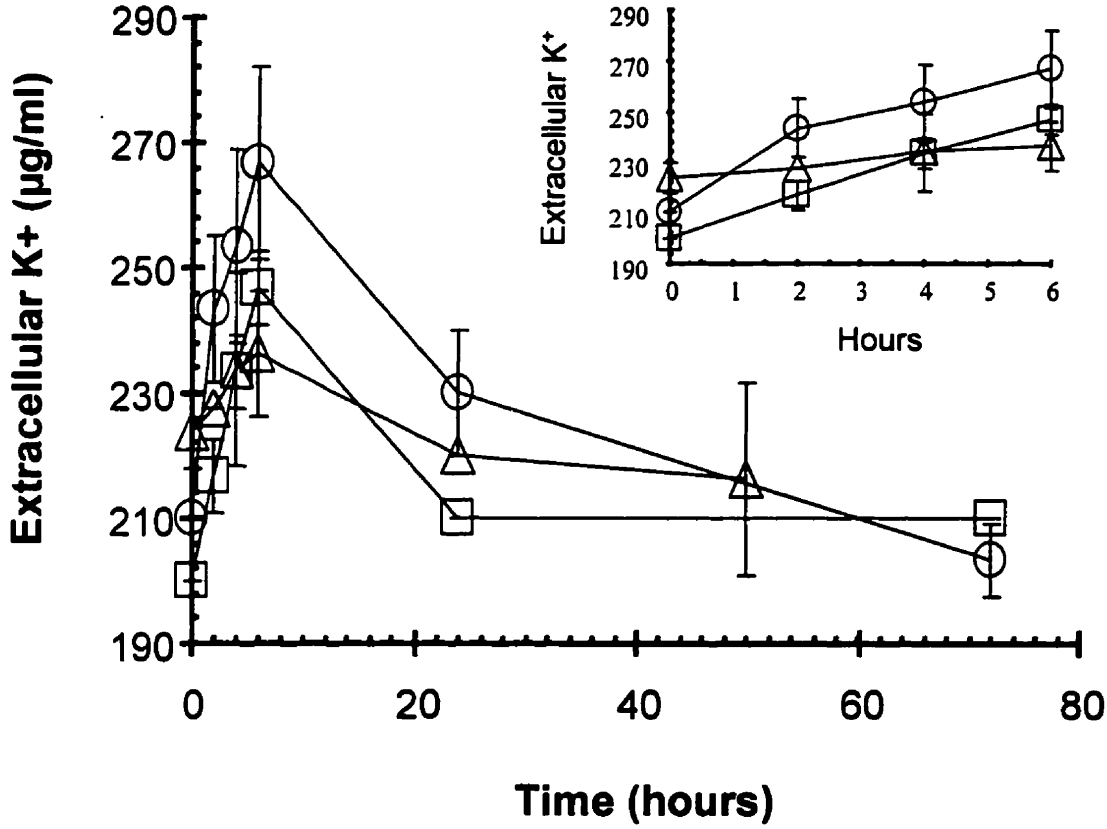

10.00
1.00
0.10
0
50
100
150
200
250
300
350
Hours

Figure 3. Changes in extracellular potassium (K^+) concentration after a 25°C to 5°C cold shock. Insert shows first 6 h following down shift. HH1-1 was grown in acetate (△), Tween 80 (○) or olive oil (□). Each point is the mean ± S.D. of triplicate samples.


Extracellular K+ (µg/ml)
290
270
250
230
210
190
0
20
40
60
80
Time (hours)
Extracellular K+
290
270
250
230
210
190
0
1
2
3
4
5
6
Hours

3). A similar trend was observed for Tween 80-grown cells except a decline to levels similar to the 25°C controls was not apparent at 24 h, although it was at 72 h post cold shock. At 72 h post cold shock, K^+ levels for olive oil-grown cells had declined, but remained higher than those observed for the 25°C controls.

Colony forming units (cfu) were also determined for 25°C mid-exponential control samples and cold shocked samples using the Standard Plate Count Method. A decrease in cfu/ml was observed after cold shock for all carbon sources tested. The number of cfu/ml decreased from 9.73×10^6 ($\pm 2.61 \times 10^6$) to 1.59×10^6 ($\pm 1.05 \times 10^6$) for acetate-grown cells, 5.73×10^6 ($\pm 8.08 \times 10^5$) to 1.83×10^6 ($\pm 8.74 \times 10^6$) for Tween 80-grown cells and from 2.18×10^7 ($\pm 2.77 \times 10^6$) to 3.67×10^6 ($\pm 1.42 \times 10^6$) for olive oil-grown cells. These results suggested that the increase in extracellular K^+ observed after cold shock could have been the result of cell lysis. To further examine this issue, an experiment was conducted to determine the K^+ content per cfu. The cfu/ml and K^+ concentration in the cell culture supernatant for 25°C mid-exponential phase cultures were determined. These cultures were then sonicated as previously described. Again, the cfu/ml and extracellular K^+ concentrations were determined for the sonicated cultures. The K^+ content per cfu was estimated by dividing the difference in K^+ concentrations between the 25°C mid-exponential controls and sonicated cultures by the difference in cfu/ml between these samples. Estimates of 4.26×10^{-6} µg, 2.02×10^{-4} µg and 9.4×10^{-5} µg K^+ per cfu were made for acetate-, Tween 80- and olive oil-grown cells, respectively. Based on these estimates, if the decrease in cfu/ml represented cell lysis, then the increase in extracellular K^+ should have been on the order of 76 µg/ml for acetate-grown cells, 788 µg/ml for Tween-grown cells and 1,701 µg/ml for olive oil-grown cells. These values are considerably higher than the measured K^+

concentration in the cold-shocked samples suggesting that the increase in K^+ was likely due to cell leakage. Because cells were debilitated after cold shock, diluting and plating of samples onto solid media could have resulted in a reduction in numbers.

The increase in K^+ concentration measured in cell culture supernatant indicated that cell membrane permeability properties were compromised following cold shock. The larger increases in K^+ levels and the longer time interval for K^+ levels to decrease again suggested that cells grown with Tween 80 and olive oil were more susceptible to cold shock.

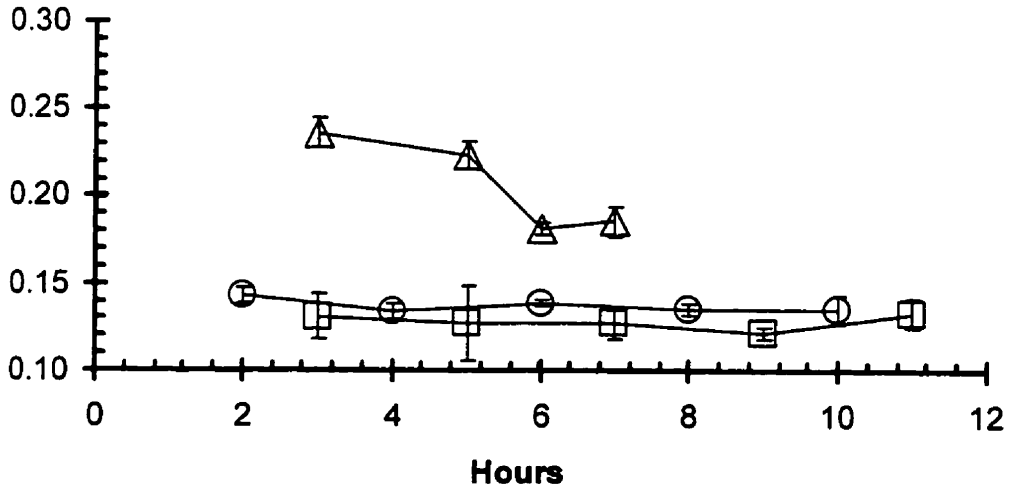
Fluorescence Polarization Measurements

Cell membrane fluidity is an important factor for the normal functioning of many cellular processes. Cells must maintain a relatively constant fluidity despite different external stimuli for the continued and efficient functioning of the cell. To study changes in membrane fluidity during growth at 25°C, 5°C and after cold shock, fluorescence polarization of the membrane probe parinaric acid (9,11,13,15-octadecatetraenoic acid) was examined (Appendix III).

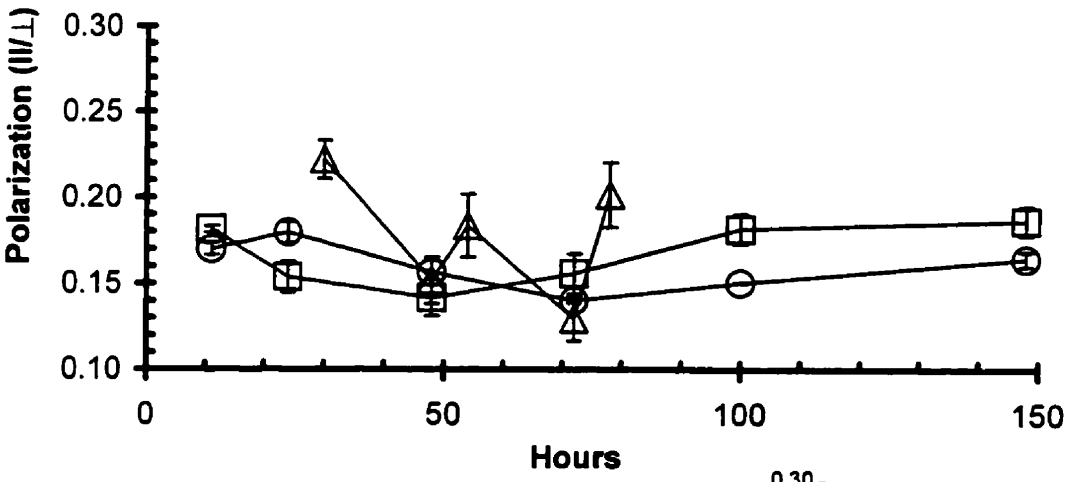
Fluorescence polarization of the *trans* isomer over time in cells from 25°C, 5°C and cold-shocked cultures are depicted in Figures 4 a-c. The polarization ratio of the *trans* isomer was significantly higher for acetate-grown cells compared to Tween 80- and olive oil-grown cells under all treatments. At 25°C, polarization decreased over time ($p<0.01$) reflecting an increase in membrane fluidity (Figure 4a). A similar trend was also observed when acetate-grown cells were incubated at 5°C, with the exception of an increase at early-maximum stationary phase (80 h) (Figure 4b). No significant changes in polarization ratios were observed for either Tween 80- or olive oil-grown cells at 25°C. But at 5°C, an initial

Figure 4. Fluorescence polarization ratios (II/⊥) versus time for *trans* parinaric acid. At various time points, the free fatty acid was added to washed cell suspensions obtained from cultures grown at a) 25°C b) 5°C or c) following a 25°C to 5°C cold shock. Cells were grown on acetate (△), Tween 80 (○) or olive oil (□). Insert shows first 6 h after cold shock. Each data point is the mean ± S.E. of triplicate samples.

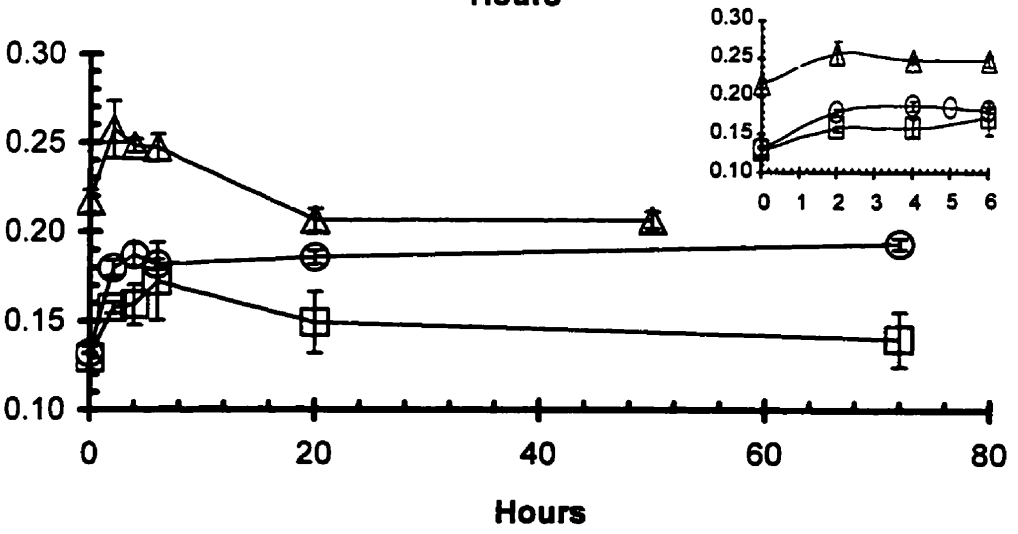
a)


0.30
0.25
0.20
0.15
0.10
0
2
4
6
8
10
12
Hours

b)


Polarization (II/⊥)
0.30
0.25
0.20
0.15
0.10
0
50
100
150
Hours

c)


0.30
0.25
0.20
0.15
0.10
0
20
40
60
80
Hours
0.30
0.25
0.20
0.15
0.10
0 1 2 3 4 5 6

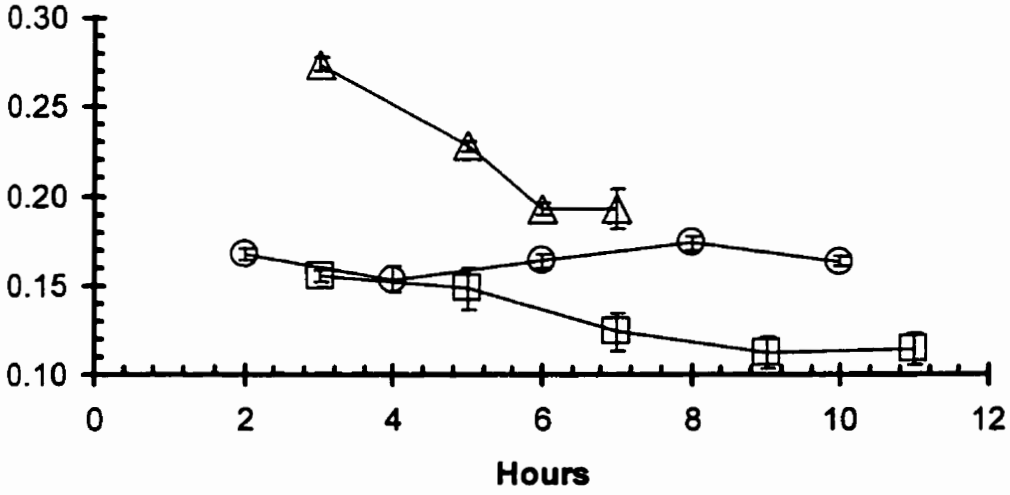
decrease in polarization followed by an increase in polarization by early-maximum stationary for Tween 80-grown cells and by mid-exponential growth for olive oil-grown cells was observed ($p<0.01$).

Polarization ratios significantly increased at 2 h post cold shock regardless of carbon source supplied ($p<0.01$) (Figure 4c) suggesting an immediate loss in membrane fluidity. Acetate-grown cells appeared to restore membrane fluidity quickly, indicated by a decline of polarization by 4 h post cold shock. After 4 h, ratios continued to decline to values similar to those exhibited by the 25°C control cells. In contrast, polarization ratios continued to increase up to 4 h post cold shock for Tween 80-grown cells and up to 6 h post cold shock for olive oil-grown cells (Figure 4c). Ratios remained high for Tween 80-grown cells and although a decrease was observed by 72 h for olive oil-grown cells, ratios were still higher than the 25°C controls.

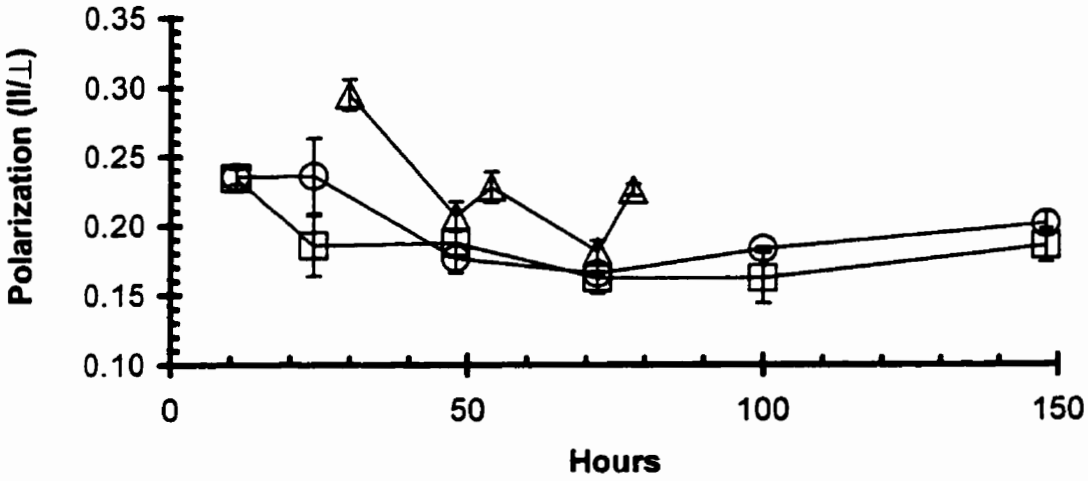
Figures 5a-c show fluorescence polarization of *cis* parinaric acid. As with the *trans* isomer, polarization ratios of the *cis* isomer measured for acetate-grown cells were significantly higher than for Tween 80 and olive oil-grown cells ($p<0.01$). The trends in polarization ratios for acetate-grown cells were similar to trends observed for the *trans* isomer, decreasing over time at 25°C and 5°C. Polarization ratios measured at 25°C for cells supplied Tween 80 showed a significant decrease in polarization at early-exponential phase followed by a significant increase in polarization by mid-exponential phase rather than remaining constant as was observed for the *trans* isomer. Also, a significant steady decrease in polarization over time was observed for cells grown in olive oil at 25°C ($p<0.01$) (Figure 5a). Changes in fluorescence polarization measured at 5°C for Tween 80 and olive oil-grown cells are similar to those reported for the *trans* isomer (Figure 5c).

Figure 5. Fluorescence polarization ratios (II/⊥) versus time for *cis* parinaric acid. At various time points, the free fatty acid was added to washed cell suspensions obtained from cultures grown at a) 25°C b) 5°C or c) following a 25°C to 5°C cold shock. Cells were grown on acetate (△), Tween 80 (○) or olive oil (□). Insert shows first 6 h post cold shock. Each data point is the mean ± S.E. of triplicate samples.

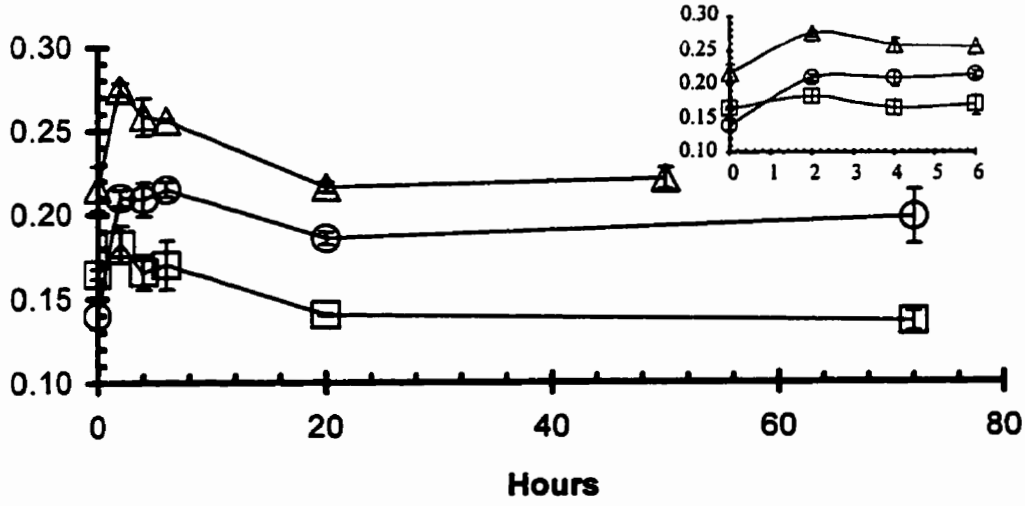
a)


0.30
0.25
0.20
0.15
0.10
0
2
4
6
8
10
12
Hours

b)


Polarization (II/⊥)
0.35
0.30
0.25
0.20
0.15
0.10
0
50
100
150
Hours

c)


0.30
0.25
0.20
0.15
0.10
0
20
40
60
80
Hours
0.30
0.25
0.20
0.15
0.10
0
1
2
3
4
5
6

Polarization ratios of the *cis* isomer significantly increased ($p<0.01$) 2 h post cold shock for all carbon sources confirming a decrease in membrane fluidity after a decrease in growth temperature. Changes in fluorescence polarization of the *cis* isomer for acetate- and Tween 80-grown cells were identical to those noted for the *trans* isomer. For cells supplied olive oil, polarization ratios had significantly decreased ($p<0.01$) between 2 h and 4 h post cold shock and continued to decline to values lower than those reported for the 25°C mid-exponential control cells (Figure 5c).

Differences in polarization ratios between incubation treatments as well as between isomers were observed. For example, fluorescence polarization ratios of both isomers were always significantly higher at 5°C compared to 25°C for Tween 80- and olive oil-grown cells ($p<0.01$). Differences between 25°C and 5°C acetate-grown cells were only sometimes significant, depending on the time of assay. Polarization ratios for the *cis* isomer were significantly higher than the *trans* isomer under all incubation conditions for both acetate- and Tween 80-grown cells. This was not observed for cells supplied olive oil as sole source of carbon. Polarization ratios for the *cis* isomer were significantly higher early in growth and then became significantly lower late in growth under all incubation conditions ($p<0.01$).

Results suggested that an increase in membrane permeability after cold shock may have been due to a decrease in membrane fluidity. Again, data suggest that acetate-grown cells respond most rapidly to cold shock indicating that these cells may be better suited for growth at low temperatures.

Fatty Acid Analysis

Since membrane fluidity is directly affected by the composition of fatty acids in phospholipids, the fatty acid compostion of HH1-1 was monitored during constant growth at

25°C or 5°C and following a 25°C to 5°C cold shock. Eight fatty acids were monitored, myristic acid (tetradecanoic acid-14:0), palmitic acid (hexadecanoic acid-16:0), palmitoleic acid (*cis*-Δ9-hexadecenoic acid-16:1), stearic acid (octadecanoic acid-18:0), oleic acid (*cis*-Δ9-octadecenoic acid-18:1), linoleic acid (*cis*-Δ9,*cis*-Δ12-octadecadienoic acid-18:2), linolenic acid (*cis*-Δ9,*cis*-Δ12,*cis*-Δ15-octadecatrienoic acid-18:3) and arachidic acid (eicosanoic acid-20:0). Of these eight fatty acids, only five fatty acids (16:0, 16:1, 18:0, 18:1 and 18:3) were found consistently to comprise greater than 2% of the total fatty acids (Tables 3-5).

The fatty acid composition of acetate-, Tween 80-, and olive oil-grown cells observed during growth at 25°C and at 5°C is summarized in Tables 3 & 4, respectively. Fatty acids 16:0, 16:1, 18:0, 18:1 and 18:3 accounted for 30-35% of the total fatty acids in acetate-grown cells, 40-50% in Tween 80-grown cells and 50-70% in olive oil-grown cells. Similar percentages were obtained regardless of incubation regime. The degree of fatty acid unsaturation, calculated as (16:1+18:1+18:3)/(16:0+16:1+18:0+18:1+18:3), varied slightly with cell type: 55-70% for acetate-grown cells, 70-80% for Tween 80-grown cells and 75-90% for olive oil-grown cells, but remained relatively stable between incubation treatments.

Fatty acid C16:0 content did not significantly change with carbon source, growth temperature or over time. Fatty acid C16:1 content did significantly increase over time ($p<0.01$), at both 25°C and 5°C for all carbon sources. At 25°C, no differences in C16:1 content were observed between cells grown on the different substrates, but at 5°C C16:1 content was significantly higher in cells supplied olive oil ($0.01<p<0.05$) compared to cells grown in the presence of acetate and Tween 80. Although a higher C18:0 content was found in acetate-grown cells ($0.01<p<0.05$), the content of this fatty acid remained stable over time,

for all carbon sources, and at both 25°C and 5°C. The content of fatty acid C18:1 was significantly higher at all sampling times and both incubation temperatures for Tween 80- and olive oil-grown cells compared to acetate-grown cells (acetate vs. olive oil-$p<0.01$; acetate vs. Tween 80-$0.01<p<0.05$). As well, olive oil-grown cells had significantly more fatty acid C18:1 than Tween 80-grown cells ($p<0.01$). Fatty acid C18:1 content was relatively constant over time for acetate grown cells at both 25°C and 5°C. In contrast, significant increases in C18:1 content were observed at both 25°C and 5°C for cells supplied Tween 80 (25°C: $0.05<p<0.10$ and 5°C: $0.01<p<0.05$). For cells supplied olive oil, a significant increase of this fatty acid was observed over time at 25°C ($0.05<p<0.10$) while a significant decrease over time was noted at 5°C ($0.01<p<0.05$). No changes in fatty acid C18:3 content were detected at 25°C, however, at 5°C a significant decrease over time was observed for both acetate-grown cells ($0.01<p<0.05$) and Tween 80-grown cells ($0.05<p<0.10$).

To determine fatty acids potentially important for growth at low temperatures, changes in fatty acid content following cold shock (Table 5) were compared to fatty acid profiles at 25°C and 5°C (Table 3 & 4). The content of fatty acid C16:1 also increased over time following cold shock. A significant increase in this fatty acid was observed at 2 h post cold shock for acetate-grown cells ($0.05<p<0.10$) compared to 24 h post cold shock for Tween 80- and olive oil-grown cells ($p<0.01$). However, in the cold-shocked cells, the increase in fatty acid C16:1 was accompanied by a decrease in fatty acid C16:0, observed at 4 h post cold shock for Tween 80-grown cells ($p<0.01$) and at 72 h post cold shock for olive oil-grown cells ($0.01<p<0.05$). In addition to changes in fatty acid C16:1 and C16:0 content,

a significant increase in fatty acid C18:1 content was also observed 2 h post cold shock for olive oil grown cells ($p<0.01$).

Results showed that the fatty acid composition of cells varied with available carbon source, although fatty acid profiles of Tween 80- and olive oil-grown cells were relatively similar relative to profiles observed for cells grown in acetate. This may be due to the similarity in the chemical nature of Tween 80 and olive oil. The observed increase in fatty acid C16:1 content following cold shock suggests that changes in the content of this fatty acid may be critical for adjusting to growth at low temperatures. The time at which the increase in this fatty acid takes place following cold shock suggests that acetate-grown cells respond faster to cold shock. Also, alterations in the content of fatty acids C16:0 and C18:1 in conjunction to changes in fatty acid C16:1 observed for olive oil-grown cells suggests that these cells may be under greater stress at lower temperatures and require modifications in the content of these additional fatty acids.

TABLE 3. Fatty acid composition of *Acinetobacter* sp. HH1-1 grown with different carbon sources. Cells were incubated at 25°C. Values are mean ± S.D. of triplicate samples.

Carbon Source	Fatty Acids (%total fatty acids)				
Acetate	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
2 h[1]	7.03 (2.09)	6.07 (0.04)	5.42 (1.80)	3.50 (0.01)	10.57 (2.80)
3 h[2]	6.84 (0.88)	5.26 (5.20)	4.58 (0.93)	2.83 (0.04)	8.17 (1.46)
5 h[3]	6.55 (1.18)	8.46 (1.72)	3.89 (0.80)	4.19 (0.56)	9.60 (1.89)
7 h[4]	7.03 (1.22)	8.44 (0.70)	4.13 (1.26)	4.18 (0.97)	5.70 (2.23)
Tween 80	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
2 h[1]	7.45 (1.75)	5.88 (0.01)	4.05 (0.32)	9.55 (4.32)	14.26 (3.13)
4 h[2]	7.15 (0.15)	7.79 (1.88)	3.39 (0.12)	16.26 (3.43)	9.96 (4.72)
6 h[3]	6.60 (0.89)	7.02 (1.70)	3.86 (0.65)	15.81 (2.46)	9.54 (2.65)
9 h[4]	6.52 (0.39)	7.80 (0.55)	3.48 (0.19)	21.3 (4.05)	7.37 (3.49)
Olive Oil	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
2 h[1]	7.63 (1.60)	7.09 (0.01)	4.86 (1.36)	22.20 (4.66)	7.91 (0.57)
4 h[2]	7.17 (1.65)	6.15 (3.90)	3.41 (0.73)	32.91 (12.62)	6.31 (1.26)
6 h[3]	6.62 (1.17)	8.18 (1.88)	3.01 (0.47)	34.80 (18.29)	6.50 (1.01)
9 h[4]	8.02 (1.13)	11.52 (1.01)	2.04 (0.08)	45.74 (3.18)	4.56 (0.34)

[1], early-exponential; [2], mid-exponential; [3], late-exponential; [4], early stationary phase.

TABLE 4. Fatty acid composition of *Acinetobacter* sp. HH1-1 grown with different carbon sources. Cells were incubated at 5°C. Values are mean ± S.D. of triplicate samples.

Carbon Source	Fatty Acids (% total fatty acids)				
Acetate	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
24 h[1]	8.17 (2.09)	8.41 (0.03)	7.48 (0.01)	UD	16.28 (6.38)
48 h[2]	7.15 (1.52)	10.07 (1.47)	4.14 (1.16)	5.07 (1.37)	11.31 (1.68)
58 h[3]	8.19 (0.37)	9.90 (1.24)	4.62 (1.10)	4.84 (0.94)	11.42 (4.10)
72 h[4]	6.15 (1.83)	10.11 (3.21)	4.18 (0.80)	5.19 (1.61)	8.71 (1.88)
Tween 80	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
10 h[1]	8.54 (5.96)	7.04 (0.32)	5.47 (0.02)	11.13 (1.52)	12.70 (0.03)
24 h[2]	6.43 (0.89)	9.07 (3.20)	3.90 (0.47)	11.35 (1.89)	9.4 (0.92)
72 h[3]	5.70 (0.68)	9.00 (2.16)	3.68 (0.38)	13.34 (2.96)	9.39 (2.39)
140 h[4]	5.25 (0.76)	7.2 (1.65)	2.87 (0.61)	20.20 (8.35)	10.26 (1.14)
Olive Oil	**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
10 h[1]	8.63 (0.57)	8.40 (2.23)	3.77 (0.02)	42.17 (11.93)	7.17 (2.83)
24 h[2]	6.30 (1.86)	9.99 (2.27)	2.93 (0.29)	34.74 (3.71)	8.62 (1.22)
72 h[3]	6.63 (1.10)	13.67 (4.72)	2.72 (0.11)	30.78 (6.15)	6.63 (1.32)
140 h[4]	5.92 0.18)	13.22 (0.85)	2.35 (0.14)	40.36 (2.06)	3.71 (1.39)

[1], early-exponential; [2], mid-exponential; [3], late-exponential; [4], early-stationary phase; UD, undetected

TABLE 5. Fatty acid composition of *Acinetobacter* sp. HH1-1 grown with different carbon sources. Cells were subjected to a 25°C-5°C cold shock. Values are mean ± S.D. of triplicate samples.

Carbon Source		Fatty Acids (% total fatty acids)				
Acetate		**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
25°C Mid-Log		6.84 (0.88)	5.25 (2.46)	4.58 (0.92)	2.83 (0.04)	8.17 (1.46)
post cold shock	2h	7.47 (1.72)	7.39 (1.80)	4.60 (0.77)	4.20 (0.78)	7.22 (0.82)
	4h	6.55 1.13)	7.77 (1.78)	4.01 (0.52)	3.63 (0.54)	6.66 (0.88)
	6h	7.58 (1.56)	9.61 (1.77)	4.81 (1.82)	3.80 (0.03)	6.11 (6.12)
	24h	6.52 (1.62)	8.46 (2.85)	4.05 (1.12)	4.11 (1.33)	6.54 (8.77)
	50h	6.68 (2.35)	9.87 (2.46)	4.16 (1.30)	5.43 (1.64)	6.19 (3.65)
Tween 80		**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
25°C Mid-Log		7.15 (0.16)	7.79 (1.88)	3.39 (0.11)	16.26 (3.40)	9.96 (4.71)
post cold shock	2h	6.96 (1.20)	8.41 (0.81)	3.59 (0.16)	14.12 (0.85)	6.68 (4.96)
	4h	5.45 (0.89)	7.28 (2.59)	3.14 (0.85)	18.92 (1.61)	7.60 (5.52)
	6h	5.54 (0.43)	6.95 (1.23)	4.27 (0.72)	17.60 (4.50)	10.55 (11.01)
	24h	6.85 (1.09)	10.13 (3.57)	4.60 (0.72)	16.27 (0.60)	10.34 (5.52)
	72h	5.68 (0.79)	10.36 (2.73)	4.01 (0.55)	12.60 (1.80)	10.28 (1.02)
Olive Oil		**16:0**	**16:1**	**18:0**	**18:1**	**18:3**
25°C Mid-Log		7.17 (1.65)	6.15 (3.93)	3.41 (0.73)	32.91 (10.91)	6.31 (1.26)
post cold shock	2h	8.32 (1.91)	6.43 (3.80)	3.47 (0.62)	48.42 (18.20)	4.43 (1.98)
	4h	8.90 (1.32)	5.14 (2.47)	3.00 (0.42)	49.07 (16.61)	5.80 (2.87)
	6h	8.25 (1.76)	5.59 (1.76)	3.45 (0.14)	45.75 (11.66)	4.09 (0.25)
	24h	7.53 (1.30)	11.65 (1.76)	3.50 (1.15)	37.6 (9.67)	6.06 (1.71)
	72h	5.87 (1.46)	13.87 (3.37)	2.83 (1.05)	36.07 (7.36)	6.03 (2.24)

Discussion

HH1-1 is of interest because of its potential uses in industry and biotechnology. For example, it may be used for bioremediation and used for the treatment of waste water at ambient temperatures. It is a lipolytic *Acinetobacter* sp. capable of growing at 0°C. Based on the definition first proposed by Morita (1975), HH1-1 is a psychrotrophic bacterium with a growth temperature range that extends from 0°C to 30°C and an optimal growth temperature occurring at 25°C. Preliminary studies showed that the growth of HH1-1 at low temperatures was different when grown with acetate relative to the other substrates. For instance, although HH1-1 was able to grow at 25°C and 5°C, it was unable to grow at 0°C when supplied olive oil as the sole carbon source. In addition, cells grown in the presence of Tween 80 or olive oil were typically more coccoid in shape compared to rod-shaped observed for acetate-grown cells.

An increase in extracellular K^+ was observed after cold shock regardless of carbon source. Potassium is a highly regulated intracellular cation required by bacterial cells to maintain internal osmotic pressure, as an essential cofactor for enzymes and required for protein synthesis (Kawano et al., 1998). Efflux of K^+ from cells into the extracellular medium is thus indicative of a leaky cell membrane (Heipieper et al. 1991). An increase in extracellular K^+ in response to cold shock has also been reported by Haest et al. (1972) after subjecting a mesophilic *E. coli* auxotroph (strain K1060) to sudden drops in temperature.

Potassium levels in the extracellular medium of cold-shocked HH1-1 cultures eventually decreased, presumably as K^+ was moved back into recovering cells and as the number of dividing cells increased. The decrease in K^+ levels for acetate-grown cells

occurred prior to an increase in cell numbers, indicating that these cells adapted quickly to the decrease in incubation temperature. In contrast, decreases of K^+ concentrations in the supernatant, observed for cells supplied with Tween 80 or olive oil, was concurrent with an increase in cell numbers. In addition, the extent of leakiness was greater for cells grown on Tween 80 and olive oil relative to cells grown with acetate. The larger increases in K^+ levels and the longer time intervals that elapsed before K^+ levels decreased indicated that cells grown with Tween 80 and olive oil were more susceptible to cold shock. Haest et al. (1972) also reported differences in the concentration of K^+ released into the extracellular medium, and determined that this response was dependent on the fatty acid supplied in the growth medium. Their auxotrophic bacterium incorporated the supplied fatty acid directly into the membrane. Cells supplied oleic acid rapidly released K^+ into the medium at temperatures below 16°, but when supplied palmitoleic acid, K^+ was not released until temperatures dropped below 13°C.

Maintenance of membrane integrity, including permeability properties, relies strongly on the degree of fluidity. Fluorescence polarization of the membrane probe parinaric acid (9,11,13,15-octadecatetraenoic acid) has been used in previous studies to investigate the physical properties of membranes (Rintoul & Simoni 1977). It has been extensively used to determine the phase transition temperature ™ of different phospholipid dispersions. Sklar et al. (1979) have also used parinaric acid to assess lipid fluidity. They found that the polarization ratios of *cis* and *trans* parinaric acid, after partitioning into an aqueous dispersion of solid-phase lipid (dipalmitoyl-phosphatidylcholine) and fluid-phase lipid (palmitoyldocosa-hexaenoyl-phosphatidylcholine), were higher for lipid dispersions containing greater amounts of solid-phase lipid. The higher polarization ratios found for parinaric acid were related to a decrease in the motional freedom of the probe. Fluorescence

polarization of parinaric acid was used in this study to assess changes in HH1-1 membrane fluidity in response to growth temperature. The method was modified so that polarization measurements were recorded over time at one temperature rather than over a large temperature range.

The temporal changes in fluorescence polarization observed during growth of HH1-1 at 25°C and 5°C may have reflected changes in both the proportion of different fatty acids in phospholipids, and in the protein-to-phospholipid ratio. An increase in the number of unsaturated fatty acyl chains in phospholipids will increase membrane fluidity, causing a decrease in polarization. In contrast, cell membranes obtained from stationary-phase cells will have increased stability due to increased interactions between proteins and lipids, causing an increase in polarization ratios (Souza et al., 1989). Differences in polarization between acetate-grown cells, and Tween 80- and olive oil-grown cells reflect the differences in the kinds of fatty acids that make up the phospholipids associated with the cells. The lower polarization ratios observed for Tween 80- and olive oil-grown cells suggest that a higher proportion of fluid-phase lipid is associated with these cells.

Higher polarization ratios observed 2 h post cold shock for all cells, suggested that there was an immediate decrease in membrane fluidity. The changes in polarization ratios following cold shock are consistent with changes that occur when phospholipids undergo a thermal phase transition (Sklar et al., 1977; Souza, 1986). The decrease in membrane fluidity likely caused the temporary leakage of K^+ that was observed after cold shock. These findings are consistent with the study conducted by Haest et al. (1972) who noted that the maximal release of K^+ monitored from synthetically-prepared liposomes (dimyristoyllecithin phosphatidic acid) was consistent with the liquid-crystalline phase transition temperature for this lipid (as measured using differential scanning calorimetry). The acetate-grown cells

appeared to readjust more quickly after cold shock, as indicated by a rapid decline of polarization at 4 h post cold shock to values similar to those exhibited by the 25°C mid-exponential controls. The greater proportion of fluid-phase lipid associated with Tween 80 and olive oil-grown cells may have interfered with the cell's ability to adjust to large variations in temperature.

Differences in the fluorescence polarization ratios of the *trans* and *cis* isomers in the various carbon and incubation treatments reflect the environment in which the isomers have partitioned. The linear structure of the *trans* probe permits it to intercalate with solid-phase lipids more liberally, while the bent configuration of the *cis* isomer causes it to be relatively excluded from solid phases (Sklar et al. 1979). Generally, both isomers will behave in a similar manner. However, the *trans* isomer will be more sensitive to changes in fluid-phase lipid while the *cis* isomer will detect changes equally, in fluid- or solid-phase lipid (Welti & Silbert, 1982). Differences in polarization between the isomers are similar for both acetate- and Tween 80-grown cells under all incubation conditions, but for cells supplied with olive oil, a distinct trend was observed. This suggested that a lipid component is associated with these cells that is not present in cells grown with acetate or Tween 80. This lipid component may inhibit the ability of olive oil-grown cells to respond to cold shock and affect the ability to sustain growth at low temperatures.

In the present study, changes in the content of individual fatty acids were observed over time during growth of HH1-1 at 25°C. Differences were observed between carbon treatments, with the exception of an increase in the content of fatty acid 16:1 that was observed for all carbon sources. At 25°C, an increase in the content of fatty acid 16:1 occurred from mid-exponential to early-maximum stationary phase (acetate-grown cells) or from early-exponential to mid-exponential phase (Tween 80 and olive oil-grown cells). An

increase in fatty acid 16:1 during the transition from mid-exponential phase to early-maximum stationary phase has also been reported in a psychrophilic marine *Vibrio* sp. by Hamamoto et al. (1994). A greater content of 16:1 chain fatty acid in olive oil-grown cells when growing at 5°C and an increase in fatty acid 16:1 concurrent with a decrease in 16:0 chain fatty acid following cold shock suggested that changes in the content of these fatty acids may be important for the growth of HH1-1 at low temperatures. This response has also been observed in *Acinetobacter calcoaceticus* 69-V following decreases in growth temperature (Loffhagen et al., 1995). The delayed increase in fatty acid 16:1 observed after cold shock for Tween 80- and olive oil-grown cells indicated that these cells were slower to respond to a down-shift in temperature. An increase in the content of unsaturated fatty acids concurrent with a decrease in the content of saturated fatty acids has also been observed in *Serratia marcescens* (Gill & Suisted 1978) and *Pseudomonas* spp. (Paton et al. 1978; Kropinski et al. 1987). These changes allow the cell membrane to remain fluid at low temperatures.

Studies by Bhakhoo & Herbert (1980) evaluating the effect of growth temperature on psychrotrophs yielded different results. The fatty acid and phospholipid compositions for five psychrotrophic *Pseudomonas* sp. growing at 0°C, 8°C or 20°C were determined. The fatty acid composition of these bacteria changed little with incubation temperature. These bacteria already had high proportion of unsaturated fatty acids (hexadecenoic acid + octadecenoic acid) ranging from 60.9% to 78.6% total fatty acids suggesting that a change in the fatty acid composition was not necessary. Similar proportions of unsaturated to saturated fatty acids were also found for HH1-1. The high proportion of unsaturated fatty acids in HH1-1 (ranging from 55-90%) may also limit its need to drastically alter the content of fatty acids during growth at lower temperatures.

The ecological importance of *trans* unsaturated fatty acids has only recently been recognized (Heipieper et al. 1994). These fatty acids appear to be important for bacteria that degrade environmental pollutants. They may also be important in the adaptation to a change in growth phase and increases in growth temperature (Moritan et al. 1993). For example, in the presence of a solvent such as phenol, bacteria considered to be solvent-tolerant increased the content of *trans*-unsaturated fatty acids concomitantly with a decrease in *cis*-unsaturated fatty acids as a means to restore membrane fluidity (Heipieper et al. 1992). Similarly, *Vibrio* sp. ABE-1 was found to increase the amount of *trans*-Δ9-hexadecanoic acid (with a corresponding decrease in *cis*-Δ9-dexadecenoic acid) to correct the increase in membrane fluidity that resulted from an increase in growth temperature (Moritan et al. 1993). In the present study, the retention times for the *trans* isomers of C16:1 and C18:1 fatty acids (palmitelaidic acid: *trans*-Δ9-hexadecenoic acid and elaidic acid: *trans*-Δ9-octadecenoic acid, respectively) were determined so that the presence or absence of these fatty acids could also be monitored. However, the retention times for these isomers were too similar to those of the *cis* isomer for quantitative assessment of the *trans* fatty acid content to be made.

Analysis of the variation in fatty acid content from isolated cytoplasmic lipids and from lipids of the outer membranes may provide additional information that can be used to assess the effect of cold shock and low temperatures on cell membranes. Alterations in the lipid composition of outer membranes have been observed for *Pseudomonas aeruginosa* (Kropinski et al. 1987). Changes in the lipid composition of the outer membrane during shifts in growth temperature may be critical for offsetting a change in membrane fluidity for psychrotrophic bacteria, which do not appear to drastically alter the fatty acid composition of their cytoplasmic membranes.

In the present study, a larger amount of the fatty acid C18:1 was found in both Tween 80- and olive oil-grown cells compared to acetate-grown cells under all incubation conditions. In a study conducted by Cronan & Vagelos (1972), the amount of lipid and fatty acid composition in *E. coli* was independent of the carbon source (glucose, glycerol, acetate, fatty acids, succinate and amino acids). There are examples, however, where switching from one sugar to another in culture medium affects the fatty acyl composition of cellular lipids (Rose, 1989). Changes in the fatty acyl composition have been found to be even greater when different alkane substrates are supplied. Thorpe and Ratledge (1972) reported changes in the fatty acid composition of lipids, isolated from different yeasts, which varied according to the chain length of the substrate provided. The results obtained in the present study are therefore consistent with other studies that show that lipid and fatty acid composition are dependent on the growth substrate.

The primary component of Tween 80 and olive oil is oleic acid (C18:1). The high concentration of oleic acid available to the cells could lead to the direct incorporation of this fatty acid into the membrane. If not incorporated into membranes, the excess oleic acid that is not metabolized may be stored inside the cell. The uptake and storage of wax esters commonly occurs with *Acinetobacter* sp. when cells are grown on an excess of alkane carbon (Fixter & Sherwani, 1991). Similarly, hexadecane-membrane inclusions were found to form inside a hydrocarbon-oxidizing *Acinetobacter* sp. after growing on hexadecane, as the sole source of carbon (Scott et al. 1976). This additional content of fatty acid C18:1 observed in olive oil-grown cells compared to acetate-grown cells, whether directly incorporated into membranes or stored, may be interfering with normal phospholipid synthesis and regulation. These conditions could affect the ability of these cells to respond quickly to cold shock and to grow at low temperatures.

Conclusion

These data suggest that olive oil, provided as a sole source of carbon, may be affecting the growth of HH1-1 at low temperatures. Results show that the loss in membrane permeability immediately after cold shock is greatest for olive oil-grown cells, and is likely due to the loss in membrane fluidity. The observed changes in fatty acid C16:1 content suggest that this fatty acid may play an important role in restoring membrane fluidity after cold shock and may be critical for low-temperature growth. All of the data collected in this study indicated that olive oil-grown cells responded more slowly to a decrease in temperature. The additional C18:1 fatty-acid content measured in olive oil-grown cells resulted in lower polarization ratios suggesting increased membrane fluidity. In addition to increased membrane fluidity, its presence in the cell may inhibit the normal *de novo* synthesis and regulation of fatty acids that would normally allow the cell to adjust the fluidity of the membranes when exposed to cold shock or during continuous growth at low temperatures.

Results for Enzyme Experiments

Since lipase activity is required to metabolize olive oil, the effects of low temperatures on lipase activity was monitored during growth at 25°C, 5°C and following a 25°C to 5°C cold shock (Appendix IV). For comparison, activities of isocitrate lyase and esterase were monitored under the same incubation conditions. Isocitrate lyase and esterase are induced when cells are grown on acetate or Tween 80, respectively (Appendix IV). Since a close association has been observed between *Acinetobacter* biosurfactant activity and their lipolytic enzymes, effects of low temperatures on biosurfactant activity was also investigated.

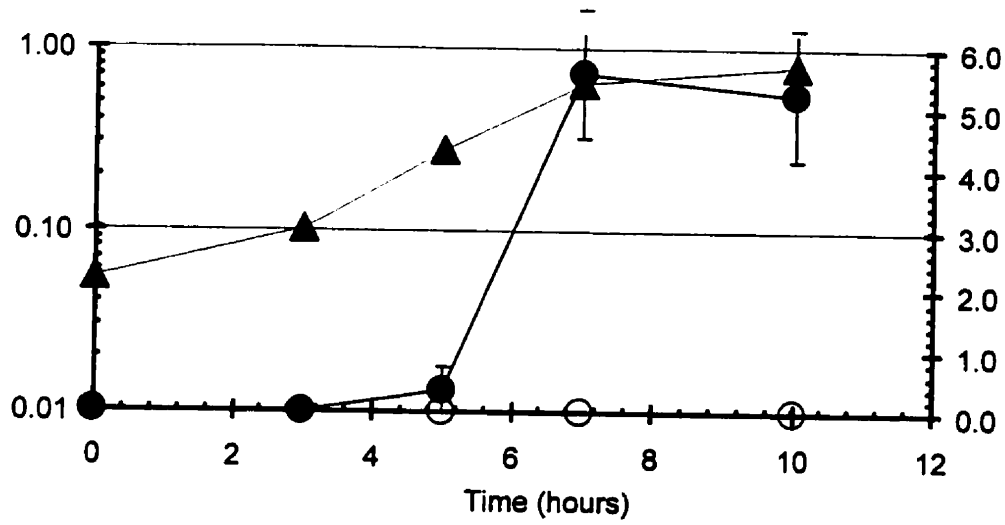
Isocitrate Lyase Activity

Isocitrate lyase was monitored in cell culture supernatant and the soluble fraction of cell sonicate samples of acetate-grown cells incubated at 25°C, 5°C and after a 25°C to 5°C cold shock (Figure 6a-c). At 25°C, isocitrate lyase activity was detected in cell sonicate only (Figure 6a). Cell-associated enzyme activity increased over time reaching a maximal activity (5.5 U/ml) by 7 h. During growth at 5°C, isocitrate lysase activity was measured primarily in culture supernatant samples (Figure 6b). Although activity was not detected until 72 h (3.5 U/ml), an additional increase in activity was observed (5.5 U/ml) by 80 h. Isocitrate lyase is a cell-associated enzyme. Activity monitored in cell culture supernatant suggests that this protein is leaking out of the cell during incubation at low temperatures.

These results were further substantiated by cold shock experiments. Little activity was detected in the 25°C mid-exponential controls, but after cold shock, activity was measured in both cell culture supernatant and cell sonicate (Figure 6c). At 2 h after cold

Figure 6. Isocitrate lyase activity measured in cell culture supernatant (○) and cell sonicate (●). Cells were grown in acetate and incubated at a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (▲). Isocitrate lyase data are the mean ± S.D. for triplicate samples.

a)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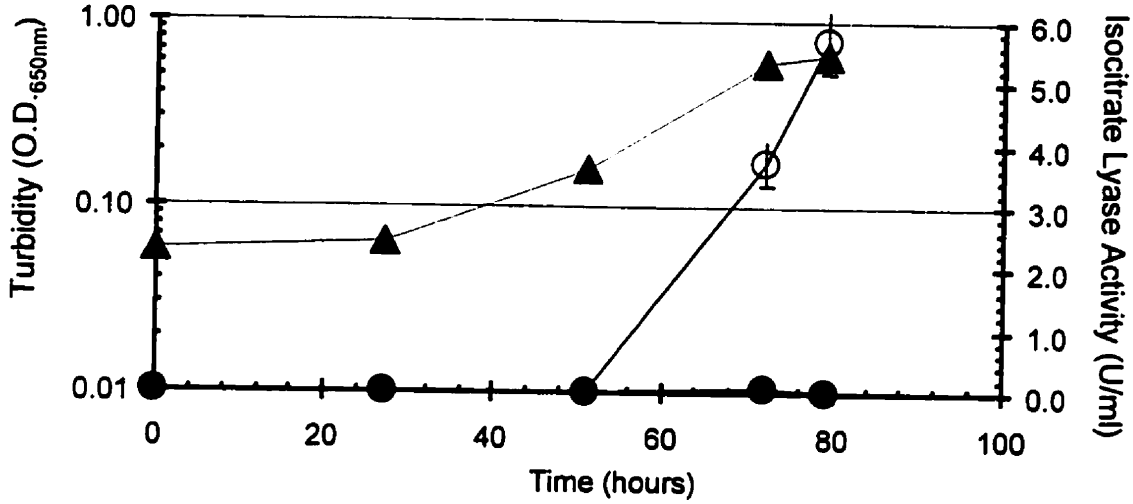
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
2
4
6
8
10
12
Time (hours)

b)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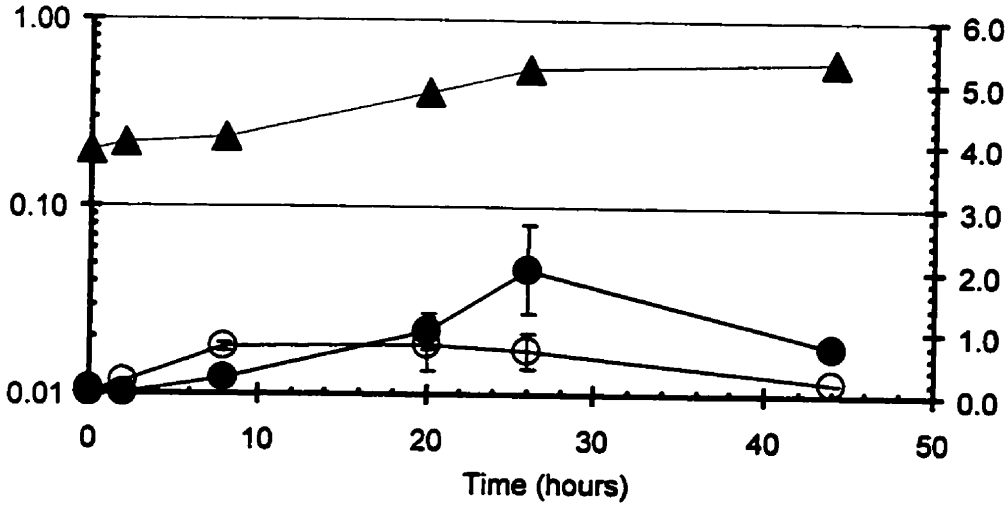
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Isocitrate Lyase Activity (U/ml)
0
20
40
60
80
100
Time (hours)

c)


1.00
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Time (hours)

shock, cell-associated activity measured in cell sonicate was less than that observed for the 25°C mid-exponential controls and activity also measured in cell culture supernatant. Although the cell sonicate activity then increased later after the cold shock reaching 2.0 U/ml by 26 h post cold shock, activity in supernatant was still measureable 50 h after cold shock.

Esterase Activity

Esterase activity was measured in both cell culture supernatant and cell sonicate. Activity, in both fractions, varied with incubation condition and carbon source provided. Figure 7a-c shows esterase activity when cells were grown with acetate during the different incubation treatments. At 25°C, extracellular esterase activity was higher than activity measured in cell sonicate at all times assayed. Extracellular activity increased over time from 0.0 U/ml at 0 h up to 2.5 U/ml at 10 h (Figure 7a). Esterase activity was not detected in cell sonicate samples until early-maximum stationary phase (10 h-1.0 U/ml).

At 5°C, esterase activity measured in cell culture supernatant remained relatively constant over time (2.0-3.0 U/ml) (Figure 7b). After 20 h of growth, esterase activity measured in cell sonicate began to increase, eventually reaching levels higher than those observed for supernatant samples (from 0.0 U/ml at 24 h up to 4.0 U/ml at 80 h) and higher than activity measured for cells grown at 25°C.

Similar trends were observed following cold shock (Figure 8c). Esterase activity in cell culture supernatant decreased from 1.5 U/ml to 1.0 U/ml 2 h post cold shock. Activity increased to 2.5 U/ml 20 h post cold shock, but by 44 h post cold shock values dropped to values similar to those observed for the 25°C mid-exponential control. Cell-associated

Figure 7. Esterase activity measured in cell culture supernatant (◇) and cell sonicate (◆). Cells were grown in acetate and incubated at a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (▲). Esterase data are the mean ± S.D. for triplicate samples.

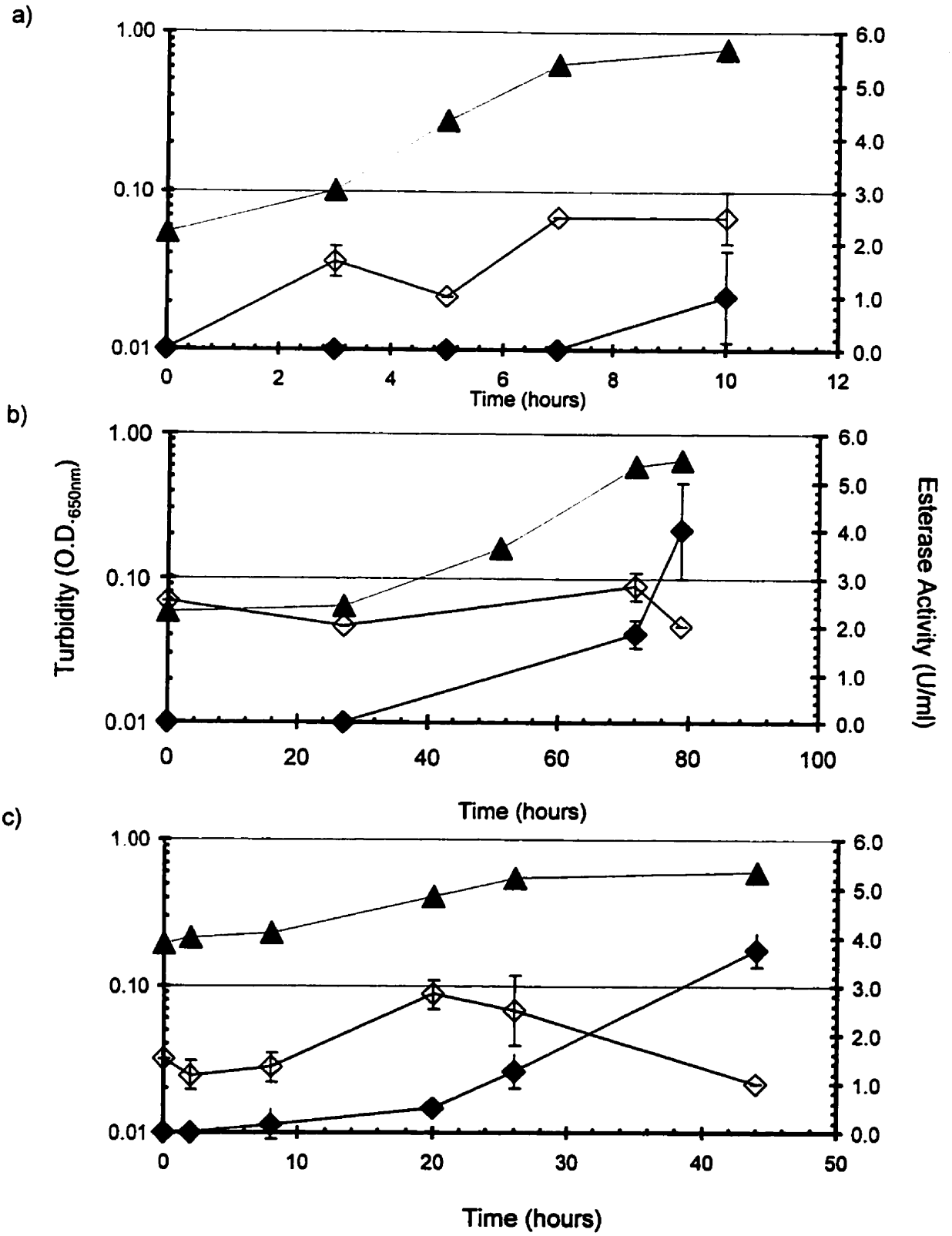
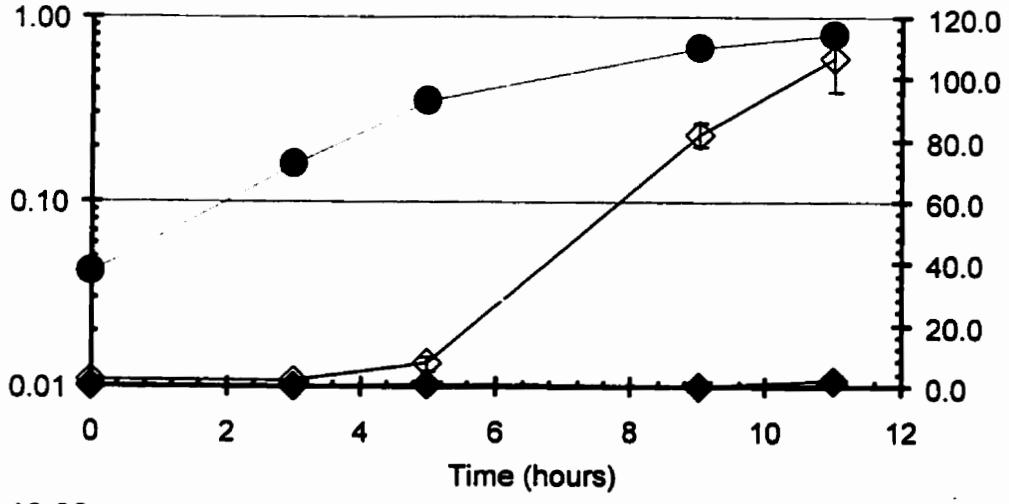
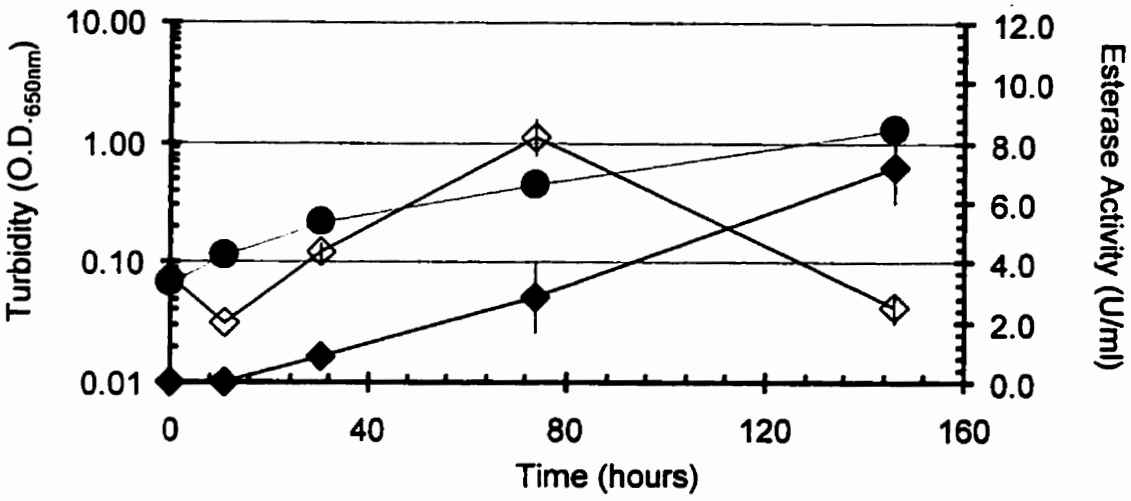

a)
1.00
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
2
4
6
8
10
12
Time (hours)
b)
Turbidity (O.D.650nm)
Esterase Activity (U/ml)
0
20
40
60
80
100
Time (hours)
c)
0
10
20
30
40
50
Time (hours)

Figure 8. Esterase activity measured in cell culture supernatant (◇) and cell sonicate (◆). Cells were grown in Tween 80 and incubated at a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (●). Esterase data are the mean ± S.D. for triplicate samples.

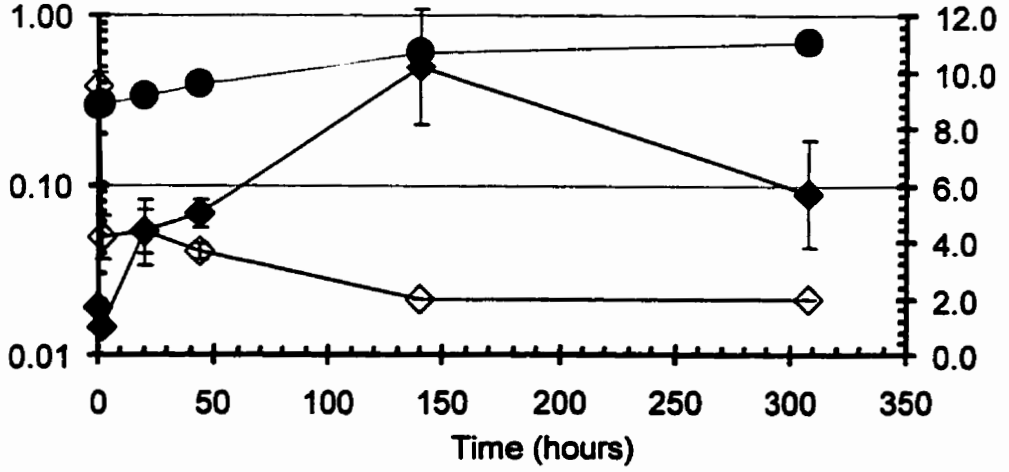
a)


1.00
0.10
0.01
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
2
4
6
8
10
12
Time (hours)

b)


Turbidity (O.D.650nm)
10.00
1.00
0.10
0.01
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Esterase Activity (U/ml)
0
40
80
120
160
Time (hours)

c)


1.00
0.10
0.01
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
50
100
150
200
250
300
350
Time (hours)

esterase activity was detected at 8 h post cold shock (0.4 U/ml) and continued to increase up to 4.0 U/ml at 44 h post cold shock.

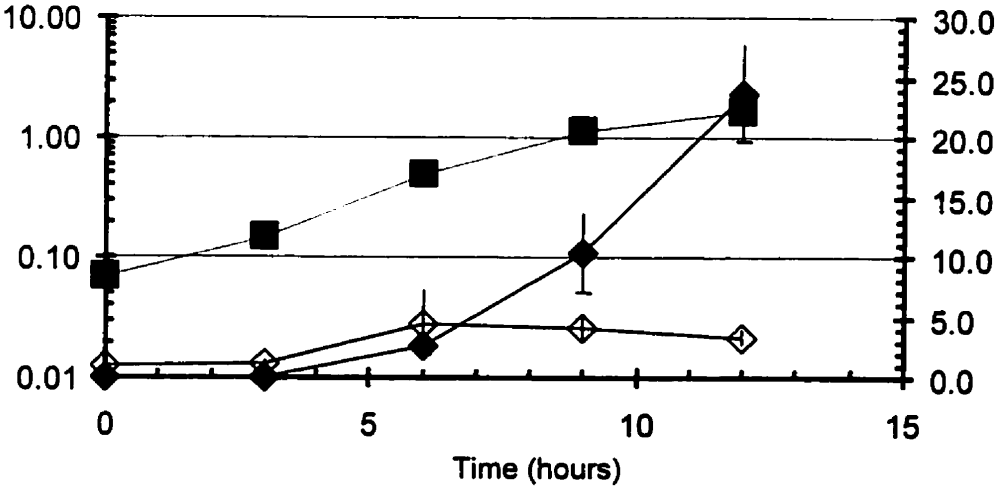
Esterase activity measured in cell culture supernatant and cell sonicate for Tween 80-grown cells was higher than activity measured for acetate-grown cells (Figure 8a-c), but trends in esterase activity were generally the same. At 25°C, esterase activity was measured primarily in cell culture supernatant (Figure 8a). Extracellular activity increased over time with an initial slow increase (undetected up to 4 U/ml) observed from 0 h to 5 h. This was followed by a sharp increase in activity (114 U/ml) at 11 h. Cell-associated esterase activity remained undetected up to 9 h but by 11 h, activity was measured at 1.0 U/ml.

Extracellular esterase activity was 10-fold lower when cells were grown at 5°C compared to growth at 25°C (Figure 8b). Activity measured in culture supernatant samples steadily increased up to 8.5 U/ml at 74 h. At 144 h activity dropped to 2.5 U/ml. A steady increase in cell-associated esterase activity was observed over the 144 h, from undetected to 7.5 U/ml.

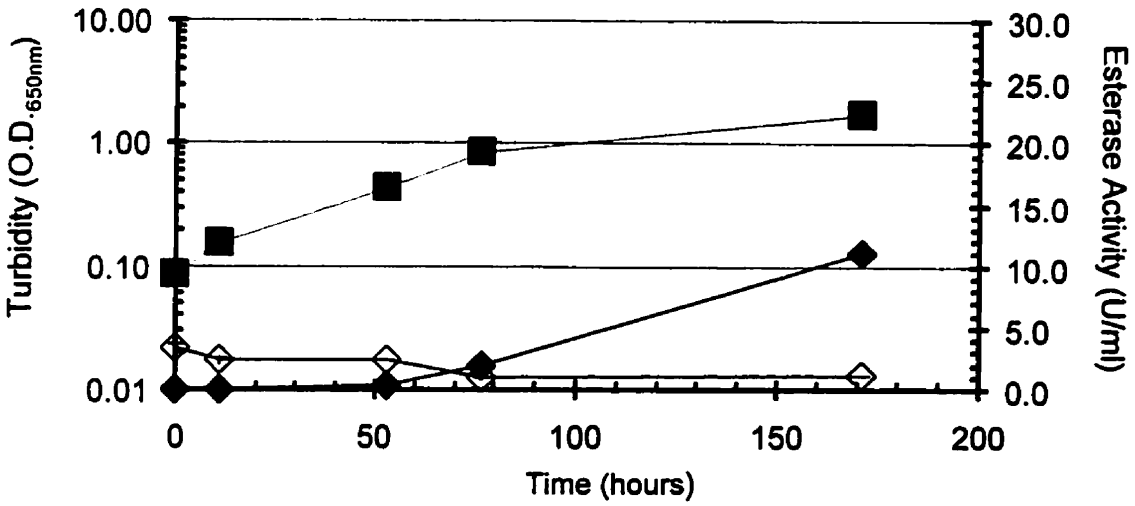
Trends in esterase activity after cold shock obtained for Tween 80-grown cells were also similar to those observed for acetate-grown cells. Following cold shock, a rapid decrease in extracellular activity was noted 2 h post cold shock (9.5 U/ml down to 4.5 U/ml) (Figure 8c). Activity continued to decline, to 2.0 U/ml at 308 h post cold shock. Although cell-associated esterase activity decreased slightly at 2 h post cold shock, by 8 h post cold shock, activity had increased 4-fold. Activity continued to increase to 10 U/ml by 140 h post cold shock. The reduction of extracellular esterase activity suggests that the activity is impaired at low temperatures. The increase in cell-associated esterase activity can be interpreted as either inefficient transport of an extracellular esterase out of the cell or increased synthesis of a cell-associated esterase(s) at low temperatures.

Figure 9. Esterase activity measured in cell culture supernatant (◇) and cell sonicate (◆). Cells were grown in olive oil and incubated at a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (■). Esterase data are the mean ± S.D. for triplicate samples.

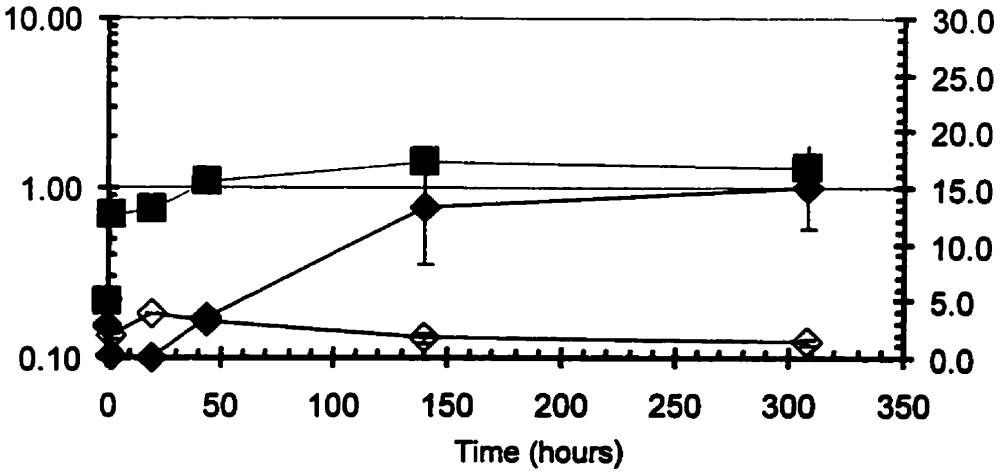
a)


10.00
1.00
0.10
0.01
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
5
10
15
Time (hours)

b)


Turbidity (O.D.650nm)
10.00
1.00
0.10
0.01
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Esterase Activity (U/ml)
0
50
100
150
200
Time (hours)

c)


10.00
1.00
0.10
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250
300
350
Time (hours)

Esterase activity at 25°C in olive oil-grown cells was greater than activity measured for acetate-grown cells but was considerably lower than activity measured in Tween 80-grown cells. The results obtained for olive oil-grown cells at 25°C and 5°C were quite different from those obtained for acetate- and Tween 80-grown cells. Esterase activity, measured in cell sonicate, exceeded activity measured in cell culture supernatant under all incubation treatments (Figures 9a-c). At 25°C, cell-associated esterase activity increased with time (undetected up to 24.0 U/ml) while extracellular activity remained constant between 1.0 and 4.0 U/ml (Figure 9a). Similar results were observed when cells were grown at 5°C; but, activity was 2.5-fold lower (Figure 9b).

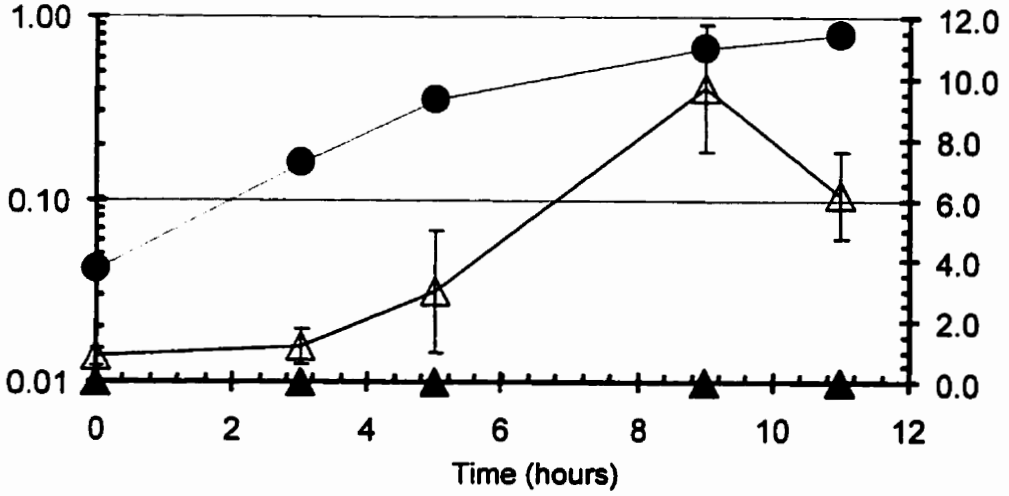
Changes in esterase activity following cold shock were similar to those observed for cells grown in acetate and Tween 80 (Figure 9c). A small decrease in cell-associated esterase activity was observed up to 20 h after cold shock followed by a steady increase up to 15.0 U/ml at 308 h after cold shock. Extracellular esterase activity appeared to be unaffected by cold shock remaining steady between 2-5 U/ml. These results indicate that there is a greater cell-associated esterase activity different from acetate- and Tween-grown cells regardless of incubation temperature.

Lipase Activity

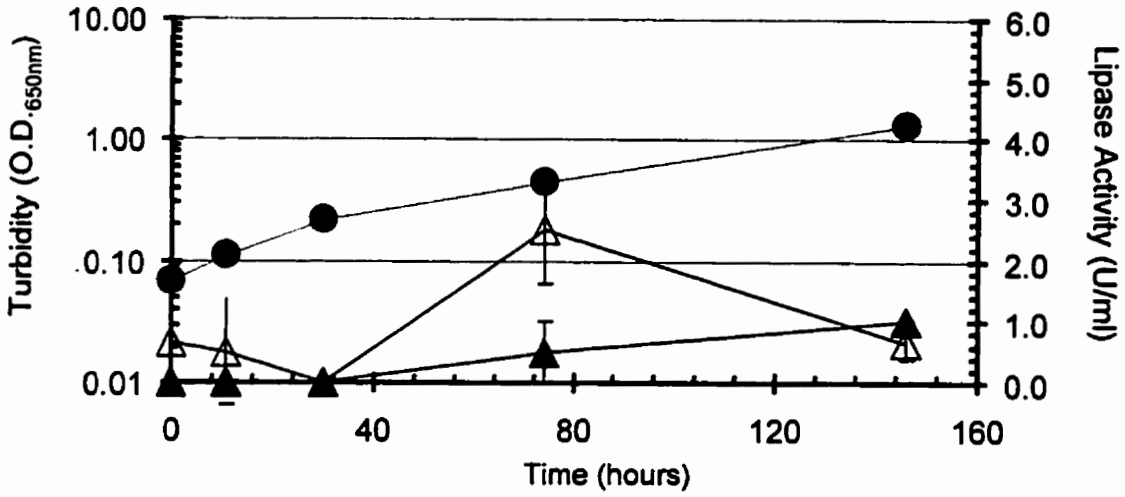
Lipase activity was observed in both cell culture supernatant and cell sonicate when cells were grown in the presence of either Tween 80 or olive oil. Activity was affected by both incubation treatment and carbon source provided (Figures 10 & 11). At 25°C, lipase activity in supernatant samples increased over time for both Tween 80- and olive oil-grown cells with slightly higher activity observed for olive oil-grown cells (Figures 10a & 11a).

Figure 10. Lipase activity measured in cell culture supernatant (△) and cell sonicate (▲). Cells were grown in Tween 80 and incubated at a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (●). Lipase data are the mean ± S.D. for triplicate samples.

a)


1.00
0.10
0.01
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
2
4
6
8
10
12
Time (hours)

b)


Turbidity (O.D.650nm)
10.00
1.00
.0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Lipase Activity (U/ml)
0
40
80
120
160
Time (hours)

c)

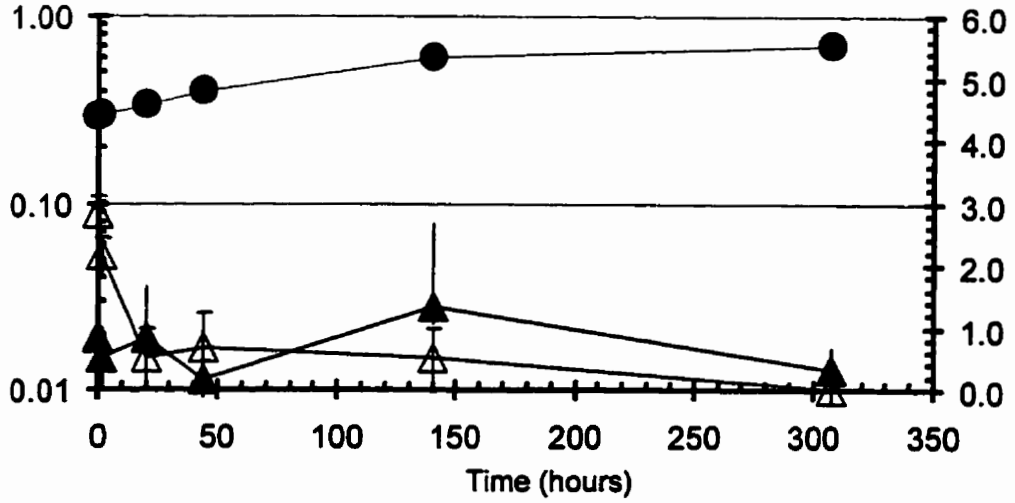
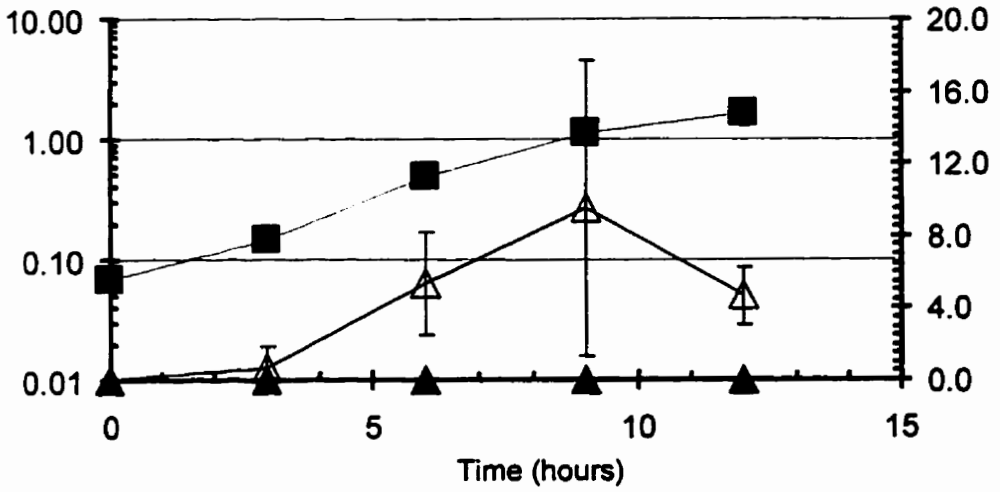
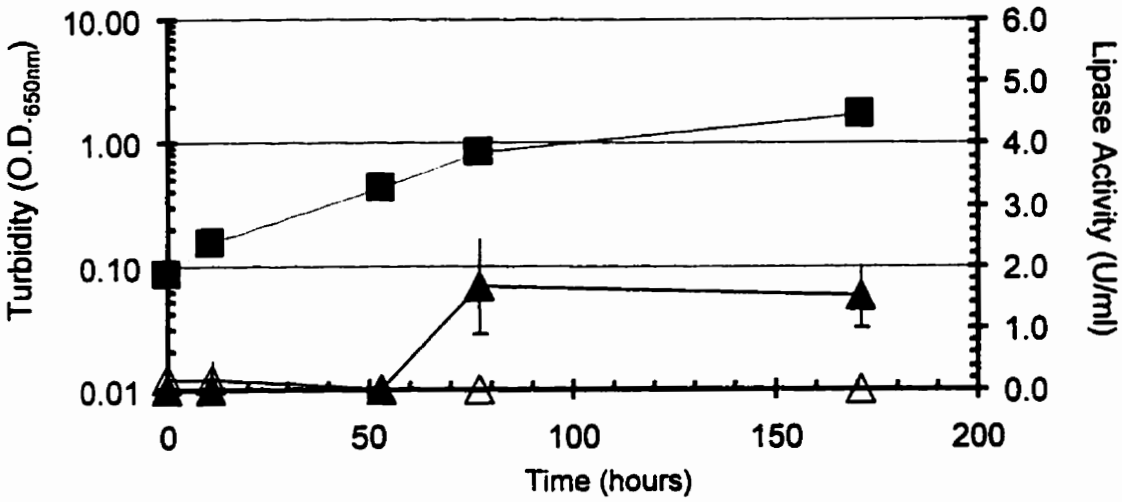

1.00
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
Time (hours)

Figure 11. Lipase activity measured in cell culture supernatant (△) and cell sonicate (▲). Cells were grown in olive oil and incubated at either a) 25°C, b) 5°C or c) following a 25°C to 5°C cold shock. Growth is indicated by (■). Lipase data are the mean ± S.D. for triplicate samples.

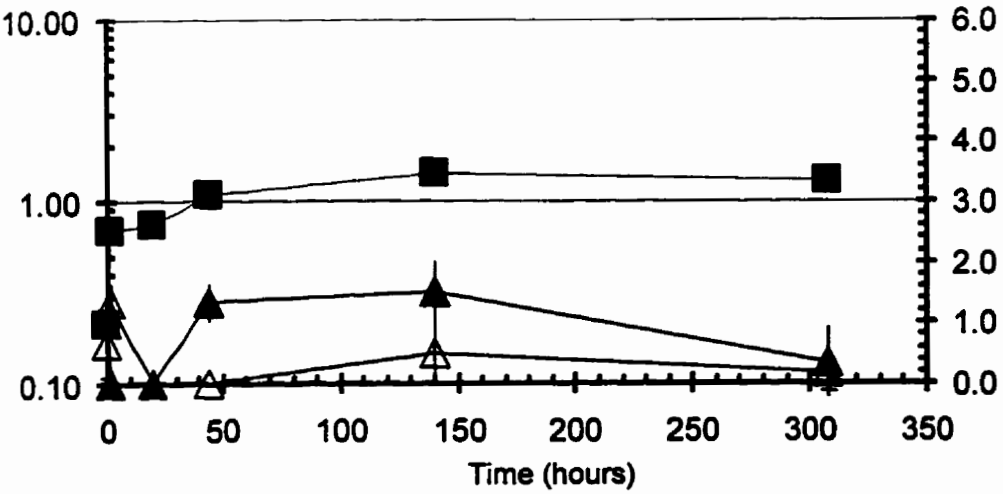
a)


10.00
1.00
0.10
0.01
20.0
16.0
12.0
8.0
4.0
0.0
0
5
10
15
Time (hours)

b)


Turbidity (O.D-650nm)
10.00
1.00
0.10
0.01
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Lipase Activity (U/ml)
0
50
100
150
200
Time (hours)

c)


10.00
1.00
0.10
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
Time (hours)

Activity was maximal (9.0 U/ml) by late-exponential phase (9 h). Lipase activity was not detected in cell sonicate samples for either carbon source at 25°C.

At 5°C, extracellular lipase activity measured in supernatant for cells grown in Tween 80 also increased over time. Activity was 5-fold lower at late-exponential phase (74 h) than was observed during growth at 25°C (Figure 10b). Activity was also measurable at this temperature and increased over time from 0.0 U/ml at 0 h to 1.0 U/ml at 144 h in cell sonicate. Lipase activity of cells grown in olive oil at 5°C was found primarily in cell sonicate (Figure 11b). Although activity was not detected up to 53 h, it had increased to 1.5 U/ml by 77 h.

After cold shock, lipase activity measured in cell culture supernatant decreased in both Tween 80- (from 3.0 U/ml at 0 h to 0.5 U/ml at 300 h) and olive oil-grown cells (from 1.5 U/ml at 0 h to 0.5 U/ml at 300 h) (Figures 10c & 11c). Lipase activity measured in these fractions, remained lower than cell sonicate activity. During the first 20 h (olive oil-grown cells) and 44 h (Tween 80-grown cells) after cold shock, lipase activity in cell sonicate decreased. However, by 150 h post cold shock, activity increased from undetected to 1.0-1.5 U/ml.

Like esterase, lipase activity is reduced at 5°C. Because lipase is typical of an extracellular enzyme requiring transport across both the cellular and outer membranes, the observed response may be the result of inefficient transport of the protein at low growth temperatures.

Changes in Protein Content

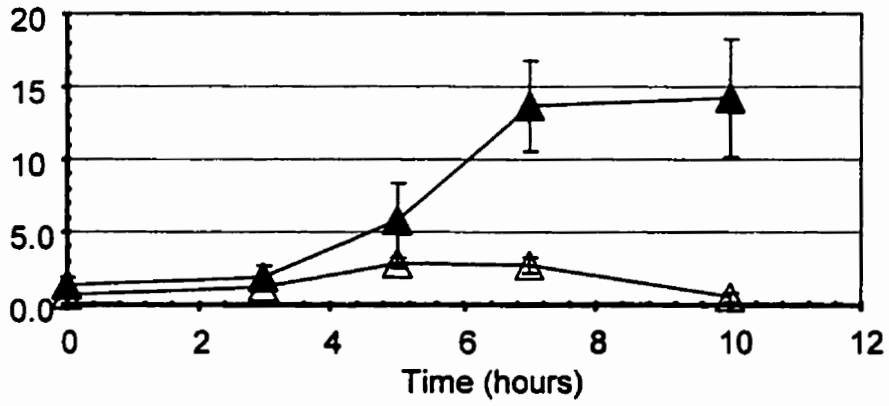
During growth at 25°C, protein content increased in cell sonicate regardless of carbon source (Figures 12a, 13a & 14a). Protein content was similar for cells grown in acetate and Tween 80 (Figures 12a & 13a) and was highest for cells grown with olive oil (Figure 14a). An increase in protein content with time was also measured in cell culture supernatant. However, levels were much lower than those observed for cell sonicate samples. By early-maximum stationary phase (10 h) protein content measured in supernatant from cells grown in acetate decreased to levels equivalent to those measured at time 0 h (Figure 14a). Protein content remained steady in cells grown with Tween 80 and olive oil after 6 h (Figures 13a &14a).

Similar trends were observed during growth at 5°C (Figures 12b, 13b & 14b). Protein measured in cell sonicate increased over time for all carbon sources but protein content was reduced compared to protein content measured at 25°C. Protein was negligible in supernatant with cells grown with acetate (Figure 12b) and olive oil (Figure 14b). Protein measured in supernatant from Tween 80-grown cells increased slightly during 11 h to 30 h of growth, then decreased thereafter (Figure 13b).

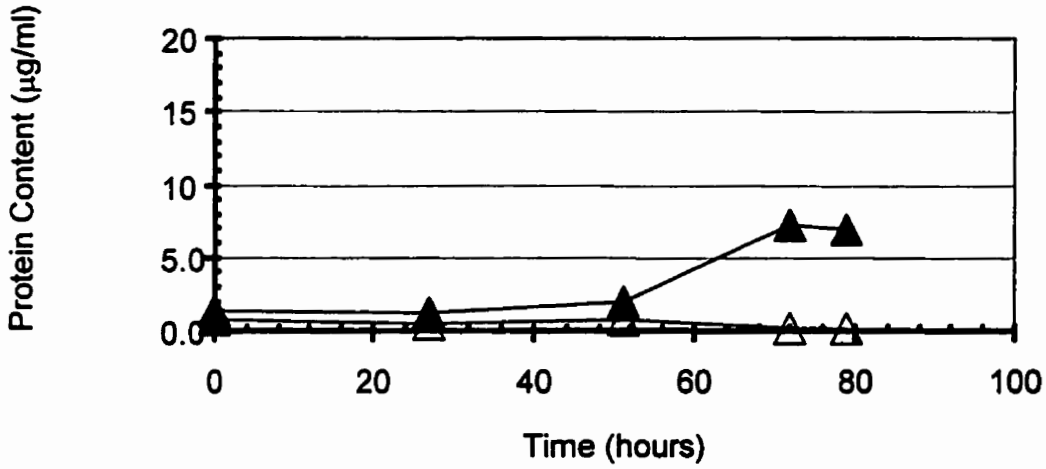
After cold shock, protein decreased in both cell sonicate and cell culture supernatant for cells grown with acetate and Tween 80 (Figures 12c & 13c). After 20 h post cold shock (acetate-grown cells) and at 308 h post cold shock (Tween 80-grown cells), an increase in protein was apparent in cell sonicate (Figure 12c & 13c). Unlike acetate- and Tween 80-grown cells, protein content observed in cell sonicate from olive oil-grown cells remained constant up to 20 h post cold shock. By 44 h post cold shock, protein increased in this

Figure 12. Protein content (μg/ml) measured in cell sonicate (▲) and cell culture supernatant (△). Cells were grown with acetate at a) 25 °C, b) 5°C and c) following a 25°C to 5°C cold shock.

a)


20
15
10
5.0
0.0
0
2
4
6
8
10
12
Time (hours)

b)


Protein Content (µg/ml)
20
15
10
5.0
0.0
0
20
40
60
80
100
Time (hours)

c)

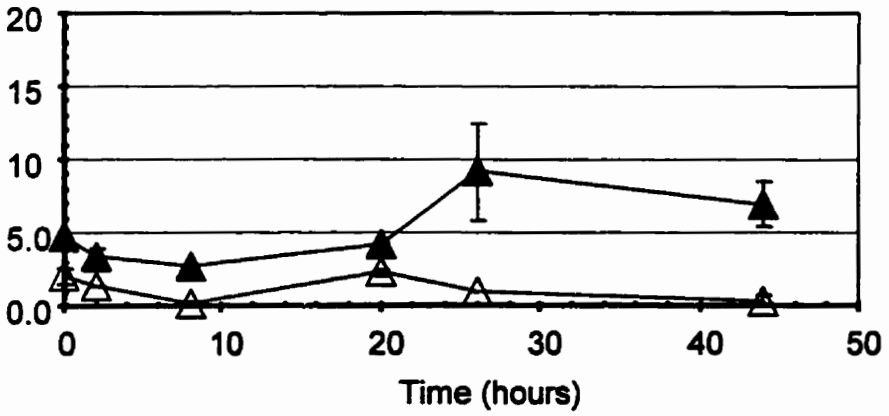
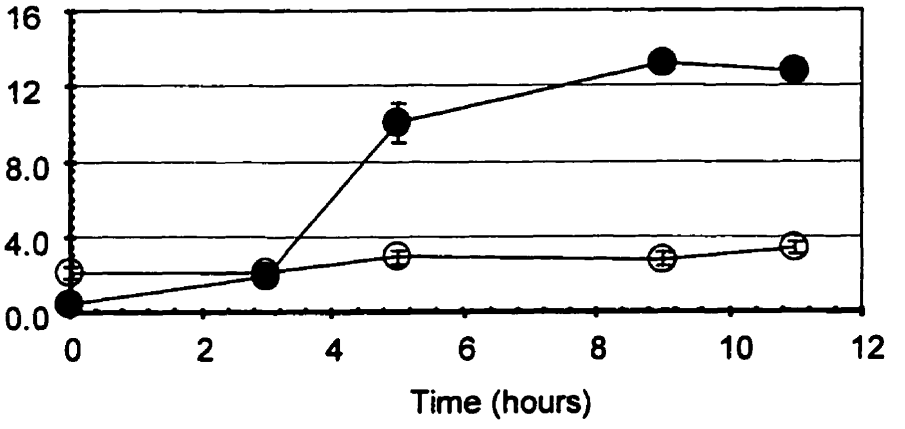
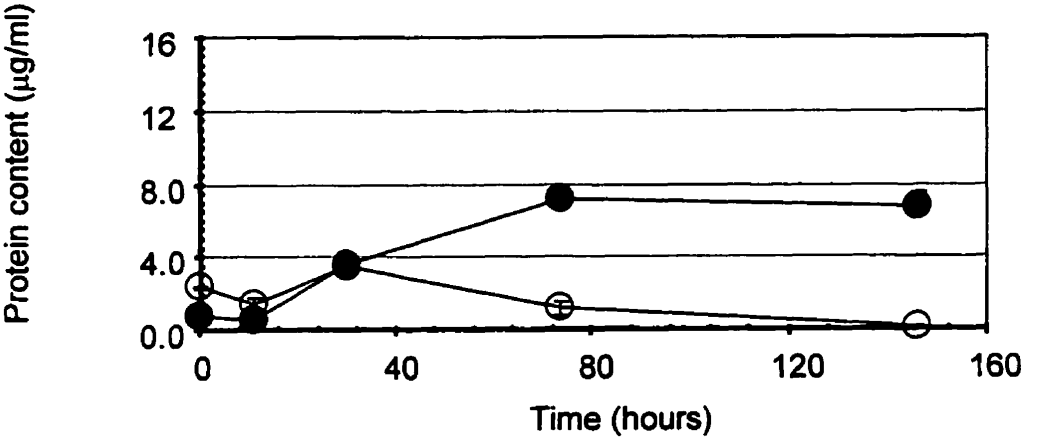

20
15
10
5.0
0.0
0
10
20
30
40
50
Time (hours)

Figure 13. Protein content (µg/ml) measured in cell sonicate (●) and cell culture supernatant (○). Cells were grown with Tween 80 at a) 25 °C, b) 5 °C and c) following a 25 °C to 5 °C cold shock.

a)


16
12
8.0
4.0
0.0
0
2
4
6
8
10
12
Time (hours)

b)


Protein content (µg/ml)
16
12
8.0
4.0
0.0
0
40
80
120
160
Time (hours)

c)

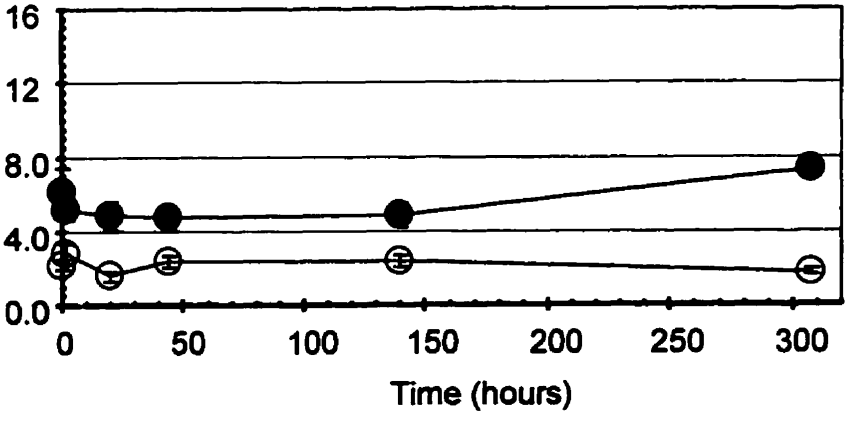
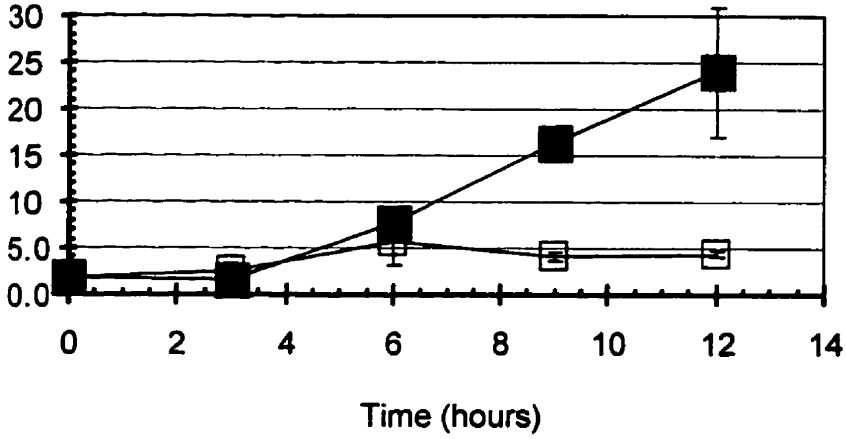
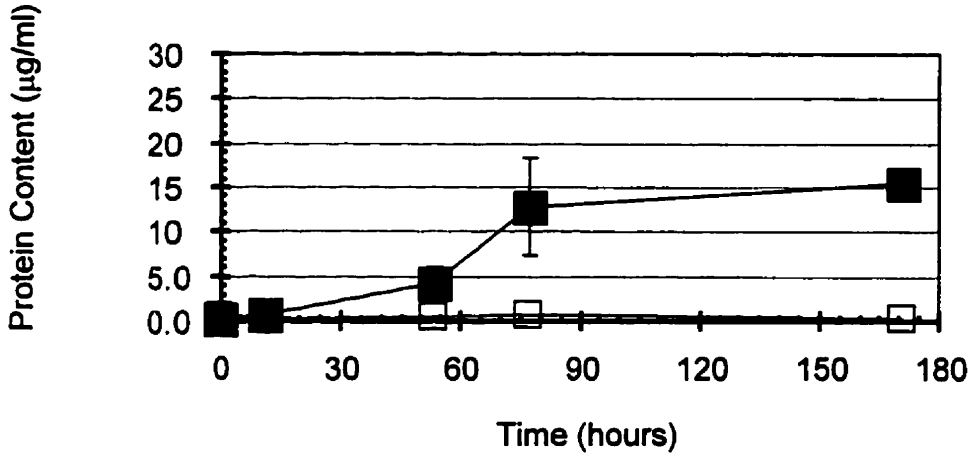

16
12
8.0
4.0
0.0
0
50
100
150
200
250
300
Time (hours)

Figure 14. Protein content (µg/ml) measured in cell sonicat( ) and cell culture supernatant (□). Cells were grown with olive oil at a) 25°C, b) 5°C and c) following a 25°C to 5°C cold shock.

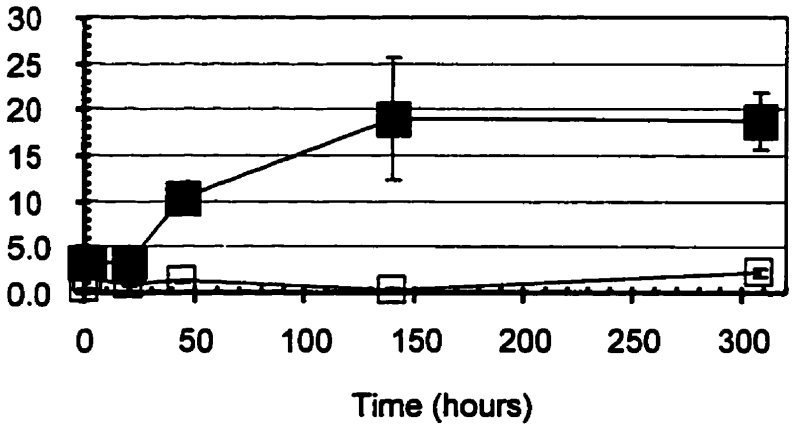
a)


30
25
20
15
10
5.0
0.0
0
2
4
6
8
10
12
14
Time (hours)

b)


Protein Content (µg/ml)
30
25
20
15
10
5.0
0.0
0
30
60
90
120
150
180
Time (hours)

c)


30
25
20
15
10
5.0
0.0
0
50
100
150
200
250
300
Time (hours)

fraction. Protein content in cell culture supernatant remained low regardless of carbon source.

Esterase Substrate Specificity

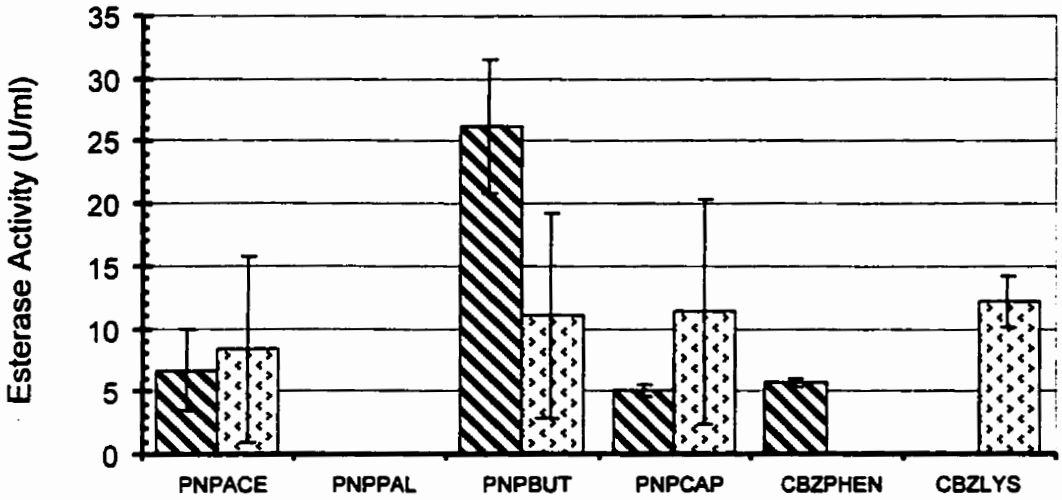
p-Nitrophenol substrates

Esterase activities against various substrates were measured in cell sonicate and cell culture supernatant of cells growing in Tween 80. Cell sonicate and supernatant samples were obtained from mid-exponential phase cultures incubated at 25°C and from the same cultures 50 h after being subjected to a 25°C to 5°C cold shock. The substrates tested were mostly fatty acids ester-linked to *p*-nitrophenol (PNP). The substrates tested included PNP-acetate (PNPACE), palmitate (PNPPAL), butyrate (PNPBUT), caprylate (PNPCAP), N-carboxybenzyloxy-phenylalanine (PNP-CBZPHEN) and N-carboxybenzyloxy-lysine (PNP-CBZLYS). Activity was determined spectrophotometrically by monitoring PNP release.

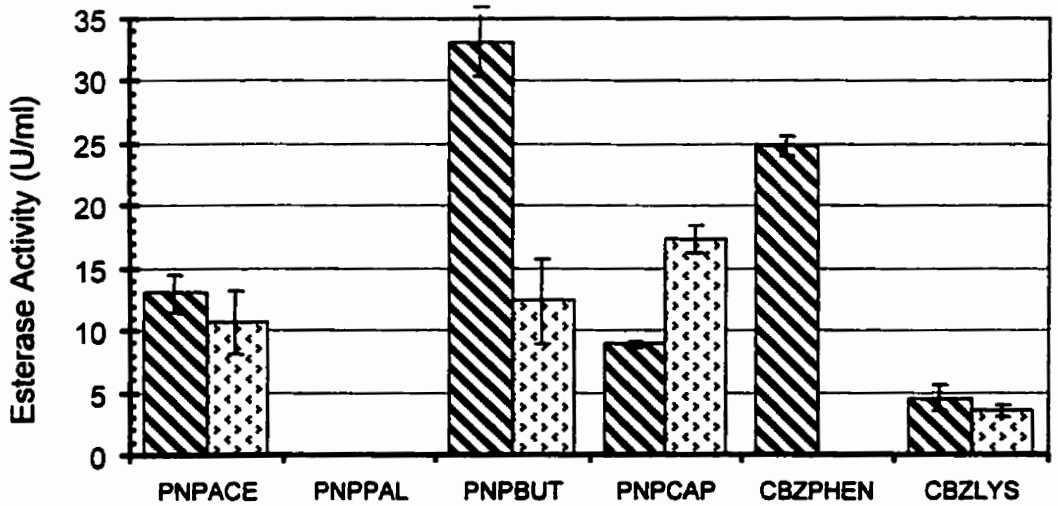
Cell sonicate and cell culture supernatant obtained from 25°C mid-exponential cultures hydrolyzed PNPACE, PNPBUT, and PNPCAP (Figure 15a). Activity against PNPACE and PNPCAP was similar in both fractions, wherase activity measured against PNPBUT in cell sonicate was two-fold greater (26 U/ml) than activity observed for cell culture supernatant (11 U/ml). Cell culture supernatant obtained from 25°C mid-exponential cells was unable to hydrolyze PNP-CBZPHEN wherase esterase activity was measured against this substrate in cell sonicate (6.0 U/ml). In contrast, cell sonicate was unable to hydrolyze PNP-CBZLYS substrate but an activity of 12 U/ml against this substrate was measured in cell culture supernatant (Figure 15a). These differences in substrate specificities suggest that the extracellular esterase is different than the cell associated esterase.

Figure 15. Substrate specificity of esterase measured in cell sonicate () and cell culture supernatant () samples. Cells were grown in Tween 80. Samples represent a) 25°C mid-log controls and b) 50h post 25°C to 5°C cold shock. Substrates tested were *p*-nitrophenol acetate (PNPACE), palmitate (PNPPAL), butyrate (PNPBUT), caprylate (PNPCAP), N-carboxybenzyloxy-phenylalanine (CBZPHEN) and N-carboxybenzyloxy-lysine (CBZLYS). Data are mean ± S.D. of triplicate samples.

a)


Esterase Activity (U/ml)
35
30
25
20
15
10
5
0
PNPACE
PNPPAL
PNPBUT
PNPCAP
CBZPHEN
CBZLYS

b)


Esterase Activity (U/ml)
35
30
25
20
15
10
5
0
PNPACE
PNPPAL
PNPBUT
PNPCAP
CBZPHEN
CBZLYS

After cold shock, both fractions maintained the ability to hydrolyze PNPACE, PNPBUT and PNPCAP. Activities against these substrates in both sonicate and supernatant samples were higher compared to the 25°C mid-exponential cells (Figure 15b). Activity measured against PNP-CBZPHEN was 5-fold higher compared to 25°C mid-exponential samples (Figure 15b). Unlike the 25°C mid-exponential samples, esterase activity measured against PNP-CBZLYS was detected in both cell sonicate and cell supernatant fractions. Increased activity in cell sonicate samples indicates an increased synthesis of cell-associated esterase activity following a decrease in growth temperature. PNPPAL hydrolysis was undetected in both fractions regardless of incubation treatment (Figure 15 a & b).

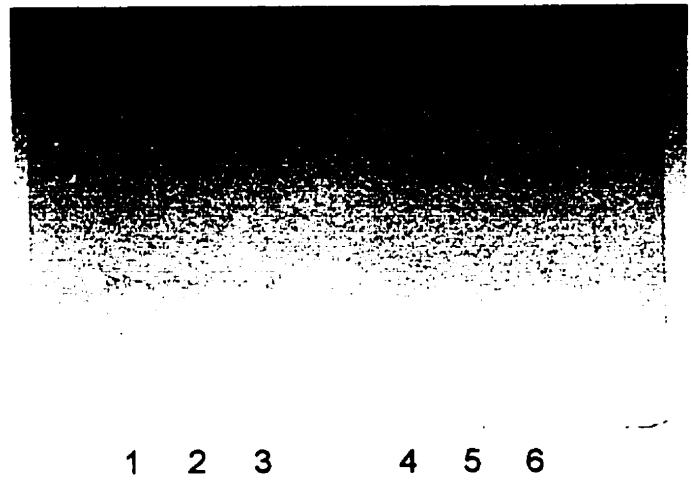
α & β- Naphthyl Ester Substrates

Cell sonicate and cell culture supernatant samples were obtained from cells grown with acetate, Tween 80 or olive oil. Culture samples were removed at 25°C mid-exponential phase and at 50 h after cold shock, subjected to electrophoresis on non-denaturing polyacrylamide gels and placed in a substrate-coupler staining solution. This technique permitted the testing of substrate specificity and visualization of esterase band(s). Naphthyl ester substrates tested included α & β- naphthyl acetate ester, α & β-naphthyl butyrate ester and α & β-naphthyl laurate ester.

Figures 16a-c show an example of results for Tween 80-grown cells. Three principal bands of enzymatic activity were distinguished (bands A, B & C). Bands A & B were found only in cell sonicate samples and band C was found only in cell culture supernatant samples. All three bands were observed for 25°C mid-exponential samples. Band A was active against α-naphthyl acetate ester only (Figure 16a) and band B was active against α-naphthyl butyrate

Figure 16. Polyacrylamide gels showing esterase bands. Cells were grown with Tween 80 as sole source of carbon. a) shows band A, a 19-kDa cell-associated esterase; gel was treated withα-acetate substrate (cold shocked samples), b) shows band B, a 53-kDa cell-associated esterase; gel was treated with α-butyrate substrate (cold shocked sample) and c) shows band C, a 200-kDa extracellular esterase; gel was treated with β-acetate substrate (25°C mid-exponential control). Lanes 1-3 represent cell sonicate samples and lanes 4-6 represent cell culture supernatant samples. Triplicate cultures were assayed.

a)



19 kDa

1 2 3 4 5 6

b)

53 kDa

1 2 3 4 5 6

c)

200 kDa

1 2 3 4 5 6

ester only (Figure 16b & c). These two bands were not detected when cells were grown at 25°C with either acetate or olive oil as sole carbon sources (Table 6). Band C isolated from culture supernatant for Tween 80-grown cells was active against a number of substrates which included α-naphthyl acetate ester, β-naphthyl acetate ester and β-naphthyl butyrate ester (Figure 16a). The same band found in olive oil-grown cells was active against α-naphthyl butyrate ester only (Table 6). Band C was not detected in supernatant obtained from acetate-grown cell samples.

Bands A and B were also present in samples obtained 50 h after cold shock from Tween 80-grown cells (Table 6). The intensity of band B was greater in cold-shocked samples compared to 25°C mid-exponential controls. Band B was also found in cold-shocked cells grown in acetate and olive oil (Table 6). Band C found in supernatant from 25°C cultures grown with Tween 80 was not present 50 h post cold shock. Similarly, this band was not detected in samples obtained from cold-shocked cells grown in acetate (Table 6). However, band C was detected in supernatant from cold-shocked cells grown in olive oil but the substrate specificity of this band was different from that observed for 25°C mid-exponential samples (Table 6).

Protein gels stained with Coomassie Blue were run parallel with substrate gels in order to determine the molecular weight of all proteins active against the ester substrates utilized (Figure 17 a,b). The molecular mass for bands A, B and C were estimated to be 19, 53 and 200-kDa, respectively.

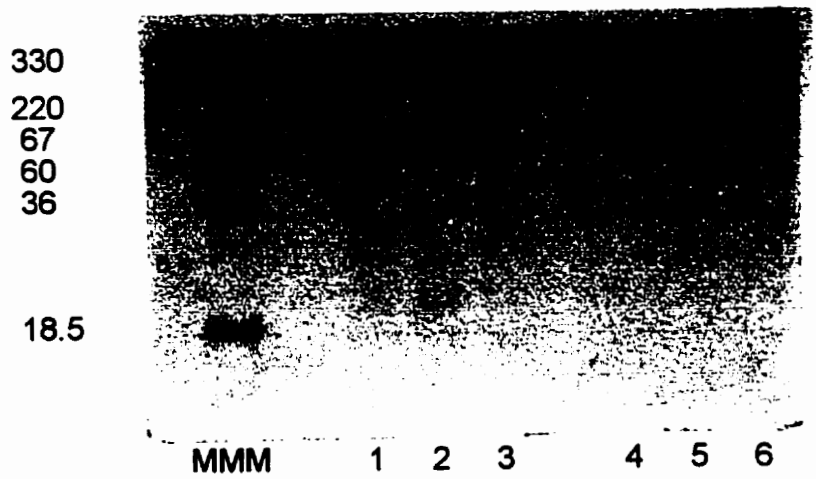
Table 6. Esterase identification and substrate specificity against naphthyl ester substrates when strain HH1-1 was grown with acetate, Tween 80 or olive oil.

		25°C Mid-log				50h Post Cold Shock		
		Sonicate		Supernatant		Sonicate		Supernatant
	Carbon Source	19 kDa	53 kDa	200 kDa		19 kDa	53 kDa	200 kDa
	Acetate							
Ester Substrates	α-acetate	-	-	-		-	-	-
	β-acetate	-	-	-		-	-	-
	α-butyrate	-	-	-		-	+	-
	β-butyrate	-	-	-		-	-	-
	α-laurate	-	-	-		-	-	-
	β-laurate	-	-	-		-	-	-
	Tween 80							
Ester Substrates	α-acetate	+	-	+		+	-	-
	β-acetate	-	-	+++		-	-	-
	α-butyrate	-	+	-		-	+++	-
	β-butyrate	-	-	+		-	-	-
	α-laurate	-	-	-		-	-	-
	β-laurate	-	-	-		-	-	-
	Olive Oil							
Ester Substrates	α-acetate	-	-	-		-	-	-
	β-acetate	-	-	-		-	-	-
	α-butyrate	-	-	+		-	+	+
	β-butyrate	-	-	-		-	-	+
	α-laurate	-	-	-		-	-	-
	β-laurate	-	-	-		-	-	-

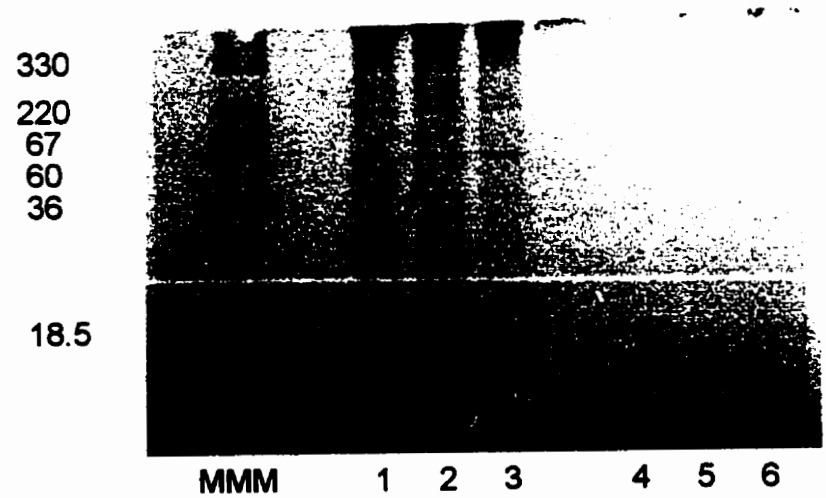
+, slight; ++, moderate; +++ strong; -, not present

Figure 17. Polyacrylamide gels stained with Coomassie Blue. Gels show protein Bands from a) 25°C mid-exponential controls and b) 50 h post cold shock cultures. Molecular mass marker lane (MMM) show proteins Thyroglobulin, 330 Kda; Ferritin (half unit), 220 kDa; Albumin, 67 kDa; Catalase, 60 kDa; Lactate dehydrogenase, 36 kDa; and Ferritin, 18.5 kDa. Lanes 1-3 represent cell sonicate samples and lanes 4-6 represent cell culture supernatant samples. Triplicate cultures were assayed.

a)



b)



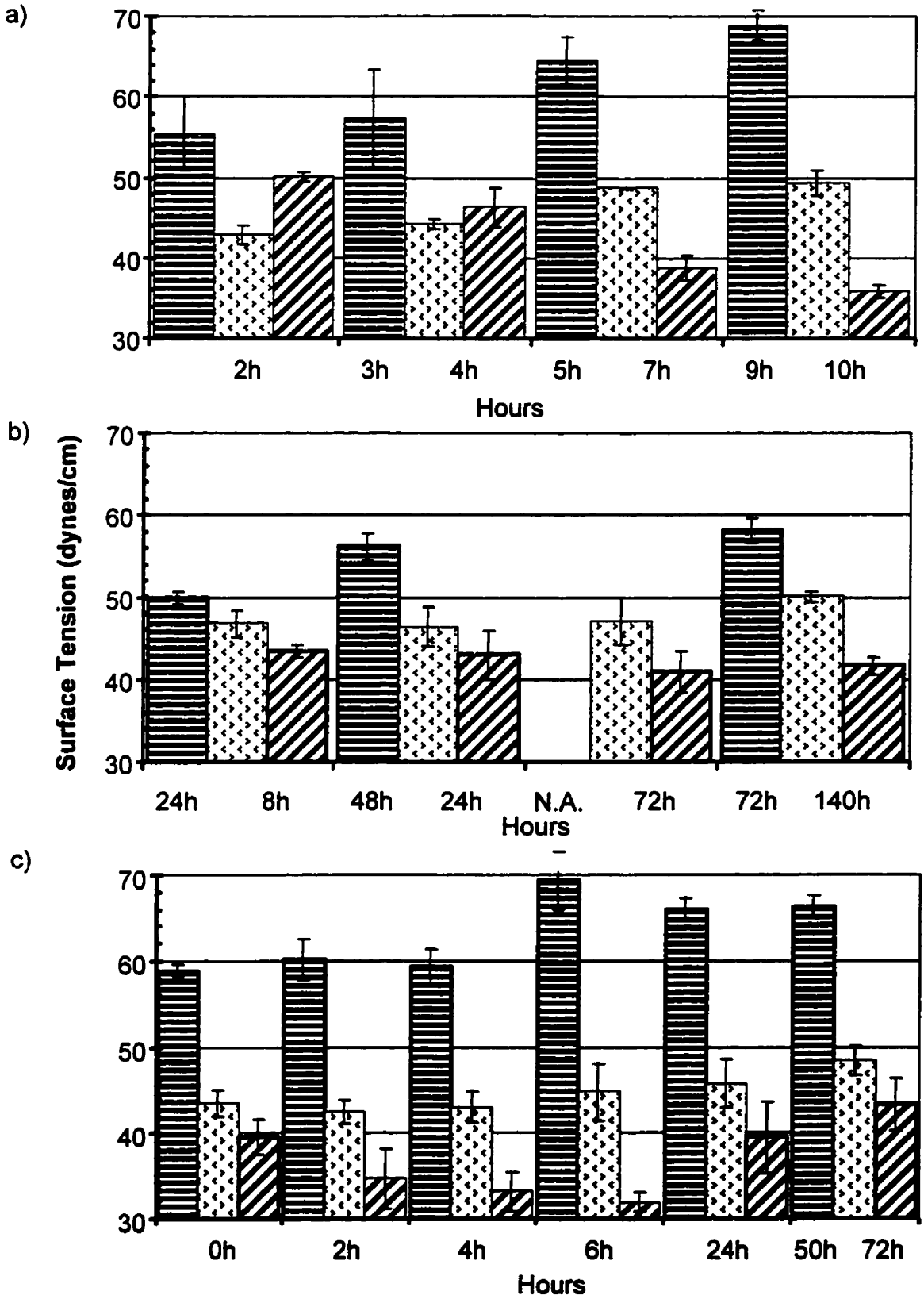
Surface Tension

Biosurfactant activity is often determined by measuring changes in surface tension (Hommel 1990). The surface tension of distilled water is 72 dynes/cm, and in the presence of a bioemulsifier, surface tension is lowered (Desai & Banat 1997). Figure18a-c shows surface tensions measured in culture supernatants during batch culture growth at 25°C, 5°C and after cold shock in the presence of acetate, Tween 80 or olive oil.

Little change in medium surface tension occurred under any incubation condition for acetate- and Tween 80-grown cells. Surface tension measured in supernatant samples obtained from olive oil cultures incubated at 25°C decreased from 50 dynes/cm at 2 h to 36 dynes/cm by 7 h (Figure 18a). During incubation at 5°C, surface tension of medium remained constant suggesting that this temperature affected the production of a surface reducing exopolymer produced by olive oil-grown cells (Figure 18b). In contrast, following cold shock, surface tension measurements decreased from 40 dynes/cm to 32 dynes/cm by 6 h post cold shock (Figure 18c). No further decrease in surface tension was observed and by 24 h post cold shock, surface tension measurements had increased to values similar to the 25°C mid-exponential controls (39 dynes/cm).

These results show that the production of an extracellular exopolymer capable of reducing the surface tension of the culture medium by HH1-1 was not induced in the presence of acetate or Tween 80. Production was induced, however, in the presence of olive oil, but production was reduced at the lower growth temperature. The reason for the decrease in surface tension following cold shock for olive oil-grown cells is less clear. The reduction of surface tension may be the result of the leakage of cellular constituents causing a temporary decrease in surface tension.

Figure 18. Surface tension of cell culture supernatant at a) 25°C b) 5°C and c) following a 25°C to 5°C cold shock. Cells were grown in acetate ▤, Tween 80 ⊡ or olive oil ▨. Each bar represents mean ± S.D.of triplicate samples. N.A., sample was not analyzed.

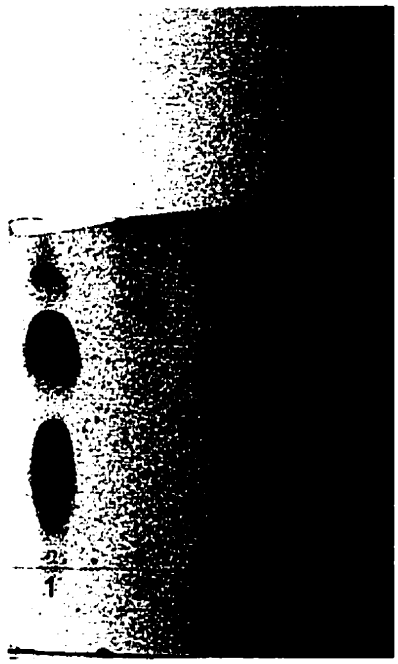

a)
70
60
50
40
30
2h
3h
4h
5h
7h
9h
10h
Hours
b)
Surface Tension (dynes/cm)
70
60
50
40
30
24h
8h
48h
24h
N.A.
72h
72h
140h
Hours
c)
70
60
50
40
30
0h
2h
4h
6h
24h
50h
72h
Hours

Partial Characterization of Surface-Active Exopolymer

In order to isolate and characterize the biosurfactant that seemed to be produced by olive oil-grown cells, cell culture supernatant was extracted with chloroform:methanol and analyzed by thin layer chromatography (TLC) on silica gel plates. Once the plates were developed, they were exposed to different reagents in order to visualize the presence of sugars (Molish reagent), lipids (iodine vapours) and proteins (orcinol reagent) (Figure 19a-c). The HH1-1 extract was spotted onto lanes 2 and 3. For comparison, pure olive oil was spotted onto lane 4 and purified rhamnolipid extracted from *Pseudomonas aeruginosa* UW-1 (Sim et al., 1997) was spotted onto lane 1. After the application of Molish reagent, three purple bands at Rf 0.5, 0.7 and 0.85, indicative of a sugar-containing compound, were visualized in lanes 2 and 3 (Figure 19a). Similarly, three yellow bands at the same Rf values were observed with iodine vapour indicating the presence of a lipid component (Figure 19b). No bands were observed after application of orcinol (Figure 19c).

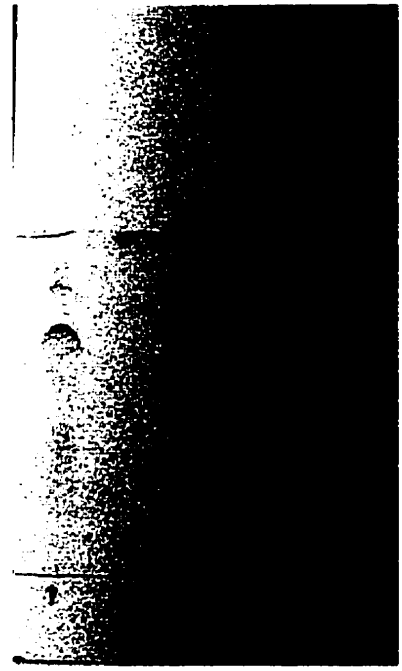
Figure 19. Thin layer chromatography characterization of HH1-1 biosurfactant. Surface-active extract was spotted onto silica gel plates then developed in a chloroform:methanol:water solvent sytem (65:15:2). Developed plates were exposed to a) Molish reagent to visualize the presence sugars, b) iodine vapours to visualize the presence of lipids and c) orcinol to visualize the presence of amino groups. Lane 1, rhamnolipid control; Lanes 2 & 3 HH1-1 biosurfactant extract; and lane 4,olive oil.

a)



b)

Rf .85
.7
.5



c)

Discussion

A growing interest in lipolytic enzymes has developed, in part because of the versatility of the enzymes in industrial applications, waste water treatment, and bioremediation schemes. One important characteristic of lipolytic enzymes, particularly for applications in industry, is their temperature stability characteristics (Choo et al., 1998). Studies with *Acinetobacter* spp. have focused primarily on characterizing and elucidating the molecular and physiological mechanisms governing lipolytic enzymes (Kok et al. 1995: Kok et al. 1993). Although some studies have investigated the effects of high temperatures on the activity of these enzymes, few have evaluated the effects of low temperatures on their production and activity (Breuil et al., 1975; Breuil et al., 1975a).

In the present study, lipase activity was detected in the cell culture supernatant when cells were grown with either Tween 80 or olive oil at 25°C, with the maximal activity occurring at late-exponential phase. It is typical to measure lipase activity in cell culture medium late in growth under batch growth conditions (Feller et al., 1990; Gilbert et al., 1991; Kok et al., 1995; Dharmsthiti et al., 1998). At the lower growth temperature, site of action and degree of lipase activity were affected. A reduction of lipase activity was observed in the supernatant of cells grown in Tween 80, with an accompanying increase in measureable activity detected in the cell sonicate. Activity, measured in the supernatant, remained higher than the activity measured in cell sonicate. In contrast, lipase activity, measured from cells grown in olive oil at the lower growth temperature, was detected in cell sonicate only, and at considerably lower levels than those observed at 25°C. Cold-shock experiments confirmed a decrease in the activity in culture supernatant. The subsequent increase in activity measured

in cell sonicate suggests that the production and/or secretion of lipase was reduced by low temperatures.

The factors that affect lipase production continue to be under investigation (Kok et al., 1996). Studies investigating the effects of temperature on the activity of purified lipase show that lipases remain active through a large temperature range that is typically related to the cell's own growth temperature range (Feller et al., 1990; Dharmsthiti et al., 1998; Choo et al., 1998). The presence of long-chain fatty acids in growth medium has been found to inhibit lipase production (Gilbert et al., 1991; Jaegar et al., 1994). Kok et al. (1996) have proposed that long-chain fatty acids (in particular, oleic acid) taken up by the cell interact with a regulator which binds upstream of *lipA* (the gene encoding lipase), preventing its transcription. Hydrolysis of olive oil will result in the liberation of oleic acid, and to a lesser extent palmitic and linoleic acids, because these are the primary fatty acids comprising this substrate (84.5%, 9.4% and 4.0% respectively) (Windholz, 1976). As has been previously noted in this study, cells grown in olive oil have a significantly greater content of 18:1 fatty acid. The effect of additional oleic acid associated with olive oil cells may affect lipase production at the molecular level.

The increase in lipase activity observed in cell sonicate at the lower temperature may also be indicative of inefficient transport of the enzyme across membranes. This is particularly true for cells grown with olive oil. Feller et al. (1990) reported an inhibition in lipase excretion when evaluating the effect of temperature on four psychrotrophic strains of *Moraxella* spp. However, in contrast to the situation for HH1-1, temperatures above optimum were inhibitory, and the greatest lipase excretion was observed as low as 3°C. A loss of cell membrane integrity, as indicated by a reduction in membrane fluidity, may be

preventing the transport of this enzyme across the membranes of HH1-1 into the extracellular medium.

The two-step transport pathway reported for *Acinetobacter* spp. entails excretion across the cytoplasmic membrane mediated by the Sec system, and transport across the outer membrane mediated by a system similar to the Xcp system found in *Pseudomonas* spp. (Kok et al., 1995; Jaeger et al., 1994). The transport system relies on different intracellular, periplasmic, and integral-membrane proteins. Changes in membrane fluidity have been found to alter the functioning of membrane-embedded proteins (Sikkema et al., 1995). In the present study, proteins responsible for transporting lipase across the membranes may have been affected by the change in membrane fluidity. Olive oil is an insoluble triacylglycerol, consequently, extracellular lipase is required to hydrolyze this substrate. These results provide additional support for the conclusion that a reduction in the activity and production of this enzyme will have a greater effect on cells grown with olive oil relative to cells grown with either Tween 80 or acetate.

Esterases of *Acinetobacter* spp. are typically cell-associated during early growth and are then detected in the extracellular medium late in growth (Breuil & Kushner, 1975a; Shabtai & Gutnick, 1985). Tween compounds are often used to induce the production of esterase enzymes. In this study, esterase activity was monitored in both cell culture supernatant and cell sonicate when HH1-1 was grown on all three substrates. Activity and the site of action of esterase were similar under all incubation conditions when grown with acetate or Tween 80. As was observed with lipase, an increase in cell sonicate esterase activity after cold shock and during growth at 5°C suggested that a loss in cell membrane integrity may be interfering with the transport of this enzyme. However, because bacteria can possess a number of different esterases (Picard & Goullet, 1990; Goullet and Picard,

1991), cell-associated and/or extracellular (Kok et al., 1993), inactivation of an extracellular esterase concurrent with induction of a cell-associated esterase may be responsible for the observed response. These results suggested that the cell-associated esterase may be adapted to function at low temperatures.

Different esterase substrates were used to determine the number and specificities of esterases produced by HH1-1. Both techniques used in this study showed that cell sonicate and cell culture supernatant esterase activity are the result of different esterases. In addition to determining substrate specificity, the use of naphthyl esters with gel electrophoresis showed the production of at least two different cell-associated esterases and the production of one extracellular esterase. An increase in activity of the cell-associated esterase capable of hydrolyzing *p*-nitrophenol-cbz phenylalanine, and the presence of cell-associated esterase that hydrolyzes α-naphthyl butyrate after cold shock indicated that the induction of a cell-associated enzyme had occurred at low temperature. A decrease in extracellular esterase activity responsible for hydrolyzing *p*-nitrophenol-cbz lysine and β- and α-napthyl acetate indicated that this enzyme was affected at low temperatures.

Cell-associated esterase activity was greater than extracellular activity under all incubation conditions for cells grown in olive oil. The difference in activity and site of action of esterase between olive oil-grown cells and the other carbon sources suggests that cell-associated esterase is important for growth under all incubation conditions when olive oil is used as the sole carbon source. This is particularly evident during cold shock and growth at 5°C where cell-associated esterase activity is higher for olive oil-grown cells relative to acetate- and Tween 80-grown cells. Induction of a cell-associated esterase during cold shock or growth at 5°C that is capable of hydrolyzing olive oil may compensate for the loss in

lipase activity. As well, a cell-associated esterase may also be required to adjust membrane fatty acids in response to a loss in membrane fluidity after cold shock and to maintain optimal membrane fluidity during growth at low temperatures.

In this study, the activity of isocitrate lyase was also monitored under the three incubation treatments. Isocitrate lyase is an enzyme that is not transported across the membranes but is cell associated. The glyoxylate bypass pathway is active when cells are grown on acetate as the sole carbon source. This pathway is required to regenerate carbon for the synthesis of cellular components that would be quickly removed from the cell in the form of CO_2 if only the tricarboxylic acid cycle were functioning (Cozzone, 1998). Isocitrate lyase is the first enzyme in the bypass pathway that removes isocitrate from the tricarboxylic cycle by converting it to glyoxylate. Like the other enzymes associated with the tricarboxylic cycle, isocitrate lyase is an intracellular enzyme. The effect of low growth temperature on this enzyme was used to compare the effect of low growth temperature on the activity and site of action of esterases and lipases. As expected, when growing at 25°C isocitrate lyase activity was measured in cell sonicate only. Activity was maximal at late–exponential growth suggesting an increased requirement for the synthesis of 4-carbon intermediates. A drop in cell sonicate isocitrate lyase activity, with an increase in cell culture supernatant activity, was observed following cold shock, and although there was a small amount of activity in the cell sonicate, isocitrate lyase activity at 5°C was measured primarily in cell culture supernatant. The increase in isocitrate lyase activity measured in cell culture supernatant suggests that leakage of isocitrate lyase out of the cell and into the supernatant occurs after a drop in temperature and during growth at 5°.

The leakage of cellular components after cold shock was also reported by Leder (1972). It was suggested that one effect of an iso-osmotic cold shock on bacterial

membranes was the crystalization of lipids in membranes causing the formation of hydrophilic channels that facilitated the efflux of cellular components. The degree to which compounds leaked out of the cell, in part, was dependent on the carbon source (Leder, 1972). The response of isocitrate lyase activity in HH1-1 to cold shock supports the conclusion that a loss in membrane integrity occurs after a rapid down shift in growth temperature. The reason for leakage of isocitrate lyase into the extracelluar medium during growth at 5°C is less clear. Although the leakage of isocitrate lyase may affect growth rate at 5°C, cell yield is not affected. Protein content was monitored in cell sonicate and supernatant as a means of assessing changes in the activity and site of action of these enzymes. However, no apparent relationship between protein content and the other parameters was evident.

Microbial bioemulsifiers are important because they have many potential uses. Presently, the physiological functions of bioemulsifiers are not fully understood (Lin 1996), and very little is known about the effects of low growth temperatures on the production and activity of these compounds. The proposed functions for bioemulsifiers include enhanced solubilization of hydrocarbon substrates, hydrocarbon transport, cell adhesion, and antimicrobial agents (Desai & Banat 1997; Lin 1996). *Acinetobacter* is known for the production of exoploymers with powerful emulsifying activity. The production of surface-active exopolymers can occur on the microbial cell surface, or the exopolymer may be excreted into the extracellular medium. Shabtai & Gutnick (1985) found that although the bioemulsifier of *A. calcoaceticus* RAG-1 is isolated from the extracellular medium, it accumulates on the cell surface as a mini-capsule prior to being released. The release of surfactant was dependent on the activity of an extracellular esterase.

Results obtained in the present study show that the production of possibly a biosurfactant by HH1-1 is dependent on the carbon source supplied. Carbon source is known

from other studies to affect yield and composition of biosurfactants (Lin 1996). For example, although a *Pseudomonas* sp. produced biosurfactant while growing on water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol, the resulting surfactant was not as effective compared to the surfactant produced when this strain was supplied with water-insoluble substrates such as n-alkanes and olive oil (Desai & Banat, 1997). In contrast, *A. calcoaceticus* RAG-1 produces bioemulsifier when growing on hexadecane, ethanol, or acetate (Rosenberg et al., 1979). Surfactant production and effects of pH and cation concentration on its activity were similar in each case. Partial characterization of HH1-1 bioemulsifier, using TLC, suggested that it is comprised primarily of sugars and lipids. Many of the microbially-synthesized surface-active compounds that have been identified contain a lipid moiety consisting of unsaturated or saturated fatty acids (Desai & Banat, 1997). In addition, Belsky et al. (1979) suggested that the fatty acids bound to *Acinetobacter* RAG-1 emulsifier play an important role its function.

Duvnjak et al. (1982) examined the effect of varying cultivation temperature (23°C-32°C) on biosurfactant production by *Arthrobacter paraffineus*. These investigators found that surfactant biosynthesis decreased substantially when *A. paraffineus* was grown at 32°C, with the greatest amount of surfactant produced at 27°C. In the present study, surface tension remained constant during growth of HH1-1 at 5°C, suggesting that the surface tension reducing exopolysaccharide produced by HH1-1 was affected at this temperature. This may be due to the failure of the cells to release the emulsifier from the cell surface, because extracellular esterase activity was also reduced at the lower growth temperature. These data suggest therefore that the decrease in surface tension observed during cold shock

may have been due to release of intracellular-related proteins rather than an increase in exopolysaccharide production.

Conclusions

The enzyme studies indicate overall that membrane integrity is compromised when HH1-1 cells are incubated at low temperatures. These observations are consistent with the conclusion of the previous chapter. The activity of isocitrate lyase was unaffected by the lower growth temperature, but this typically cell-associated enzyme was detected in the extracellular medium when the cells were incubated at 5°C. This demonstrates that membranes are leaky at the lower growth temperature. Leakage of intracellular constituents may be molecule specific, or possibly confined to a certain size of molecule, so that losses of other important intracellular proteins under these conditions was minimized. This permitted the growth of these cells at low temperatures despite the loss in membrane integrity.

The effects of low temperature on esterase and lipase were considerably different than those observed for isocitrate lyase and acetate-grown cells. For instance, lipase which is an extracellular enzyme, was predominately measured in cell sonicate after cold shock and growth at 5°C. These results again indicate that a loss of cell membrane integrity was influencing the transport of lipase. Transport inhibition may result from the changes in membrane fluidity that occur at 5°C. Because of the inefficient transport of lipase across membranes, and the possible repression of lipase production due to the presence of oleic acid, the ability of olive oil-grown cells to grow at low temperatures was severely impaired. Although lipase was also produced in the presence of Tween 80, esterases were likely responsible for its hydrolysis. As a result, cells grown with Tween 80 were not affected by low growth temperatures to the same extent as olive oil-grown cells.

HH1-1 produced at least three esterases, a 19-kDa cell associated esterase, a 53-kDa cell-associated esterase, and a 200-kDa extracellular esterase. Although the activity of the extracellular esterase was inhibited at low temperatures, the 19-kDa cell-associated esterase was still active, and an apparent increase in induction of the 53-kDa cell associated enzyme was observed after cold shock. The reasons for an increased induction of the 53-kDa cell associated esterase after cold shock are not clear, but because this enzyme was observed after cold shock for all carbon sources, it may play another role in addition to substrate hydrolysis.

Restricted exopolysaccharide production during growth at 5°C also suggested that olive oil-grown cells are most affected by low temperatures. A failure to release surfactant from the cell may have resulted in the failure of cell culture supernatant to reduce surface tension. Again, the inability of the cells to release surfactant may be related to a loss of cell membrane integrity. In addition, reduction of extracellular esterase activity at the lower temperature may have affected the release of the surfactant from the cell surface.

The cold shock response of HH1-1 was characterized in terms of changes in cell protein. Olive oil-grown cells display a number of properties indicative of a greater cold sensitivity than is shown by acetate- or Tween 80-grown cells as shown in earlier chapters. Protein profiles for cells grown with olive oil were therefore compared to the protein profiles obtained for cells grown in the presence of acetate and Tween 80, to determine if protein synthesis was inhibited to a greater extent in cells grown in the presence of olive oil.

Cold shock and Cold Acclimation Proteins in HH1-1

The induction of csps following a 25°C to 5°C cold shock by HH1-1 growing with acetate, Tween 80 or olive oil was investigated. To determine the time at which the induction of csps first occurred following cold shock, a time-course study was conducted. Proteins were radiolabelled 1 h, 2 h, 5 h, and 6 h after cold shock, subjected to 1-D SDS PAGE and compared to protein profiles obtained for the 25°C mid-exponential controls. Induction of csps occurred 2 h after cold shock for all carbon sources (data not shown). In subsequent experiments, csps proteins were analyzed 2 h after cold shock using 2-D SDS-PAGE and computing scanning laser densitometry. Protein profiles were compared to 25°C mid-exponential controls and protein profiles produced from radiolabelled proteins at 72 h (acetate-grown cells) and 140 h (Tween 80- and olive oil-grown) post cold shock (5°C acclimated cultures).

At 2 h post cold shock, 3 csps (89a, 36a and 18) were synthesized by HH1-1 on all tested carbon sources (Figure 20b, 21b, 22b). Two of these proteins, csp18 and csp89a, were

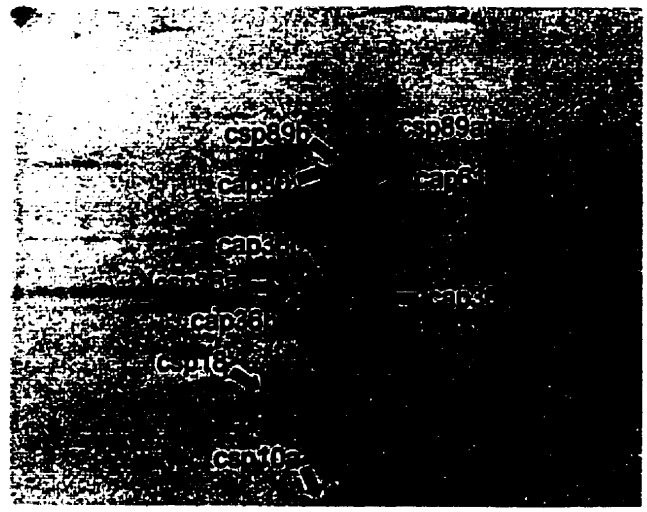
transiently induced. The greatest increase was observed for csp18 with a 40.2 fold-increase for acetate-grown cells, 16.8 fold-increase for olive-oil grown cells and 92.4 fold-increase for Tween 80-grown cells. Increases of csp 89a ranged from 2.7-fold (acetate-grown cells) to 6.9-fold (olive oil-grown cells) (Table 7). Although the induction level of csp36a decreased 72 h (acetate-grown cells) and 140 h (Tween 80- and olive oil-grown cells) after cold shock, its density was still elevated at these times compared to the 25°C mid-exponential controls (2.8-5.7-fold increase). This indicates that csp36a also functions as a cap (Figures 20c, 21c and 22c). Induction of all other csps 2 h after cold shock was dependent on carbon source. Csp10a, another transiently synthesized protein, was present only in acetate- and Tween 80-grown cells with increases of 14.2 and 12.0, respectively. Although csps 27 and 25a were common to all three carbon sources, levels increased in Tween 80-grown cells only (4.1 and 5.3-fold increase, respectively) and csp 25b, found only in Tween 80- and olive oil-grown cells, was induced 27.3-fold in olive oil-grown cells only. Finally csp12 was transiently induced at 2 h in olive oil-grown cells only (fold-increase of 18.6).

Similar trends were observed for cold acclimation proteins. Cap61 was the only protein with increased synthesis at 72 h (acetate-grown cells) or 140 h (Tween 80- and olive oil-grown cells) post cold shock for all carbon sources (Figures 20c, 21c and 22c). Increases of the protein ranged from 4.5-fold (acetate-grown cells) to 14.6-fold (Tween 80-grown cells) (Table 7). In addition to cap61, increased synthesis of caps 80, 36b, 36c and 30 was observed for acetate-grown cells, caps 44, 38a, 38b, 30, 10b for Tween 80-grown cells and caps 90, 68, 44, 38a, 36b, 36c for olive oil-grown cells.

Generally, few differences were observed between protein profiles for HH1-1 acetate-grown cells with vitamin supplementation incubated at 30°C, 25°C, 20°C, 15°C, 10°C, and 5°C (Figure 23 a-f). Protein profiles of HH1-1 grown at 25°C and 5°C were similar to those

Figure 20. Induction of csps and caps in HH1-1 following a 25°C to 5°C cold shock. Cells were grown with acetate as the sole source of carbon. Fluorograms represent a) 25 °C mid-exponential controls b) 2 h after cold shock and c) 72 h growth at 5°C. Location of csps and caps are identified by their molecular mass (kDa).

a)


csp89b
csp89a
csp36a
csp18
csp10a

b)


csp89b
csp89a
csp36a
csp18
csp10a

c)

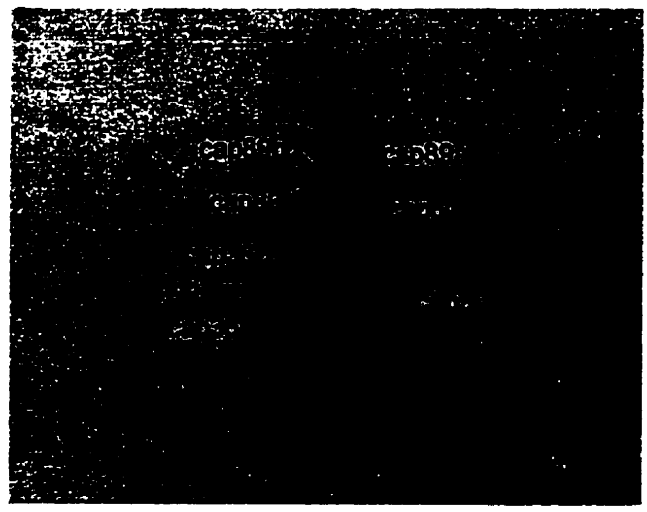
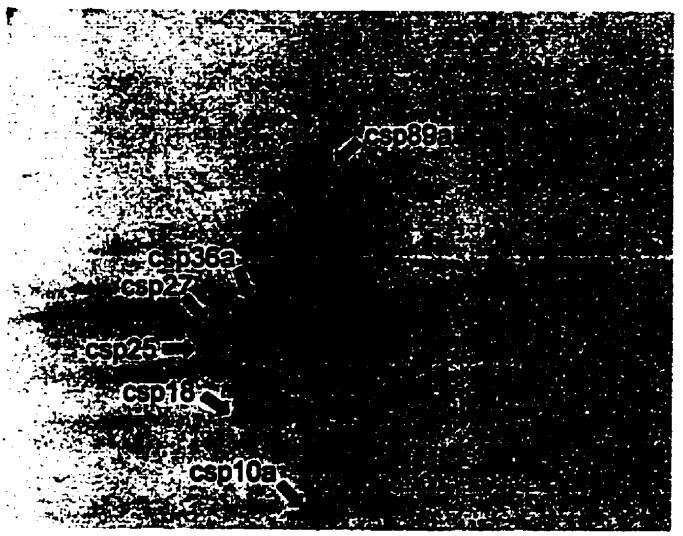


Figure 21. Induction of csps and caps in HH1-1 following a 25°C to 5°C cold shock. Cells were grown with Tween 80 as the sole source of carbon. Fluorograms represent a) 25°C mid-exponential controls b) 2 h after cold shock and c) 140 h growth at 5°C. Location of csps and caps are identified by their molecular mass (kDa).

a)

b)



c)

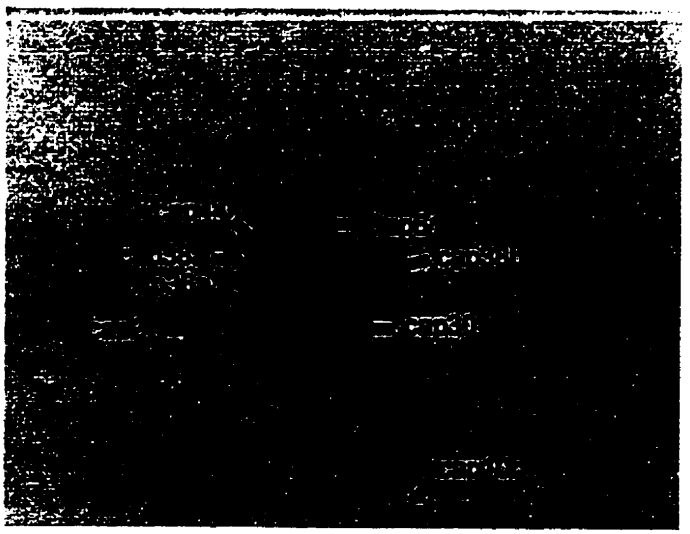
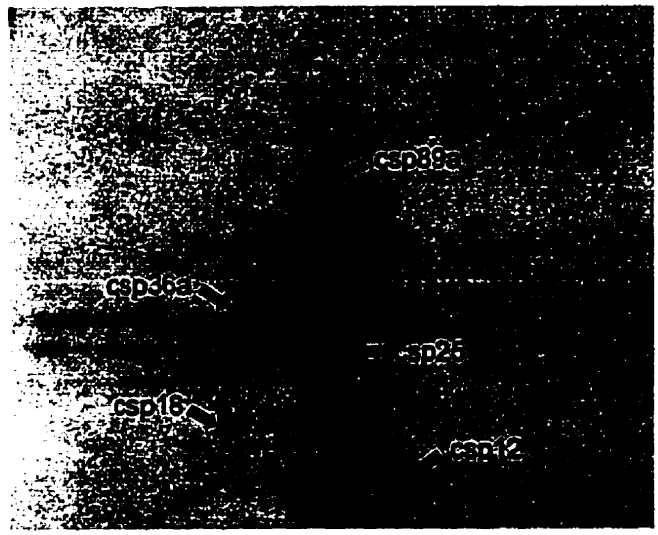


Figure 22. Induction of csps and caps in HH1-1 following a 25°C to 5°C cold shock. Cells were grown with olive oil as the sole source of carbon. Fluorograms represent a) 25°c mid-exponential controls b) 2 h after cold shock and c) 140h growth at 5°C. Location of csps and caps are identified by their molecular mass (kDa).

a)

b)



c)

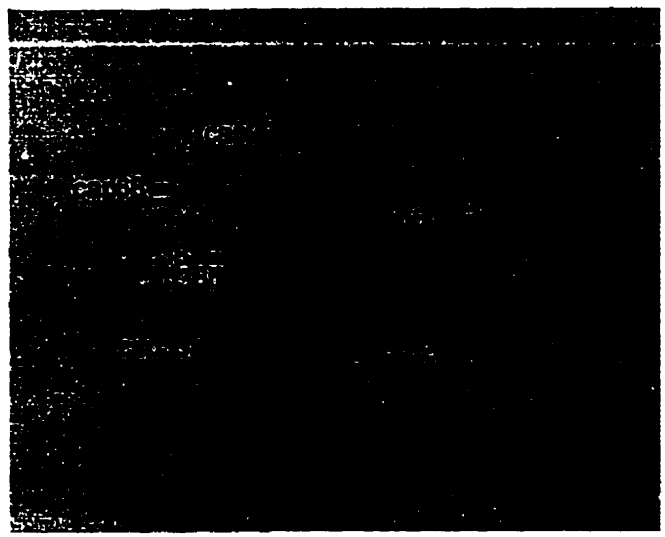


Table 7. Induction of csps and caps in HH1-1 following a 25°C-5°C cold shock. Cells were grown in acetate, Tween 80 or olive oil. The fold-increase in proteins was determined using Computer Scanning Laser Densitometry.

	Acetate		Tween 80		Olive Oil	
Protein	Fold Increase					
csps	2h	72h	2h	140h	2h	140h
89a	2.7	--	4.8	--	6.9	--
89b	1.5	1.9	--	--	--	--
36a	1.9	3.1	2.5	5.7	1.7	2.8
27	--	--	4.1	5.0	NP	NP
25a	--	--	5.3	5.0	NP	NP
25b	NP	NP	NP	NP	27.3	7.6
18	40.2	--	92.4	--	16.8	--
12	NP	NP	NP	NP	18.6	--
10a	14.2	--	12.0	--	NP	NP
caps	2h	72h	2h	140h	2h	140h
90	NP	NP	--	--	--	6.4
80	--	2.7	NP	NP	NP	NP
68	NP	NP	NP	NP	NP	6.8
61	--	4.5	--	13.5	--	14.6
44	NP	NP	--	2.8	--	4.8
38a	NP	NP	--	3.4	--	4.4
38b	NP	NP	--	52.2	NP	NP
36b	--	2.0	--	--	--	10.6
36c	--	4.2	--	--	--	7.6
30	--	4.8	--	3.1	--	--
10b	--	--	43.1	--	NP	NP

(--) less than 1.5-fold induction, (NP) protein not present

obtained at these temperatures for acetate-grown cells (Figure 20 a,c) during the cold shock experiments discussed above. Cells grown at 30°C synthesized an acidic protein found to have the same molecular mass as csp12, synthesized 2 h post cold shock in olive oil-grown cells (Figure 23a). This protein may be a stress protein, rather than strictly a csp protein, since 30°C is close to the maximum growth temperature for this bacterium.

Cold shock experiments were repeated and an attempt was made to separate radiolabelled proteins in the supernatant, periplasm, cell envelope and intracellular fraction and subject those in each fraction to 2-D SDS PAGE. However, the absence of detectable protein on fluorograms for the supernatant, cell envelope, and intracellular fractions indicated the methodology was not adequate. The problem may have been due to a small cell mass used, so that protein recoveries in the various fractions were too small to allow detection. To further increase the cell mass subjected to fractionation would have necessitated the development of a new protocol for processing the biomass, so this approach was not pursued further. Six proteins were detectable on fluorograms representing the periplasmic fraction. The six proteins were found at the basic end of the fluorogram at 25°C for all carbon sources (Figure 25a-c) and were still present after growth had resumed at 5°C. However, at 140 h additional proteins were observed for olive oil-grown cells (Figure 24c). This result suggests that olive oil-grown cells may be required to synthesize additional caps at the lower growth temperature.

Presence of a Homologous E. coli csp A Gene

To determine if a gene homologous to the *E. coli cspA* gene is encoded on the chromosome of HH1-1, PCR using primers specific for the *csp*A gene was conducted. The

Figure 23. Protein profiles for HH1-1 when grown in BSM with vitamin supplementation and acetate as the sole source of carbon. Cells were incubated at a) 30°C b) 25°C c) 20°C d) 15°C e) 10°C and f) 5°C.

a)

b)

Basic

Acidic

c)

d)

e)

f)

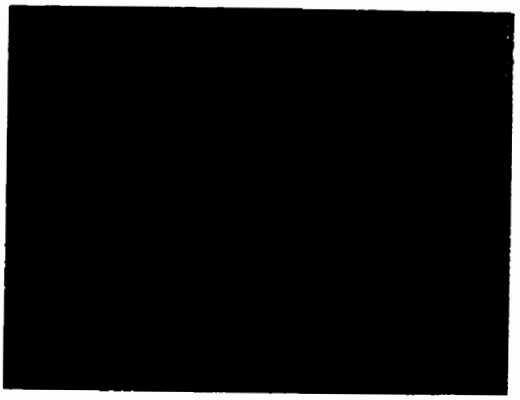


Figure 24. Isolation of periplasmic proteins following a 25°C-5°C cold shock. Cells were grown in olive oil as the sole source of carbon. Proteins were radiolabelled at a) 25°C mid-exponential growth b) 2 h post cold shock and c) 140 h post cold shock.

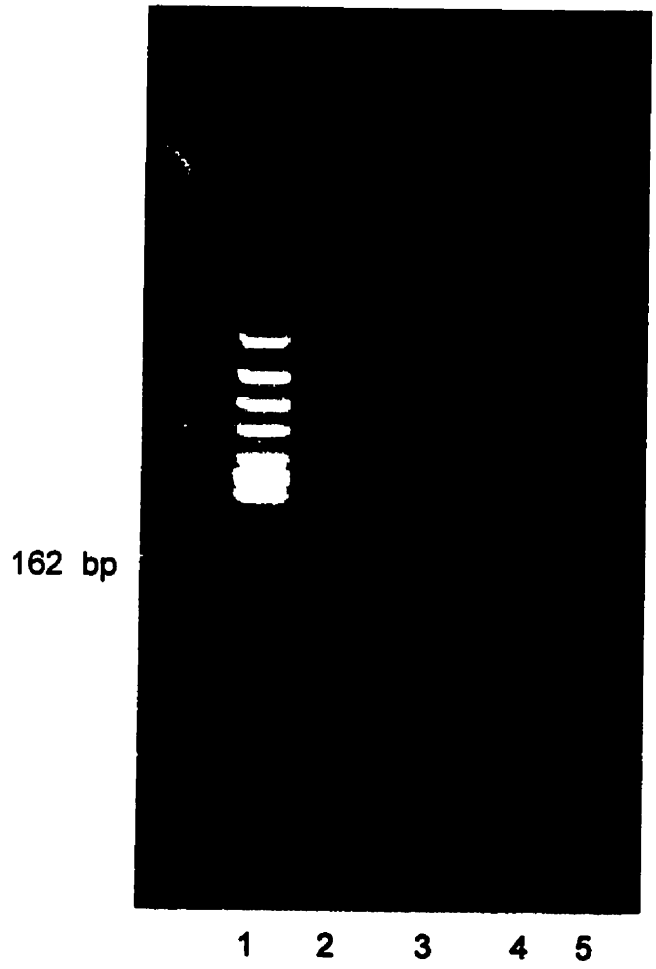
a)

b)

c)

resulting product would produce an expected DNA fragment of 162 base pairs. Figure 25 shows the results of PCR following gel electrophoresis. Lane 1 represents bands associated with Hinc II digested ϕX174 DNA. Ten bands were observed. The first five molecular markers represent 1057, 770, 612, 495, and 392 kbp linear DNA. The sixth band represents fragments 345, 341, and 335 kbp. The seventh band represents fragments 297 and 291 kbp. The last three bands represent fragments 210, 162 and 79 kbp. A 162-kbp fragment seen in lane 2 was produced in the positive control tube (*csp*A-pUC9). No bands were observed in lanes 3, 4 and 5 representing HH1-1 DNA (lanes 3 & 4) and the negative control (lane 5). The absence of a 162-kbp fragment indicates that a gene homologous to *cspA* is not present in HH1-1.

Figure 25. Polymerase chain reaction analysis and gel electrophoresis used for the detection of the *csp*A gene in HH1-1. Lane 1, Hinc II digested ϕX174 DNA; Lane 2, Amplified *Escherichia coli csp*A gene from pUC9 plasmid containing *csp*A gene-positive control; Lane 3 & 4, HH1-1 amplified DNA; Lane 5, H_2O-negative control.


162 bp
1
2
3
4
5

Discussion

The physiological characteristics that allow psychrotrophic bacteria to grow at low temperatures are currently not well understood. The induction of proteins in response to cold shock and the increased synthesis of proteins during sustained growth at low temperatures have been implicated as one mechanism for continued growth at near 0°C temperatures. The most extensive work on the cold shock response and protein synthesis has been conducted using the mesophilic bacterium *E. coli* (Jones et al., 1987). Studies have further examined this response in psychrotrophic bacteria (Whyte & Inniss, 1992; Cloutier et al., 1992; Berger et al., 1996) and psychrophilic bacteria (Araki, 1991a). In each case, a decrease in growth temperature resulted in an increased synthesis of certain proteins. A similar cold shock response has been observed in the present study for *Acinetobacter* HH1-1. A notable increased synthesis of 21 proteins was observed after HH1-1 was subjected to a 25°C to 5°C cold shock. These results provide additional evidence that this response may be ubiquitous and supports the idea that there is a distinct cold shock response used by bacteria to survive relatively rapid decreases in temperature.

Induction of csps first occurred at 2 h post cold shock for each carbon source. Induction occurred during the lag phase prior to the resumption of growth for both acetate- and Tween 80-grown cells. Because an emulsion was formed immediately after cold shock, the time at which protein synthesis occurred in relation to the lag period could not be determined for olive oil-grown cells. Induction of csps generally occurs prior to the resumption of growth (Colucci and Inniss, 1996; Jones et al., 1987). The number of csps and caps induced and the level of induction of these proteins were dependent on carbon source. Three csps induced 2 h after cold shock were found in HH1-1 regardless of carbon source.

Of these three, two proteins, csps18 and 89a, were transiently produced. Csp18 was consistently induced to the greatest levels compared to all other proteins. One other protein, csp10a, was also transiently induced; however, this protein was associated with acetate- and Tween 80-grown cells only. The pattern of induction of these proteins is similar to that described for the major cold shock protein of *E. coli* (Goldstein et al., 1990). It is thought that transiently-produced proteins are critical for cells in adjusting to growth at new lower temperatures (McGovern & Oliver, 1995). The lack of induction of two csps in a cold-sensitive mutant of *B. psychrophilus* was implicated as a possible cause for the mutant's inability to grow at lower temperatures (Whyte & Inniss, 1992). Similarly, in the present study the absence of csp10a in olive oil-grown cells might contribute to their inability to grow at 0°C.

Csp36a, the third protein common to all cells, was also present at higher levels during sustained growth at 5°C relative to growth at 25°C. It is considered a cap as well. Csps are frequently designated as caps. For instance, Berger et al. (1996) reported that nine out of a total of 28 csps expressed by *A. globiformis* SI55 following a 25°C to 4°C cold shock were also caps. In addition, Whyte and Inniss (1992) reported the presence of 8 caps similar to csps. In the present study, 14 additional caps were reported at 5°C. Only 1 protein, cap61, was induced to higher levels than the 25°C control for all three carbon sources. The other 13 proteins were either unique to 1 carbon (for example, cap38b-Tween 80-grown cells) or found in only 2 of the 3 carbon sources. Csp36a and cap61 may therefore represent the only proteins directly related to sustaining the growth of cells at low temperatures. What particular role these proteins play in low temperature growth is not clear. In fact, the structure and function of currently identified bacterial caps have yet to be elucidated (Bayles

et al., 1996). Because enzyme function and protein conformation can be affected at low temperatures, cells will increase the synthesis of cold-sensitive proteins to compensate for a decrease in activity and stability (Margesin & Schinner, 1994). The other caps identified in this study may represent this type of cold-sensitive protein. To maintain housekeeping functions of the cell, such as metabolism, energy transduction, and cell membrane and wall integrity at low temperatures, concentrations of these proteins were increased in response to cold shock.

Protein profiles of HH1-1, when incubated over a wide range of temperatures, were prepared to determine changes in protein content, and to identify the synthesis of new proteins. It has been shown that the synthesis of new proteins varies little for mesophilic bacteria growing in their normal growth temperature range, but at temperatures above or below this range, newly synthesized proteins appear (Herendeen et al., 1979). It has been suggested that temperature regulation by psychrotrophic bacteria is unique (Gugi et al. 1991). Therefore, it was of interest to evaluate protein profiles for HH1-1, particularly at 0°C and 30°C, because these temperatures are close to its minimal and maximal growth temperatures. Quantification of individual proteins was not performed, so an assessment of the changes in the levels of synthesized proteins could not be verified. However, the presence of new proteins was examined. Protein profiles were similar for all growth temperatures with the exception of growth at 30°C. An additional protein, with a molecular mass of 12 kDa was observed at this temperature. Interestingly, the molecular mass of this protein is identical to csp12 induced exclusively in olive oil-grown cells 2 h after cold shock. A wide variety of stresses can induce the synthesis of stress proteins (Watson, 1990). Thus, it is conceivable that csp12, found in olive oil-grown cells after cold shock and during growth at 30°C, is a

general stress protein rather than one associated strictly with high or low temperature growth. The presence of csp12 in olive oil-grown cells emphasizes the finding these cells are under greater stress relative to acetate- and Tween 80-grown cells in response to cold shock.

A cell fractionation protocol was conducted to further characterize the proteins induced following cold shock and during growth at 5°C. This protocol was done to separate intracellular, membrane associated, periplasmic and extracellular proteins. Cold shock proteins that have been identified and their functions determined are intracellular proteins. Examination of the different fractions of the cell were expected to reveal additional cold-associated proteins located in different compartments of the cell. However, problems with the technique caused poor separation of the different fractions. On the other hand, fluorograms showing labeled periplasmic proteins did provide some interesting results. Regardless of sole carbon source, at least six proteins were visualized on the fluorograms of the 25°C controls. An apparent decrease in the level of these proteins was observed on the fluorograms 2 h after cold shock, but the same proteins were still present at 5°C. For cells grown in the presence of olive oil, additional proteins were observed at 140 h post cold shock. The presence of these additional proteins suggests that cells grown with olive oil may synthesize distinct proteins during sustained growth at 5°C. However, it is also possible that olive oil-grown cells were more susceptible to chloroform extraction. Because these cells were stressed and a loss of cell membrane integrity was observed after cold shock, they may have been particularly sensitive, resulting in the release of additional proteins that otherwise would not have been released.

The rapid induction of cspA following a down-shift in growth temperature and the absence of this protein at normal growth temperatures (37°C) has resulted in its designation

as a major cold shock protein in *E. coli* (Goldstein et al., 1990). Since the cloning and sequencing of the *cspA* gene, comparable genes have been found in a number of bacteria. Protein sequencing of cold shock induced acidic proteins of similar molecular weight to the major csps has verified the presence of homologous proteins in *Bacillus subtilis* (Graumann et al., 1996), *Bacillus cereus* (Mayr et al., 1996) and *A. globiformis* SI55 (Berger et al., 1997). In the present study, the polymerase chain reaction (PCR) using primers constructed from the known seqence of the *csp*A gene, was performed to determine the presence of the *csp*A gene in HH1-1. The forward primer (F1) consisted of 24 nucleotides with the sequence 5'-GTTCAACGCTGACAAAGGCTTCGG-3', and was located at position 648 on the sequenced *csp*A gene. The reverse primer consisted of 29 nucleotides with the sequence 3'-GTTTCGCCGCGATTTCCGGGCCGTCGACC-5', and was located at position 810 on the sequenced *csp*A gene (Appendix II). The control lane, containing the *csp*A gene encoded on the pUC9 plasmid, showed the expected 162-kbp band. No bands were detected for the lanes containing HH1-1 DNA. The prevalence of cspA and closely related proteins in various bacteria makes it difficult to conclude that no such proteins exist in *Acinetobacter* spp. In fact, the presence of these proteins in *Acinetobacter* spp. has not been previously investigated. PCR amplification has been successfully used to assay *Lactococcus lactis* for *csp*A-like gene fragments (Chapot-Chartier et al., 1997). However, in that study, degenerate primers, derived from highly conserved regions of the *csp*A and related proteins, were used to successfully detect the presence of at least three related genes. It is possible that a *csp*A gene is present in HH1-1, but the gene sequence may have diverged so that the primers used in the present study did not hybridize to it.

Conclusions

A typical cold shock response with the induction of both csps and caps was observed for HH1-1 following a 25°C to 5°C decrease in temperature. While an increased synthesis of 21 different proteins was observed, not all these proteins were found for all 3 carbon sources examined. Csps common to cultures grown on all three substrates may represent proteins that are directly related to the adaptations that allow cells to survive cold shock and sustain growth at low temperatures. The increased synthesis of proteins, specifically required by cells growing on a particular carbon source, possibly represents proteins that are susceptible to a decrease in growth temperature. The synthesis of these proteins may be required to compensate for a decrease in activity and increased conformational instability. Genes with a high degree of homology to the major cold shock gene, *cspA*, are frequently detected in both mesophilic and psychrophilic microorganisms; however, one was not detected in HH1-1. The absence of at least one protein (csp10a) in olive oil-grown cells may contribute to the inability of these cells to grow at 0°C. In addition, the presence of cap12, possibly a general stress protein, may be further evidence that these cells are more sensitive to low growth temperatures than acetate- or Tween 80-grown cells.

Overall Conclusions

This present study has examined the effects of low temperatures and rapid decreases in growth temperature (cold shock) on membrane properties, enzyme activities and protein synthesis of an *Acinetobacter* sp. Results from growth-temperature studies showed that HH1-1 is a psychrotroph with a growth temperature range that extends from 0°C to 30°C. However, the ability of HH1-1 to grow at 0°C was affected when cells were grown on olive oil as the sole source of carbon. Cells became leaky after being subjected to cold shock, as indicated by an increased concentration of K^+ measured in cell culture supernatant. The leakage of K^+ was likely due to changes in membrane fluidity. Fatty acid analysis indicated that changes in the content of fatty acids C16:1 and C16:0 may be critical for correcting a decrease in membrane fluidity that occurred when the cells were subjected to cold shock and, as well, be important for maintaining membrane fluidity during growth at 5°C.

Results from the enzyme activity experiments also support the finding that cell membrane permeability was compromised at low temperatures. The typically cell associated enzyme, isocitrate lyase, was found primarily in cell culture supernatant after cold shock, and during growth at low temperatures, suggesting that this enzyme leaked out of the cell. Low temperature affected the lipolytic enzymes differently. The activity of the extracellular esterase was lower after cold shock and during low temperature growth, possibly due to inefficient transport across cell membranes. In contrast, activity of a cell associated esterase increased after cold shock and during low temperature growth. Although the exact function of this esterase was not determined in this study, the data did suggest that it may have played a role in cold adaptation in addition to substrate metabolism.

HH1-1 exhibited a cold shock response similar to that observed for other psychrotrophic bacteria. Three proteins were transiently induced 2 h post cold shock. These proteins fit the definition of csps. Transiently induced, newly synthesized proteins are believed to be critical for the survival of cells after exposed to a rapid decrease in temperature and during sustained growth at low temperatures. In addition to csps, caps were also identified following sustained growth at 5°C.

Tween 80- and olive oil-grown cells responded more slowly to cold shock than acetate-grown cells, with K^+ concentration in cell culture supernatant remaining high until cell numbers had increased. Significantly lower polarization ratios were observed for olive oil-grown cells, suggesting that a greater proportion of fluid phase lipid is associated with these cells relative to the other substrates. Fluorescence polarization data also suggested that there may be a lipid component associated with olive oil-grown cells, which is not associated with acetate- or Tween 80-grown cells, which responds to cold shock differently. This lipid component may be interfering with the ability of olive oil-grown cells to respond to rapid decreases in temperature, and to grow at low temperatures. Changes in C16:0 and C16:1 fatty acids were not observed until 24 h after cold shock for olive oil-grown cells compared to 2 h for acetate-grown cells, suggesting that olive oil-grown cells were slower to adjust these fatty acids. Moreover, a significantly higher C16:1 fatty acid content was found in olive oil-grown cells relative to acetate- and Tween 80-grown cells during sustained growth at 5°C. This suggested that there was an additional requirement for fatty acid C16:1 at the lower growth temperature.

Lipase activity was also impaired when cells were subjected to cold shock and during sustained growth at 5°C. The cause for decreased lipase activity is unclear, but the results do suggest that decreased activity resulted from an inability of the cell to transport the enzyme

across the membrane. Lipase activity may also have been affected by the additional C18:1 content associated with these cells, because this fatty acid has been shown to inhibit lipase production at the molecular level. Although olive oil-grown cells also produce esterases, these enzymes generally cannot attack insoluble triacylglycerides. Lipase must be transported to the extracellular environment and come in direct contact with the substrate to hydrolyze ester bonds. Only then can esterases attack the more soluble fatty aclyglycerides released through the action of lipase.

Protein profiles indicated that HH1-1 cells grown with olive oil are stressed to a greater extent than are acetate- and Tween 80-grown cells following cold shock. For example, csp12 was induced 2 h after cold shock by olive oil-grown cells only. This protein is similar to a protein synthesized at 30°C, the maximum growth temperature for this bacterium, suggesting that this protein may be a general stress protein rather than one specifically required for low-temperature growth. In addition, the additional periplasmic proteins that appeared during growth at 5°C in olive oil-grown cells suggested that additional mechanisms may be required to maintain sustained growth at low temperatures.

One particularly interesting result was the demonstration that the ability of HH1-1 to grow at low temperatures is dependent on the substrate available for growth. Metabolism of olive oil as the sole carbon source resulted in the inhibition of the physiological mechanisms required to restore membrane integrity, and affected lipase activity when cells were incubated at low temperatures. Overall, the study provided useful results on the physiological changes that occur during low-temperature growth of this *Acinetobcter* strain. This information will also be useful if cold-temperature growth of *Acinetobacter* is to be used in industry, biotechnology, and cold climate bioremediation schemes.

Recommendations for Future Research

More specific details regarding the changes in membrane integrity could have been obtained if the cell membrane fraction was isolated and separated into inner and outer fractions prior to examining fatty acid content and fluidity. Effects of low temperatures and cold shock on fatty acid content and fluidity may be different for each membrane fraction. As well, there are examples where changes in phospholipid composition have also been observed in bacteria in response to a decrease in growth temperature. In conjunction with determining the fatty acid content, monitoring phospholipids in each fraction could provide additional information important for determining the effects of low temperatures on HH1-1 membranes.

Another important result that requires further investigation is the apparent increased synthesis of the 53-kDa esterase in response to low temperatures. Isolation and purification of this protein would allow one to perform temperature-controlled experiments that could be used to determine whether the esterase is a cold-adapted enzyme. Elucidating the amino acid sequence in conjunction with experiments designed to assess the funtion(s) of this protein may provide insight into one mechanism used by bacteria to cope with low temperatures.

The cell-fractionation procedure used to assess the induction of intracellular, periplasmic, membrane and extracellular proteins in response to cold shock should be repeated. These experiments could show that a particular group of proteins are more important for the survival of cells at low temperatures than others. The transient nature of cold shock proteins18 and 12 suggests that they play a critical role in the survival of cells after a rapid decrease in growth temperature. As with many of the presently isolated cold shock proteins their functions are not known. Again, isolation, purification and sequencing of these proteins may provide insight into their role in cold temperature growth.

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Appendix I

Sample Calculations for Determining Generation Times.

Example: HH1-1 grown in BSM with acetate (0.1% w/v) as sole carbon source and incubated at 25°C.

Turbidity at $O.D._{650}$.

Time	Replicate 1	Replicate2	Replicate 3
0 h	0.031	0.032	0.031
1 h	0.031	0.031	0.031
2 h	0.040	0.039	0.041
3 h	0.069	0.068	0.062
4 h	0.128	0.122	0.108
6 h	0.490	0.506	0.429
7 h	0.768	0.724	0.648
24 h	0.081	0.785	0.769

Specific growth rate constant (K) was first calculated as follows:

$$K = \frac{2.303(Log[N_{t2}] - \log[N_{t1}])}{t2 - t1}$$

(Stanier et al., 1976)

Where: N_{t2}=concentration of cells at the second sampling time
N_{t1}=concentration of cells at the first sampling time
t2=second sampling time
t1=first sampling time.

Generation time (g) was then determined using the equation:

$$g = \frac{\ln 2}{K}$$

(Stanier et al., 1976).

Sample calculation for replicate 1:

$$K = \frac{2.303(Log[.490] - \log[0.069])}{6 - 3} = 0.654$$

$$g = \frac{\ln 2}{0.654} = 1.06$$

Appendix II

Equations used for the determination of standard errors (SE):

For comparison of two means at different times but at the same level of carbon, incubation or carbon x incubation:

$$\sqrt{\frac{2E_{wi}}{ir}} \qquad \sqrt{\frac{2E_{wi}}{cr}} \qquad \sqrt{\frac{2E_{wi}}{r}}$$

Where: E_{wi} = error within groups
I = number of incubation treatments
c = number of carbon treatments
r = number of replicates

For comparison of two means at the same time but at different levels of carbon, incubation or carbon x incubation:

$$\sqrt{\frac{2(t-1)E_{wi} + E_{bet}}{tr}}$$

Where: t = number of sampling times
E_{wi} = error within groups
E_{bet} = error between groups
r = number of replicates

Determination of t'_{crit}:

$$t'_{crit} = \frac{(t-1)E_{wi}t_{wi,\alpha} + E_{bet}t_{bet,\alpha}}{(t-1)E_{wi} + E_{bet}}$$

Where: $t_{wi,\alpha}$ = student t value using degrees of freedom associated with E_{wi}
$t_{bet,\alpha}$ = student t value using degrees of freedom associated with E_{bet}
α = alpha level
t = number of sampling times

Adapted from Steel & Torrie (1980).

Paired comparison sample calculations using extracellular K^+ data collected after cold shock.

Potassium Concentrations Measured in Cell Culture Supernatant

Time	Acetate	S.D.	Tween 80	S.D.	Olive Oil	S.D.
25C Control	223.75	5.80	210.00	0.00	200.00	0.00
2 h	230.00	0.00	243.33	11.55	216.67	5.77
4 h	233.75	15.28	253.33	15.28	233.33	5.77
6 h	236.25	10.00	266.67	15.28	246.67	5.77
24 h	220.00	0.00	230.00	10.00	210.00	0.00
50 h	216.25	15.28	203.33	5.77	210.00	0.00

Sample calculation for Tween 80 data, paired comparison of 25°C mid-exponential control and 2 h after cold shock.

Calculated critical *t* values:

$t'_{0.01}$=3.51 where: df_{wit}=14, df_{bet}=6, E_{wit}=35.92, E_{bet}=298.15

$t'_{0.05}$=2.36

$t'_{0.10}$=1.89

$$SE = \sqrt{\frac{2E_{wi}}{ir}} = \sqrt{\frac{2(35.92)}{3}} = 4.89$$

$$t_{calc} = \frac{243.3 - 210.0}{4.89} = 6.80$$

$t_{calc} > t_{crit}$ at α=0.01 $\therefore$ reject null hypothesis

Appendix III

Fluorescence polarization data for *trans*- and *cis*-parinaric acid. Data are means of triplicate samples.

Growth at 25°C.

Time	*trans* Acetate	STD	*cis* Acetate	STD
3 h	0.236	0.008	0.274	0.004
5 h	0.223	0.008	0.228	0.003
6 h	0.181	0.003	0.193	0.003
7 h	0.185	0.008	0.193	0.011
	Tween 80	**STD**	**Tween 80**	**STD**
2 h	0.143	0.005	0.167	0.003
4 h	0.134	0.005	0.153	0.007
6 h	0.139	0.001	0.164	0.004
8 h	0.135	0.003	0.174	0.004
10 h	0.135	0.008	0.163	0.003
	Olive Oil	**STD**	**Olive Oil**	**STD**
3 h	0.131	0.013	0.155	0.003
5 h	0.127	0.021	0.148	0.011
7 h	0.127	0.009	0.124	0.011
9h	0.122	0.003	0.112	0.008
11 h	0.131	0.008	0.114	0.009

Growth at 5°C.

Time	*trans* Acetate	STD	Time	*cis* Acetate	STD
30 h	0.222	0.011	30 h	0.295	0.011
48 h	0.152	0.014	48 h	0.207	0.010
54 h	0.184	0.018	54 h	0.228	0.011
72 h	0.130	0.013	72 h	0.182	0.008
78 h	0.202	0.019	78 h	0.226	0.004
	Tween 80	**STD**		**Tween 80**	**STD**
11 h	0.170	0.003	11 h	0.235	0.006
24 h	0.180	0.006	24 h	0.236	0.027
48 h	0.156	0.007	48 h	0.177	0.010
72 h	0.140	0.002	72 h	0.166	0.002
100 h	0.150	0.000	100 h	0.184	0.001
148 h	0.164	0.004	148 h	0.202	0.008
196 h	0.180	0.005	196 h	0.202	0.006
	Olive Oil	**STD**		**Olive Oil**	**STD**
11 h	0.181	0.002	11 h	0.234	0.009
24 h	0.154	0.009	24 h	0.186	0.022
48 h	0.142	0.011	48 h	0.188	0.010
72 h	0.156	0.012	72 h	0.163	0.011
100 h	0.182	0.009	100 h	0.163	0.018
148 h	0.187	0.008	148 h	0.186	0.012

25°C-5°C Cold Shock

Time	*Trans* Acetate	STD	*Cis* Acetate	STD
25C control	0.217	0.006	0.216	0.013
2 h	0.257	0.016	0.275	0.004
4 h	0.248	0.004	0.259	0.011
6 h	0.247	0.008	0.256	0.001
20 h	0.207	0.006	0.216	0.003
50 h	0.207	0.005	0.221	0.006
	Tween	**STD**	**Tween**	**STD**
25C Control	0.132	0.000	0.140	0.008
2 h	0.180	0.004	0.210	0.005
4 h	0.187	0.006	0.209	0.010
6 h	0.182	0.003	0.215	0.005
20 h	0.186	0.004	0.186	0.004
72 h	0.194	0.003	0.198	0.016
	Olive Oil	**STD**	**Olive Oil**	**STD**
25C control	0.129	0.007	0.165	0.003
2 h	0.157	0.003	0.183	0.011
4 h	0.159	0.011	0.166	0.010
6 h	0.172	0.022	0.170	0.014
20 h	0.149	0.017	0.140	0.001
72 h	0.140	0.015	0.136	0.006

Appendix IV

Data for isocitrate lyase, esterase and lipase activity. Data are means of triplicate samples.

Isocitrate lyase activity: growth at 25°C.

Time	Sonicate	S.D.	Supernatant	S.D.
0 h	0.00	0.00	0.00	0.00
3 h	0.00	0.00	0.00	0.00
5 h	0.37	0.38	0.00	0.00
7 h	5.57	1.07	0.00	0.00
10 h	5.23	1.08	0.00	0.00

Isocitrate lyase activity: growth at 5°C.

Time	Sonicate	S.D.	Supernatant	S.D.
0 h	0.00	0.00	0.00	0.00
27 h	0.00	0.00	0.00	0.00
51 h	0.00	0.00	0.00	0.00
72 h	0.07	0.06	3.70	0.35
79 h	0.00	0.00	5.67	0.49

Isocitrate lyase activity: 25°C-5°C cold shock.

Time	Sonicate	S.D.	Supernatant	S.D.
25C Control	0.05	0.07	0.00	0.00
2 h	0.00	0.00	0.20	0.00
8 h	0.25	0.07	0.75	0.07
20 h	1.00	0.28	0.80	0.42
26 h	2.00	0.71	0.70	0.28
44 h	0.75	0.07	0.20	0.00

Esterase activity: growth at 25°C.

Acetate				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	0.00	0.00
3 h	0.00	0.00	1.67	0.29
5 h	0.00	0.00	1.00	0.00
7 h	0.00	0.00	2.50	0.00
10 h	1.00	0.87	2.50	0.50
Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	2.00	0.50
3 h	0.00	0.00	2.17	0.29
5 h	0.50	0.87	7.50	2.18
9 h	0.00	0.00	81.67	4.04
11 h	2.17	2.02	106.33	10.56
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	1.00	0.00
3 h	0.00	0.00	1.17	0.29
6 h	2.67	0.76	4.50	2.78
9 h	10.33	3.21	4.17	1.04
12 h	23.67	4.01	3.33	0.58

Esterase activity: growth at 5°C.

Acetate				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	2.50	0.00
27 h	0.00	0.00	2.00	0.00
51 h	1.83	0.29	2.83	0.29
72 h	4.00	1.00	2.00	0.00
Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	3.50	0.50
11 h	0.00	0.00	2.00	0.00
30 h	0.83	0.29	4.33	0.29
74 h	2.83	1.15	8.17	0.58
146 h	7.17	1.15	2.50	0.50
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	3.33	0.29
11 h	0.00	0.00	2.33	0.29
53 h	0.33	0.58	2.33	0.29
77 h	2.00	1.32	1.00	0.00
171 h	11.00	0.87	1.17	0.29

Esterase activity: 25°C-5°C cold shock.

Acetate				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
25C Control	0.00	0.00	1.50	0.00
2 h	0.00	0.00	1.17	0.29
8 h	0.17	0.29	1.33	0.29
20 h	0.50	0.00	2.83	0.29
26 h	1.25	0.35	2.50	0.71
44 h	3.75	0.35	1.00	0.00
Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
25C Control	1.67	0.29	9.50	0.50
2 h	1.00	0.00	4.17	0.76
20 h	4.33	1.15	4.33	0.76
44 h	5.00	0.50	3.67	0.29
140 h	10.17	2.02	2.00	0.00
308 h	5.67	1.89	2.00	0.00
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
25C Control	2.83	0.29	5.00	0.00
2 h	0.17	0.29	1.83	0.29
20 h	0.00	0.00	3.83	0.29
44 h	3.50	0.50	3.17	0.76
140 h	13.33	5.11	1.67	0.29
308 h	15.00	3.61	1.33	0.58

Lipase activity: growth at 25°C.

Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	0.83	0.29
3 h	0.00	0.00	1.17	0.58
5 h	0.00	0.00	3.00	2.00
9 h	0.00	0.00	9.67	2.08
11 h	0.00	0.00	6.17	1.44
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	0.00	0.00
3 h	0.00	0.00	0.67	1.15
6 h	0.00	0.00	5.33	2.84
9 h	0.00	0.00	9.50	8.23
12 h	0.00	0.00	4.67	1.61

Lipase activity: growth at 5°C.

Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	0.67	0.76
11 h	0.00	0.00	0.50	0.87
30 h	0.00	0.00	0.00	0.00
74 h	0.50	0.50	2.50	0.87
146 h	1.00	0.00	0.67	0.29
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
0 h	0.00	0.00	0.17	0.29
11 h	0.00	0.00	0.17	0.29
53 h	0.00	0.00	0.00	0.00
77 h	1.67	0.76	0.00	0.00
171 h	1.50	0.50	0.00	0.00

Lipase activity: 25°C-5°C cold shock.

Tween 80				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
25C Control	0.83	0.76	2.83	0.29
2 h	0.50	0.50	2.17	0.29
20 h	0.83	0.29	0.50	0.50
44 h	0.17	0.29	0.67	0.58
140 h	1.33	0.29	0.50	0.50
308 h	0.33	0.58	0.00	0.00
Olive Oil				
Time	**Sonicate**	**S.D.**	**Supernatant**	**S.D.**
25C Control	1.00	0.50	0.67	0.58
2 h	0.00	0.00	1.33	0.29
20 h	0.00	0.00	0.00	0.00
44 h	1.33	0.29	0.00	0.00
140 h	1.50	0.50	0.50	0.50
308 h	0.33	0.58	0.17	0.29

Appendix V

DNA sequence of *cspA*:

AAGCTTCGAT GCAATTCACG ATCCCGCAGT GTGATTTGAG GAGTTTTCAA TGGAATATAA 60

AGATCCAATG CATGAGCTGT TGAGCAGCCT GGAACAGATT GTTTTTAAAG ATGAAACGCA 120

GAAAATTACC CTGACGCACA GAACAACGTC CTGTACCGAA ATTGAGCAGT TACGAAAAGG 180

GACAGGATTA AAAATCGATG ATTTCGCCCG GGTTTTGGGC GTATCAGTCG CCATGGTAAA 240

GGAATGGGAA TCCAGACGCG TGAAGCCTTC AAGTGCCGAA CTAAAATTGA TGCGTTTGAT 300

TCAAGCCAAC CCGGCAGGAA GTAAGCAGTT GATGGAATAG ACTTTATCCA CTTATGCTGT 360

TTACGGTCCT GATGACAGAC CGTTTTCCAA CCGATTAATC ATAAATATGA AAAATAATTG 420

TTGCATCACC CGCCAATGCG TGGCTTAATG CACATCAACG GTTTGAACGTA CAGACCATTA 480

AAGCAGTGTA GTAAGGCAAG TCCCTTCAAG AGTTAATCGTT GATACCCCTC GTAGTGCACA 520

TTCCTTTAAAC GCTTCAAAAT CTGTAAAGCA CGCCATATCG CCGAAAGGCA CACTTAATTA 600

TTAAAGGTAA TACACT ATGT CCGGTAAAAT GACTGGTATC GTAAAATGGT TCAACGCTGA 660

CAAAGGCTTC GGCTTCATCA CTCCTGACGA TGGCTCTAAA GATGTGTTCG TACACTTCTC 720

TGCTATCCAG AACGATGGTT ACAAATCTCT GGACGAAGGT CAGAAAGTGT CCTTCACCAT 780

CGAAAGCGGC GCTAAAGGCC CGGCAGCTGG TAACGTAACC AGCCTGTAAT CTCTGCTTAA 840

AAGCACAGAA TCTAAGATCC CTGCCATTTG GCGGGGATTT TTTTATTTGT TTTCAGGAAA 900

TAAATAATCG ATCGCGTAAT AAAATCTATT ATTATTTTTG TGAAGAATAA ATTTGGGTGC 960

AATGAGAATG CGCAACGCCG TAAAGTAAGGC GGGAATAATT TCCCGCCGAA GACTCTTACT 1020

CTTTCAATTT GCAGGCTAAA AACGCCGCCA GCTCATAACT CTCCTGTTTA ATATGCAATT 1080

CAGACAGTGA ATCTCTTATC ATCCAGGTGA AAAATAAAAG CGTGAAACAA ATCACTATTA 1140

AAGAAAGTAA TCTATATTTC TGCGCATTCC AGCTCTGTGT TGATTTCACG AGTATGTACT 1200

GCACC

Adapted from Goldstein et al. (1990)