UNIVERSITY OF CALIFORNIA, SAN DIEGO

Piezophysiology of Membrane-Based Adaptations in the Deep-Sea Bacterium *Photobacterium profundum* strain SS9

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Marine Biology

by

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DEDICATION

To Pappy

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ABBREVIATIONS

ACP ..... Acyl carrier protein

AT ..... Acyl CoA:ACP transferase

CLF ..... Chain length factor

CRP ..... cyclic AMP receptor protein

DH/I ..... β-Hydroxy-ACP Dehydratase/Isomerase

DHA ..... Docosahexaenoic acid (22:6*n*-3)

EPA ..... Eicosapentaenoic acid (20:5*n*-3)

ER ..... Enoyl reductase

FabB (KAS II) ..... β-ketoacyl-ACP synthase I

FabF (KAS I) ..... β-ketoacyl-ACP synthase II

KR ..... β-ketoacyl-ACP reductase

KS ..... β-ketoacyl-ACP synthase

MPa ..... Megapascal (0.101 MPa = 1 atmosphere)

MUFA ..... Monounsaturated fatty acid

pfaA-D ..... Polyunsaturated fatty acid biosynthetic genes

PPTase ..... Phosphopantetheinyl transferase

PUFA ..... Polyunsaturated fatty acid

RPA ..... Ribonuclease protection assay

SFA ..... Saturated fatty acid

UFA ..... Unsaturated fatty acid

LIST OF TABLES AND FIGURES

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ASTRACT OF THE DISSERTATION

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Biological membranes are highly dynamic complex structures exquisitely tuned to alterations in the state of the physical environment. Increased hydrostatic pressure and reduced temperature elicit similar physical influences on the phase and fluidity properties of biological membranes. As hydrostatic pressure is increased, or temperature is decreased, membranes undergo a reversible change from a fluid disordered state to a non-fluid ordered state resulting in membrane supraoptimal viscosity or phase transition. These deleterious consequences result to a large extent from the close packing of phospholipid fatty acyl chains within the membrane bilayer. In the absence of corrective membrane restructuring events to alleviate this perturbation, a host of membrane localized essential processes are prone to inactivation. Consequently, preservation of a suitable membrane dynamic state is of

paramount importance in allowing an organism to adapt accordingly to prevailing environmental conditions.

In this dissertation, the relative importance of unsaturated fatty acids (UFAs) to growth at high pressure and low temperature has been evaluated employing the piezophilic deep-sea bacterium *Photobacterium profundum* strain SS9. SS9 modulates the levels of numerous fatty acid species in response to pressure and temperature change resulting in enhanced levels of UFAs at high pressure and low temperature. Analysis of SS9 mutant strains exhibiting altered fatty acid profiles have revealed particular classes of UFA species important for growth at high pressure and low temperature. Furthermore, molecular genetic analyses of genes responsible for UFA synthesis have identified multiple genes required for pressure-responsive UFA production. The fatty acid biosynthetic condensing enzymes FabF (KAS II) and FabB (KAS I) are required for high pressure regulation of 18:1 synthesis and growth at elevated pressure. In addition, the genes responsible for omega-3 polyunsaturated fatty acid (PUFA) synthesis in SS9 have been characterized. PUFA levels undergo both pressure- and temperature-dependent modulations yet they are not required for growth under these conditions. Lastly, the involvement of the global transcription factor CRP is evidenced to influence UFA synthesis in SS9. Overall, these studies present a comprehensive analysis of membrane-based adaptations important for growth and survival under elevated pressure and low temperature conditions.

CHAPTER I

Introduction

Piezophiles: Microbial Adaptation to the Deep-Sea Environment

KEYWORDS

Archaea, Barophile, Cell division, Deep sea, Eicosapentaenoic acid, *Escherichia coli*, Membrane lipid, Omega-3 polyunsaturated fatty acids, Osmolytes, Outer membrane proteins, *Photobacterium*, Piezophile, Pressure, Pressure-inducible proteins, Psychrophile, *PT*k diagram, RecD, RseC, *Shewanella*, Thermophile, ToxR, Unsaturated fatty acids

GLOSSARY

Allochthonous: found in a region outside of where they originated.

Autochthonous: found in the region where they originated.

Chemosynthesis: Inorganic carbon fixation using energy obtained from chemical oxidations.

CydD: Subunit of a heterodimeric ATP-binding cassette transporter required for assembly of the cytochrome *bd* complex involved in aerobic respiration. In certain piezophilic *Shewanella* species, *cydD* has been found to undergo pressure-regulated gene expression.

Eurythermal**:** capable of tolerating a wide range of temperatures.

Oligotrophy: Characterized by low nutrient conditions.

PCR: "Polymerase Chain Reaction"; method for selectively and repeatedly replicating defined DNA sequences from a DNA mixture.

Piezophile: An organism which exhibits optimal reproduction rates at pressures greater than 1 atmosphere (0.1 MPa). Piezophiles were previously termed "barophiles".

Poikilothermic: "Cold-blooded"; an organism whose internal body temperature is environmental temperature dependent and therefore fluctuates with *in situ* temperature fluctuation.

Pressure units: 1 atmosphere (atm) = 0.101 Megapascal (MPa) = 1.01 bar. Pressure increases 1 atm (0.1 MPa) with every 10 m increase in depth in liquid water environments.

Pressure-inducible proteins: Proteins which exhibit elevated rates of synthesis relative to total protein synthesis at elevated hydrostatic pressures.

Psychrophile: An organism possessing an optimum growth temperature <15°C and an upper limit <20°C.

PTk diagram: A plot used to display experimentally derived values of k, the specific growth rate constant, of an organism as a function of pressure (P) and temperature (T) under specific nutrient conditions. Such plots are displayed either as a contour plot of constant k lines as a function of P and T or as a three-dimensional surface showing how P and T influence k. *PTk* diagrams are useful in determining the P and T for an organism at which its growth rate has its highest value under defined nutrient conditions.

RecD: Subunit of the DNA recombination and repair complex Exonuclease V. In *P. profundum* strain SS9 RecD is required for high pressure growth and is implicated in influencing cell division at high pressure.

RseC: Regulatory protein believed to function as an inner transmembrane protein which anchors protein complexes to the membrane for the transfer of electrons to certain anabolic enzymes. Required for high pressure/low temperature growth in *P. profundum* strain SS9

Stenothermal: capable of surviving over only a narrow range of temperatures.

TMAO: (Trimethylamine *N*-oxide) An organic osmolyte implicated in stabilizing proteins at elevated pressures. Increased TMAO concentrations have been found to correlate with increased depth in many deep-sea metazoan species.

ToxR: Transmembrane DNA binding protein that regulates multiple genes in response to environmental stimuli. In the moderate piezophile *P. profundum* strain SS9 ToxR regulates the inverse expression of two outer membrane proteins in response to hydrostatic pressure as well as multiple additional genes.

Unsaturated fatty acids: Membrane phospholipid fatty acyl chains which contain one or more double bonds. Fatty acids are denoted as number of carbon atoms:number of double bonds.

SUMMARY

The study of microorganisms isolated from deep-sea habitats is providing insight into the ecology and evolution of life in high pressure environments. Elevated hydrostatic pressure is an important thermodynamic parameter which has greatly influenced the physiological and biochemical adaptations of marine organisms inhabiting different depths. At the molecular level, the responses of non-high-pressure-adapted microorganisms to elevated pressures have revealed fundamental differences in cellular metabolism and regulatory processes as compared to microorganisms which specifically thrive under elevated pressures (piezophiles). Analysis of non-piezophilic microorganisms at elevated pressures hint at pressure-sensitive cellular phenomena which piezophilic microorganisms must modify for high pressure adaptation. Investigations with piezophilic isolates have identified genes required for growth at elevated pressure, pressure responsive genes and gene products, and general categories of cellular processes which, if disrupted, result in specific growth impairment at high pressure. Examples of these processes include cell division and membrane homeostasis. The focus of this chapter is to review the genetic, physiological, and biochemical evidence regarding microbial adaptation to high pressure.

INTRODUCTION

Within the last 50 years, hydrostatic pressure has emerged scientifically as a significant environmental parameter influencing the ecology and evolution of marine organisms. Whereas the roots of piezobiology began to be established in the late 19th century by the efforts of Certes and coworkers, progress in the field lay relatively dormant until the mid-20th century. Around this time, the question of whether life existed in the deepest portions of the world's oceans remained a mystery. In the early 1950s, the Danish ship *Galathea* lowered dredges into the deepest ocean trenches hauling up a variety of invertebrates. It is with credit to the *Galathea* expedition that the modern field of deep-sea microbiology was born. ZoBell and Morita, participants onboard *Galathea*, showed that bacteria could be cultivated under *in situ* pressure and temperature conditions from 10,400 meters depth (115). However despite these findings, the question of whether true high pressure-adapted bacteria existed was still unresolved. In 1979, the first pure culture of a pressure-adapted bacterium was reported by Yayanos *et al.* and served to underscore the relevance of pressure as a selective influence on the evolution of life (109). Today, improvements in oceanographic sampling and laboratory instrumentation have allowed researchers to begin to uncover molecular processes important for growth and survival under conditions present in the deep sea. In this chapter, an overview of the diversity of deep-sea environments and their resident microflora will be discussed along with physiological, biochemical, and genetic data related to mechanisms of deep-sea (primarily high pressure) adaptation.

DEEP SEA HABITATS

Terrestrial habitats with pressures of one atmosphere or lower account for less than 1% of the total volume of the biosphere. The oceans, which cover approximately 70% of the Earth's surface have an average depth of 3,800 m and an average pressure 380 times that present at the Earth's surface. Thus, high pressure and low temperature deep-sea environments occupy the largest fraction of the biosphere in terms of volume, with the notable exception of deep subsurface environments, the precise biologically relevant volume of which having yet to be determined. The deep sea can broadly be characterized by the presence of high hydrostatic pressures [up to 1,100 atm or 110 megapascal (MPa)], generally low temperatures ca. 2°C except in regions of hydrothermal activity (up to 380°C), the absence of light, and general oligotrophy. As such, the deep sea can be regarded as an extreme environment. Throughout the spectrum of physico-chemical parameters encountered in the deep sea, microbial life exists.

The nature of deep-sea habitats is determined by numerous factors including input of surface derived nutrients, geochemical and geothermal influences, and physical oceanographic and hydrological regimes in addition to the pressure and the temperature. These factors in turn govern the local community structure and biodiversity of the habitat. Microbiologically relevant high pressure environments encountered on Earth are listed in Table 1.

Chemosynthetic communities of microorganisms, often associated with invertebrate hosts, exist in the deep sea at hydrothermal vents and cold seeps (40, 56). Hydrothermal vents are fissures in the ocean floor that leak hot, acidic water. Cold seeps are additional sites of fluid release from the sea floor which are frequently present along the borders of continental plates. Adult animals living near hydrothermal vents have been found to require high pressure for survival, and their larvae require high pressure for development (25). Whale falls in the deep sea have been found to also harbor chemosynthetic communities of microorganisms and animals similar to those found at cold seeps and hydrothermal vents (90). Another high pressure environment where chemosynthetic communities are also found is that of methane hydrates. Under appropriate conditions of high pressure, low temperature and sufficient methane concentration, methane in the seafloor can combine with water to form solid methane hydrate (53). These structures support a unique microbial assemblage which is apparently capable of utilizing the methane as an energy source under anaerobic conditions.

In addition to deep-sea environments, high pressure can be considered an important environmental parameter within other habitats. For example, Lake Baikal in Siberia is the deepest surface exposed freshwater lake in the world, possessing a maximal depth of 1,600 m. Recent evidence suggests that Lake Vostok, a large freshwater lake located 3 to 4 km beneath the East Antarctic Ice Sheet contains bacteria in relatively high concentrations (45). Microbes existing in these habitats could also be adapted for optimal growth and survival under elevated pressure

conditions. If we allow our consideration of possible biospheres to extend outside of Earth, high pressure will remain an important parameter. For example, if life exists within the potentially watery environment of the Jovian moon Europa, then it may need to be adapted down to the lower liquid water depths of 80-170 km (21). Taking into account Europa's surface gravity of approximately 13% of that of Earth, hydrostatic pressures could reach 200 MPa, roughly 2-fold that of earthly deep-sea environments.

An active area of current research involves assessing the extent of the deep subsurface biosphere (79). Piezophilic sulfate reducing bacteria have been isolated from greater than 500 m below the sea floor in the Pacific Ocean, and evidence for microbial activity in deep basaltic or granitic rock has also been obtained (94). The extent to which life within the deep subsurface exists is likely to be determined not by the pressures encountered but rather the extremes of temperature. Currently, the upper limit for microbial growth is 113°C (13). For oceanic crust, where the temperature rises about 15°C per kilometer of depth, tolerance of microbial life may extend to about 7 km below the seafloor. For continental crust, where the temperature is near 20°C at the surface and typically increases by about 25°C per kilometer, life should extend approximately 4 km into the subsurface. It is predicted that the largest number of prokaryotes in the biosphere are likely to reside within the subsurface environment (99).

ISOLATION AND CHARACTERIZATION OF PIEZOPHILES

Nomenclature

ZoBell and Johnson first coined the term barophile to describe organisms which grow optimally at increased pressures. Later, the change from barophile to piezophile was proposed for etymological reasons [the prefix *baro* is from the Greek word for "weight" whereas the prefix *piezo* is from the Greek word for "pressure"] (105). Piezophiles are broadly defined as those organisms which exhibit optimal reproduction rates at pressures greater than 0.1 MPa. Hyperpiezophiles can be defined as those organisms which display optimal growth rates at pressures >60 MPa. Such definitions give an operational framework within which to describe high pressure-adapted organisms. Just as all organisms can be categorized according to their growth temperature ranges (psychro-, meso, thermo-, hyperthermo-philes), so can piezophilic species. Following the temperature convention, the vast majority of piezophilic species in culture would be classified as piezopsychrophiles or hyperpiezopsychrophiles. Currently, no examples of hyperpiezomesophiles have been characterized. The most piezophilic bacterium yet obtained is an isolate from the Mariana Trench whose pressure limit for growth is ~130 MPa (108).

Isolation and Cultivation

The majority of research conducted on deep-sea piezophiles has been performed on isolates collected from the cold deep sea. This is primarily a reflection of the relative ease with which many of these organisms are capable of laboratory based

cultivation in nutrient rich media. Hence, although significant advances have been made in the isolation and culturing of piezothermophiles, emphasis here will be placed on the piezopsychrophiles. Pressure adapted microorganisms have been successfully collected from water, sediment, and animal samples. An important consideration when sampling the deep sea is the maintenance of appropriate *in situ*-like conditions. This usually entails the use of appropriate isopiestic (pressure retaining) and isothermal (temperature retaining) samplers (see ref. (103)). The final consideration is the maintenance of collected samples in the dark. It has been shown that deep-sea bacteria are extremely sensitive to ultraviolet radiation (58). It follows to reason that autochthonous organisms present in the deep sea are specifically adapted to deep-sea conditions.

Many microorganisms which are not specifically adapted to deep-sea conditions may be introduced into this environment as a result of their association with sinking phytoplankton debris or as spores. Even piezosensitive sporeforming thermophiles have been recovered at colony forming units between 100 and 1,000 colonies per gram of dry sea mud (96). Eurythermal *Clostridium* species related to *C. bifermentans* have been obtained from sediments within the Japan Trench at a water column depth of 7.3 km (55). The vegetative cells of these microbes were very pressure sensitive, whereas their spores were very pressure resistant. It has also been possible to isolate *Clostridium perfringens* (an indicator of sewage contamination) from a temporary deep-sea dump site located off the continental slope about 200 km off the coast of New Jersey at water depths of approximately 2,500 m (36).

The isothermal recovery of deep-sea samples is the key consideration when attempting to retain autochthonous deep-sea bacteria within a sample, while limiting the proliferation of allochthonous invader species. Loss of viability of deep-sea psychrophiles due to warming must therefore be prevented. Depending on the variety of samples to be collected a host of sampling devices are available which provide ample insulation to meet these requirements (see ref. (103)). For example, Niskin bottles are ideal in the collection of water samples because of their insulation properties. In addition, numerous box and gravity coring devices to obtain sediment samples and animal traps have been used with success which are sufficiently insulated to avoid large temperatures fluctuations.

With regard to maintenance of *in situ* pressure conditions during sampling, a key question is the decompression sensitivity of the target organisms. This is somewhat problematic since the spectrum of loss of viability due to decompression of deep-sea organisms is not entirely understood (105). For example, numerous deep-sea piezophiles exhibit facultative piezophily meaning they are capable of growth at atmospheric pressure as well as increased hydrostatic pressures. For such strains, decompression is not a lethal event. However, certain isolates such as MT-41 (isolated from the Mariana Trench at a pressure of 100 MPa) do not survive decompression for extended periods of time (107). For these reasons, care should be taken to preserve deep-sea samples under *in situ* pressure conditions.

The use of pressure-retaining animal traps have been used with particular success for obtaining piezophilic isolates. The first piezophile to be characterized, strain

CNPT3, was isolated from an animal trap (109). Deep-sea metazoans appear to be particularly reliable sources for the isolation of piezopsychrophiles. These include scavenging amphipods, filter-feeding holothurians, and various fish species. Investigations of the pressure growth responses of the intestinal microflora of abyssal fish species have revealed facultative and obligate piezophiles to be the dominant representative isolates with non pressure-adapted and piezotolerant species playing a minor role (102). The use of animal traps for the recovery and isolation of deep-sea microorganisms is particularly useful in the study of metazoan/bacterial symbioses, an area of investigation yet to be fully explored.

Advances in deep-sea research technologies have greatly facilitated investigations of deep-sea bacterial adaptation. In particular, the use of manned and unmanned submersibles has notably aided in the efficient sampling of deep-sea microorganisms. Operated by the Japan Marine Science and Technology Center (JAMSTEC), the manned submersible Shinkai 6500 and the unmanned submersible Kaiko are two prime examples. Shinkai 6500 is capable of diving to a depth of 6500 m whereas Kaiko has recorded dives to nearly 11,000 m in the Mariana Trench. In 1996, Kaiko obtained sediment samples from a depth of 10,897 m from the Challenger Deep of the Mariana Trench. From this sample, numerous microbes were isolated composed of diverse lineages including actinomycetes, yeasts, and a variety of various piezophilic and non-piezophilic extremophiles (47, 95).

Laboratory cultivation and analysis of deep-sea isolates requires suitable pressure vessels for the maintenance of working cultures and experimental incubations (for

reviews see refs. (32, 103)). Commonly employed is the use of a pin-retained piston closure vessel in conjunction with a quick-connect coupler to secure the vessel to a high-pressure pump (Figure 1) . Such vessels are practical owing to their rapid compression/decompression time and their relatively inexpensive fabrication cost. Various techniques of cultivation and colony formation at high pressure have been recently reviewed by Yayanos (103). The use of heat-sealable sterile transfer pipettes has proven ideal for routine batch culturing of isolates for example in performing high-pressure growth curves.

Analysis of deep-sea thermophiles is complicated by the simultaneous requirements of high pressures, high temperatures, and strictly anaerobic conditions. Characterization of such strains has been significantly aided by the engineering of large-scale cultivation systems which are capable of maintaining these parameters (69, 71). One such system, the DEEP-BATH system (deep-sea baro/thermophiles cultivation system), has been developed by the Japan Marine Science and Technology Center. This high pressure/high temperature bioreactor is designed to work within a 0 - 300°C range and at pressures up to 68 MPa. In addition, it is suitable for continued sampling without sample perturbation. Such systems are particularly well suited for analysis of microbial assemblages isolated from hydrothermal vent habitats.

Taxonomy

The genus and species designations of a number of piezophilic isolates are presented in Figure 2 along with their pressure and temperature optima. As a general

rule piezophiles are either psychrophilic or psychrotolerant γ-proteobacteria obtained from low temperature deep-sea settings or thermophilic or hyperthermophilic Archaea obtained from deep-sea hydrothermal vents. The piezophilic bacteria tend to have pressure optima less than that present at their depth of isolation, whereas the opposite is true for the piezophilic Archaea.

The Bacteria primarily belong to a narrow cluster within the γ-proteobacteria including the genera *Shewanella*, *Photobacterium*, *Moritella*, *Colwellia*, and *Psychromonas*. However, this surely reflects a culture bias derived from selection for growth in nutrient rich peptide media. A piezotolerant *Bacillus* species and a piezophilic sulfate reducer have also been obtained (5). The fact that piezophilic and nonpiezophilic members exist in closely related taxonomic clusters (Figure 3) suggests that piezophily has evolved separately multiple times or that horizontal gene transfer has introduced piezophilic traits into assorted species. Piezophilic growth of some deep-sea bacteria have been demonstrated under extreme conditions of nutrient limited chemostat culture conditions as well as in high nutrient batch cultures, apparently reflecting the ability of deep-sea bacteria to acclimate to the "feast or famine" existence nature of life in low nutrient deep-sea waters. This feature may be another key property of many piezophiles. Most of the deep-sea microbes characterized to date are chemoorganotrophs, which use organic compounds for growth.

The piezophilic Archaea are more taxonomically diverse than the piezophilic Bacteria examined to date, containing members within both major archaeal kingdoms. Although they are all strict anaerobes, some ferment organic compounds and respire

sulfur, whereas others are H_2 and CO_2 utilizing methanogens. As with the piezophilic Bacteria there are no novel phylogenetic groups of piezophilic Archaea; they are all closely related to shallow-water species. Undoubtedly there are many more piezophilic Archaea in existence than have yet been cultured. Sequencing of small subunit ribosomal RNA genes from microorganisms collected in both the deep Pacific and Atlantic Oceans indicates that the so-called group I crenarchaeotes are ubiquitous and highly abundant (10, 65). Although these microbes have not yet been cultured they surely contain psychrophilic and piezophilic members. It has been suggested that these Archaea may be among the most abundant organisms on Earth.

Growth and Physiology of Piezophiles

Analysis of organisms inhabiting varying pressure and temperature regimes is fundamental to understanding the physical limits of life within the oceans and the deep Earth. In characterizing piezophilic isolates it is of interest to know both the pressures and temperatures to which they are optimally adapted. A physiological index of adaptation can be quantified under a given set of conditions by the specific growth rate constant, k, where k=ln2/doubling time, and is determined when cells are growing logarithmically under defined conditions. The value of k is a reproducible physiological trait of an organism under defined conditions which for piezophilic isolates most notably includes pressure (P) and temperature (T) (103). Under conditions where nutrients levels are non-limiting, k can be plotted versus P and T to yield an empirically derived graph, termed a *PTk* diagram, displayed either as a

contour plot of constant k lines as a function of P and T or as a three-dimensional surface showing how P and T influence k (104). Figure 4 shows the *PTk* diagram for the deep-sea psychrotolerant piezophile *Photobacterium profundum* strain SS9. Analyzing the effect of pressure and temperature on the rate of reproduction of deep-sea isolates has lead to many interesting conclusions regarding the ubiquity and evolution of piezophilic bacteria (103).

Nearly all isolates collected from the cold deep-sea exhibit piezophilic growth at *in situ* temperatures and, in general, the rate of reproduction at pressures approximating capture depth increases with increasing temperature. That is cells typically grow better at temperatures slightly higher than the presumed habitat temperature. In addition, piezophilic isolates exhibit obligate piezophily at temperatures above those that permit growth at atmospheric pressure. Similarly, piezophiles exhibit increased reproduction rates at lower temperatures (eg. 2°C) when the pressure is less than that of their isolation depth. The temperature range for most deep-sea piezopsychrophiles is approximately 15°C highlighting the stenothermal nature of deep-sea inhabitants. Furthermore, piezophilic isolates typically form large and abnormal cell shapes when cultivated at the limits of their pressure ranges. The profound sensitivity of deep-sea bacteria to ultraviolet radiation suggests possible diminished capacities for repair of photo-induced DNA damage which is consistent with their evolution within an environment devoid of light.

Some isolates from deep-sea hydrothermal vent environments also display adaptations to elevated pressure. Such analyses have to be undertaken carefully

because while increased pressure often increases the maximal temperature for growth and survival of a thermophile, it does not always improve the optimal growth rate. However, *Thermococcus barophilus* grows best at a pressure of 40 MPa as well as requiring high pressure for growth at supraoptimal temperatures between 95 and 100°C (63). The related species *Thermococcus peptonophilus* is also a piezophile (28). Several thermophilic or hyperthermophilic methanogens are also known to be piezophilic. *Methanococcus thermolithotrophicus* which was isolated from a shallow-water geothermally heated marine sediment exhibits piezophilic growth (37). *Methanococcus jannaschii* displays a pressure optimum under hyperbaric conditions of ~75 MPa, although the pressure present in the environment from which it was isolated is only about 26 MPa (42). Because pressure adaptation in deep-sea hyperthermophiles often exceeds the pressure of the environment from which they were isolated, some have suggested that this is evidence of a deeper biosphere within hydrothermal vents. Another complicating factor is that differences in pressure effects on deep-sea thermophiles can arise with the techniques used for pressurization and perhaps as well as from differences among these Archaea in their modes of carbon and energy acquisition (i.e. peptide transport and fermentative metabolism versus chemolithoautotrophic growth).

HIGH PRESSURE ADAPTATION MECHANISMS

Whereas molecular responses and adaptive mechanisms to physical variables such as temperature, pH, water activity, and light have been investigated with some detail,

pressure as either an environmental parameter or physical stimulus awaits detailed characterization. The fundamental physical and chemical factors that may render biological systems susceptible to pressure perturbation can be described from a theoretical approach applying physical and chemical laws. Briefly, the basis of all pressure effects arises from the change in system volume that accompanies a physiological or biochemical process. In general, elevated pressure either inhibits or favors biochemical processes which occur with an increase or reduction in system volume, respectively. However, whereas the physical influences of elevated pressure are understood to some degree, it is not a trivial task to attempt to translate these physical effects into biological consequences. Understanding the physical influences of elevated pressure upon the cell provides a theoretical framework within which the biologist is capable of understanding pressure sensitive or pressure enhanced processes. Uncovering the molecular basis of high-pressure adaptation is fundamental to understanding the ecology and evolution of deep-sea inhabitants and while existing studies are limited in breadth, they provide initial insight into general principles governing adaptation to elevated pressure in deep-sea organisms.

Metabolic Responses to Pressure

Before discussing adaptive mechanisms in deep-sea microorganisms, it is useful to describe the metabolic responses to pressure in non-high-pressure-adapted organisms. Such investigations indicate pressure-sensitive processes that deep-sea microorganisms must modify for high pressure adaptation. Numerous studies have

investigated high-pressure effects on discrete functions in non-piezophilic species, primarily in the mesophile *Escherichia coli.* Such studies have revealed large differences in sensitivity to pressure among various biological systems. Whereas the ability to respond to pressure may not be unique to deep-sea bacteria, the capacity to respond to pressure and pressure changes in an adaptive manner, as will be discussed, appears to be restricted to those organisms inhabiting high-pressure environments.

The effects of elevated pressure on *E. coli* cells are highly pleiotropic. Flagellar filament polymerization and rotation are effected at pressures well below those which affect cell growth (67). Likewise, pressures on the order of 20-50 MPa inhibit cell division causing single cells to form long filaments (116). Based on the uptake of radiolabeled nucleotides and amino acids, DNA synthesis has been shown to stop around 50 MPa, protein synthesis around 58 MPa, and RNA synthesis around 70 MPa (110). In addition, cells under high pressure expend more energy pumping protons across the membrane due to pressure sensitivity of the proton-translocating ATPase (62). Sensitivity of such processes in *E. coli* suggests that closely related piezophilic species have evolved molecular mechanisms that are capable of functioning under elevated pressures.

The stress response of microorganisms in all three domains of life have been studied following exposure to pressure upshifts. However the most information has been obtained from work with the mesophile *E. coli.* When anaerobically grown *E. coli* cells were isothermally shifted from 0.1 MPa to 53 MPa 55 pressure-inducible proteins (PIPs) were identified which were transiently induced (98). The magnitude of

the PIP response increased with increasing pressure, but was slow, generally requiring 60-90 minutes post-induction for maximal production. By comparing the 2-D gel coordinates of these proteins with those present in the *E. coli* 2-D PAGE protein database 32 of the proteins could be identified. Unfortunately, this did not include the major PIP, a highly basic protein of 16 kD which even with the complete genome sequence now available for *E. coli* has not been identified. However, for the proteins which could be identified it is interesting to note that the PIP response included a large induction of heat shock proteins, 11, as well as four cold shock proteins. No other environmental stress is known to simultaneously induce so many members of these two sets of stress response proteins. Likewise, in a separate study the steady-state levels of the heat shock protein GroES was shown to be elevated when *E. coli* was cultivated at 40 MPa (30). The connection between high pressure and high temperature has been further extended by observation of heat shock protein 70 induction following a mild pressure increase on human osteocarcinoma cells (33), and the fact that heat shock protein 104 and trehalose are both needed for thermotolerance as well as high pressure stress survival in *Saccharomyces cerevisiae* yeast cells (26). Moreover, brief heat shock induces piezotolerance allowing yeast cells to survive brief exposure to 150 MPa (38). Heat shock proteins may also be induced in piezophiles upon decompression. A stress protein showing similarity to a heat shock protein was found to be induced in the deep-sea piezophilic hyperthermophile *Thermococcus barophilus* subsequent to shift to atmospheric pressure (64).

Some of the known effects of pressure on biological systems may help explain the apparent paradox of simultaneous heat shock and cold shock stimulation. High pressure and high temperature can destabilize protein quaternary and to a lesser extent tertiary structure. On the other hand, high pressure and low temperature share similar effects on protein synthesis and membrane structure. Thus, the induction of both heat shock and cold shock proteins may help ameliorate the damaging effects of elevated pressure on membrane integrity, translation processes, and macromolecule stability (7).

While there is much interest in using high pressure for killing bacteria in food sterilization processes, there is very little information regarding molecular mechanisms of high pressure resistance among strains. Some insight has been gained by the observation that heterogeneity in the *rpoS* locus is associated with differences in high pressure resistance of natural isolates of *E. coli* (84). This sigma factor controls numerous genes involved in stress protection, particularly those associated with the onset of stationary phase. RpoS activity appears to correlate with survival under elevated pressure conditions. Strains exhibiting reduced RpoS activities exhibited the greatest pressure sensitivities. Since RpoS is known to influence the expression of more than 30 genes, delineating which one or more of these genes is essential for conferring pressure resistance will require considerable effort.

Gene expression under high pressure conditions has been investigated using *E. coli* as a model system. Gene expression initiating from plasmid encoded *lac* and *tac* promoters fused to the chloramphenicaol acetyltransferase (CAT) reporter gene

showed dramatic enhancement (nearly 90-fold induction) when cells were grown at elevated pressures, even in the absence of the chemical inducer of these promoters (48). Results suggest this induction of gene expression is the result of interference of the *lac* repressor binding to its cognate operator binding site. Negative regulation of protein abundance at high pressure has also been observed for *E. coli*. The formation of plaques by lambda phage in *E. coli* is prevented at 30 MPa hydrostatic pressure due to decreased expression of the lambda receptor protein LamB (88). In addition, elevated pressure has been shown to repress the synthesis of outer membrane proteins OmpC and OmpF in *E. coli* (73).

Protein synthesis is an essential cellular process which is abolished in mesophiles at relatively small pressures, 58 - 68 MPa in most cases for *E. coli*. *In vitro* measurements of ribosome stability under physiologically relevant magnesium concentrations indicate that the uncharged ribosome and the posttranslocational ribosome complex are both destabilized to a significant extent at about 60 MPa (29, 80). Thus, the basis of pressure sensitivity of protein synthesis could stem from effects on ribosome stability. Hybrid protein synthesis experiments using ribosomes from piezosensitive and piezotolerant bacteria as well as analyses of protein synthesis rates in a streptomycin resistant mutant of *E. coli* indicate that it is the 30S ribosomal subunit which is the determinant of pressure sensitivity (81). At pressures well beyond that at which *E. coli* can grow, in the range of 100 - 250 MPa, pressure killing correlates well with loss of intact ribosomes. This effect can be lessened during post-

pressurization recovery by the addition of $MgCl_2$ because of its stabilizing effects on ribosomes (91).

The effect of elevated hydrostatic pressure on eukaryotic cells has been examined in the yeast *Saccharomyces cerevisiae.* Increased pressure causes either a delay in growth or a complete cessation of growth at pressures greater than 40 MPa (2). An intriguing result of elevated pressure on yeast cells is the transient acidification of the vacuole (2). The vacuole is a large acidic organelle involved in the storage of ions and metabolites and the degradation of cellular proteins. Consequently, the vacuole plays an important role in cellular ion homeostasis. Elevated pressure promotes the dissociation of carbonic acid to bicarbonate and H^+ resulting in accumulation of protons within the cytoplasm. Pressure-induced inactivation of the plasma membrane H^+-ATPase results in sequestration of protons within the vacuole mediated by the vacuolar H^+-ATPase. The vacuolar H^+-ATPase is presumably less pressure-sensitive than the plasma membrane enzyme. Proton pumping into the vacuole preserves cytoplasmic pH homeostasis at the cost of vacuole acidification (1).

Membrane Proteins and Pressure Regulated Gene Expression

While pressure induced proteins (PIPs) in non-piezophilic species are generally of the stress response variety, PIPs analyzed in piezophilic microbes which can acclimate to pressure change are providing a much different picture. Such investigations began with a study of the methanogen *Methanococcus thermolithotrophicus* where it was found that upon shifting the cells from atmospheric pressure to their pressure optimum

of 50 MPa a lag in growth occurred during which changes in the abundance of numerous proteins occurred (39). Presumably many of the PIPs produced by *M. thermolithotrophicus* are important for piezophily, however this has not been verified because it is not yet possible to perform genetic experiments with this microbial species.

This experimental limitation does not hold for the PIP producing deep-sea piezophile *Photobacterium profundum* strain SS9. Although SS9 is only moderately piezophilic, growing best at ~29 MPa, genetic techniques such as transposon mutagenesis, gene insertional inactivation and complementation analysis are possible, making this microbe a useful model system for molecular investigations into pressure adaptation. SS9 responds to pressure changes by altering the abundance of membrane proteins and fatty acids (4, 6, 18). Two outer membrane proteins (OMPs) designated OmpH and OmpL undergo inverse regulation in abundance as a function of hydrostatic pressure (18). OmpH is present in high amounts at 29 MPa while OmpL dominates at 0.1 MPa. Both OMPs appear to be nonspecific porin channel proteins, but OmpH provides for the uptake of larger peptides, a feature which could be important under the low nutrient conditions prevalent in the deep sea. Although neither *ompH* nor *ompL* mutants are impaired in piezophilic growth, some regulatory mutants affecting *omp* expression in SS9 do influence growth at high pressure (18).

The regulation of OmpH and OmpL is controlled at the transcriptional level by a membrane-localized transcription factor possessing homology to the ToxR regulatory protein from *Vibrio cholerae*, which functions with an associated protein designated

ToxS (97). As in SS9, *V. cholerae* ToxR controls the inverse regulation of two *omp* genes (16), but unlike SS9 *V. cholerae* ToxR does not respond to pressure. Thus, the SS9 ToxR protein, which is 50% identical to the *V. cholerae* homologue, has evolved novel pressure sensitive properties. The location and structure of ToxR would seem to make it well suited to function as a piezometer for a cell. Membrane fluidity and phase is highly pressure-sensitive and membrane fluidizing treatments can counteract SS9 ToxR high pressure signaling (97). Another possible site of pressure influence on ToxR is between the interface of its subunits, as well as between ToxR and ToxS. *V. cholerae* ToxR/S is multimeric and many protein multimers dissociate under moderately high pressure conditions (72).

In addition to the fact that SS9 ToxR senses pressure, the expression of the *toxR* gene is itself regulated by pressure. The *htpG* gene, encoding heat shock protein 90, is immediately upstream of the SS9 *toxRS* operon and oriented in the opposite direction. This could be significant in that high pressure induction of *htpG* transcription could sterically inhibit RNA polymerase entry to the *toxRS* promoter (Figure 5). This model is analogous to one previously proposed for temperature regulation of *toxR* expression in *V. cholerae* (78).

Overexpression of *toxR* renders SS9 more pressure-sensitive. Since *ompH* is not required for piezophily, at least under standard laboratory culture conditions, other ToxR regulated genes must be required to be either turned on or off at high pressure. Recently, using the technique of RNA arbitrarily primed PCR it has been possible to identify 8 additional genes which are regulated in a positive or negative manner by

SS9 ToxR (12). The products of these genes are all predicted to alter membrane structure and/or participate in a starvation response. Which of these genes, if any, are required for piezoadaptation is not known. Since not all of these ToxR-regulated genes are regulated by pressure in a ToxR-dependent fashion, the expression of many of these genes could be complex, being controlled by multiple regulatory proteins.

Regulatory factors in addition to ToxR have been identified which influence *ompH* gene expression. *ompH* transcription also increases in response to carbon starvation and this appears to be under the control of the carbon and energy transcription factor CRP (8, 9). Another category of *ompH* regulatory mutants harbor mutations within the *rpoE* operon (17). RpoE is an alternative sigma factor which regulates gene expression in response to stress within the membrane or periplasm (70). The *rpoE* operon is comprised of *rpoE*, two downstream genes whose products modulate RpoE activity in response to environmental cues and the *rseC* gene whose product has a negligible effect on RpoE activity. RseC and related proteins are believed to function as inner transmembrane proteins which anchor protein complexes to the membrane for the transfer of electrons to key enzymes such as those involved in thiamine biosynthesis. Mutations in SS9 which decrease *rseC* expression result in both high pressure-sensitive and cold-sensitive growth phenotypes, perhaps because of low temperature and high pressure effects on membrane-based electron transport. Lack of *rseA*, *rseB* or *rseC* expression decreases *ompH* transcript levels. The basis of this effect is unknown, however, it could depend on the activity of an additional regulatory gene which is under RpoE control.

Pressure Regulated Operons in Piezophilic *Shewanella* sp.

Analysis of genes activated by high pressure within the *Shewanella* barophile (piezophile) taxonomic branch has revealed two pressure-regulated operons. This was accomplished by screening for promoter DNA from the piezophilic *Shewanella benthica* strain DB6705 capable of controlling expression of a reporter gene at moderate pressures (50). A promoter fragment was isolated which directed elevated pressure-inducible transcription both in the piezophilic DB6705 strain as well as in *E. coli* transformants containing plasmids with this promoter. Interestingly, the sequence of this promoter bears similarity to the promoter of the pressure-regulated *ompH* gene of *P. profundum* strain SS9 (6, 8). Analysis of the downstream region of this promoter identified two small open reading frames (ORFs) which comprise an operon and exhibit pressure-regulated expression. This operon was also cloned from the moderate piezophile *Shewanella violaceae* strain DSS12 (46). Electrophoretic mobility shift assays performed on cell lysates of cells grown at high and low pressure suggest that a σ^{54}-like factor as well as another regulatory element, possibly a pressure responsive DNA-binding protein, bind to the upstream region of this promoter (74). Subsequent analyses of additional members of the *Shewanella* piezophile branch have found expression of ORFs 1 and 2 controlled by elevated pressure (57). The functions of ORFs 1 and 2 have yet to be identified.

The second identified pressure-regulated operon within the *Shewanella* piezophiles is located immediately downstream of the operon containing ORFs 1 and 2 (46, 51). The first gene of this operon, designated ORF3, encodes the aerobic respiratory

protein CydD. In *E. coli*, CydD is required for assembly of the cytochrome *bd* complex . *E. coli cydD* mutants exhibit elevated pressure sensitivity which can be complemented by introduction of the *Shewanella* ORF3 gene. Purification and characterization of respiratory components from the piezophilic *Shewanella benthica* strain DB172F has revealed that pressure variation significantly alters respiratory chain composition (82, 83). It appears that the mechanism of electron transport in the *Shewanella* piezophiles is altered at elevated pressure suggesting modified bioenergetic modes under different pressure conditions.

Enzyme Stability and Activity at High Pressure

Very little work has been done addressing the biochemical adaptations to high pressure in the proteins of piezophiles. More has been accomplished studying dehydrogenase enzymes and membrane-localized Na^+-K^+-ATPases from deep-sea fish and invertebrates (for review see ref. (92)). In the case of enzymes from these sources, high pressure adaptation has been observed to be accompanied by more pressure insensitive K_ms and reduced k_{cat}s. However some studies with piezophile proteins do exist (35, 93). Hydrogenase activity from *Methanococcus jannaschii* is increased three-fold as the pressure is increased from 0.8 MPa to 25.3 MPa. The activity of a protease purified from *M. jannaschii* is likewise enhanced about three-fold as pressure is increased from 1 to 50 MPa, and both enzymes are substantially stabilized by high pressure. The primary structure of the well conserved single-stranded DNA-binding protein has been examined from closely related shallow-water

and deep-sea piezophilic bacteria within the genus *Shewanella* (19). There is a piezophilic bias for serine in place of tyrosine and a decrease in helix-destabilizing glycine residues and helix-breaking proline residues. Such changes are expected to result in proteins with low flexibility and low compressibility, characteristics which could be favored in high pressure environments.

Extrinsic factors could also play a major role in protein function at high pressure in deep-sea microorganisms. The evidence for this comes mostly from examinations of deep-sea animals. Deep-sea fish, skates and crustaceans have been found to increase their levels of the organic osmolyte trimethylamine-N-oxide (TMAO) with depth (52). Additional correlations between increased osmolyte levels and depth have been observed in amphipods from Lake Baikal (114), in deep-sea eels exposed to high pressure, and hydrothermal vent and cold seep vestimentiferan tubeworms (113). Furthermore, there is some evidence that *in vitro* protein synthesis is less pressure-sensitive in the presence of increased osmolytes (54). TMAO has been shown to decrease pressure effects on enzyme substrate binding (27). Stabilizing osmolytes such as TMAO are preferentially excluded from protein hydration layers, making it more entropically unfavorable for a protein to unfold and expose more hydrated surface area. Thus, osmolytes such as TMAO, like a reduction in helix breaking and helix destabilizing amino acid residues, could favor a more compact protein structure.

Cell Division

One of the most immediately apparent effects of exposing microbial cells to supraoptimal hydrostatic pressure is the inhibition of cell division. The result of this is that mesophiles exposed to pressures in the range of 20 - 40 MPa often form long "snake-like" single cell filaments (116). Conversely, piezophiles may exhibit a similar phenotype subsequent to decompression to atmospheric pressure (106). The basis of this phenomenon could lie in pressure modulating a process directly concerned with cell division such as septum formation and cell wall or membrane biosynthesis. Indeed, one piezophile, *Shewanella benthica* strain DB6705, has been found to pressure-regulate the expression of the *asd* gene involved in cell wall biosynthesis (49), and FtsZ ring formation, which is required for septum formation, appears to be pressure-sensitive in *E. coli* (87). However, it is also possible that pressure effects on cell division are related to DNA replication or chromosome partitioning, since the formation and separation of two chromosomes is a necessary prelude to cell division. In this context it is interesting to point out that when *E. coli* is grown at pressures up to 45 MPa less DNA per cell is present, while RNA levels increase and protein levels are unaffected . Pulse-labeling studies of DNA synthesis suggest that it is either the initiation or termination of DNA replication which is the most pressure-sensitive step in chromosome duplication (110). While the overall rate of DNA synthesis is less pressure-sensitive than that of cell division, it should be kept in mind that the termination of DNA synthesis could be more pressure-sensitive than bulk DNA

synthesis, and it is the termination of DNA replication along with the subsequent partitioning of the chromosomes which is the signal for cell division.

The possibility that an enzyme involved in chromosome partitioning is the weakest link in cell division at high pressure has been strengthened by studies of two enzymes. First, the DNA supercoiling enzyme DNA gyrase is required for chromosome decatenation. At low enzyme concentrations DNA gyrase can be completely inactivated by pressures as low as 28 MPa (20). Second, the RecD subunit of exonuclease V has also been implicated in controlling cell division at high pressure (11). RecD is a potent inhibitor of DNA recombination between plasmids following their replication and presumably serves a similar role preventing interchromosomal recombination. In SS9, the *recD* gene is required for high-pressure growth. Furthermore, transfer of the SS9 *recD* gene into an *E. coli recD* mutant enables *E. coli* to divide normally at high pressure as evidenced by normal cell morphology. The basis of this effect could be in limiting DNA recombination at high pressure. This requirement would arise if high pressure either promotes increased DNA recombination or decreased resolution of chromosome multimers.

Membrane Lipids

Increased hydrostatic pressures and reduced temperatures elicit similar physical effects on the phase and fluidity properties of biological membranes (22, 34, 60). As growth pressure is increased or temperature decreased, biological membranes undergo a reversible change from a fluid disordered state to a non-fluid ordered state. Such

changes would seem particularly problematic for life in deep-ocean environments where extremes of both pressure and temperature persist. The effective temperature of a phospholipid bilayer in a deep-sea inhabitant living near 2°C at a depth of 10,000 m is approximately –11 to –19°C (61). Such equivalency estimates highlight the strong interacting effects of high pressure and low temperature on physical properties of membranes.

Conformational changes in membrane physical structure as a function of pressure and/or temperature variation result primarily from the tighter packing of fatty acyl chains within the membrane phospholipids (14). The close packing of acyl chains result in membranes assuming an ordered array where molecular motion is restricted. One of the characteristics believed critical for life at high pressures and low temperatures is the maintenance of appropriate membrane structure. Consequently, the membrane gelling effects of pressure and temperature are predicted to provide strong inducement for adaptive membrane restructuring. Membrane transport processes, intracellular signaling and gene regulation, membrane protein dispersion, and metabolic electron transport are reliant on appropriate membrane structure (43). Thus, the maintenance of biological membranes within a narrow range of viscosity (homeoviscous response) (89) or within a liquid-crystalline phase (homeophasic response) (66) may be key to an organism's growth ability and survival. Indeed, analysis of neural and muscular functions in non-high-pressure adapted metazoan species suggests membrane-based processes are among the most susceptible to perturbation by high pressure (15).

Many poikilothermic organisms respond to decreased temperature by altering their membrane lipid composition, apparently to tailor the membrane with physical properties suited to prevailing environmental conditions . Such changes may include decreases in mean chain length, increased methyl branching, *cis/trans* isomerization of unsaturated fatty acid double bonds, increases in the ratio of *anteiso/iso* chain branching, acyl chain shuffling between the *sn*-1 and *sn*-2 positions, or phosholipid headgroup changes (34, 44, 77, 85). However, perhaps the most pervasive cellular response to temperature decrease observed among prokaryotic species is the increased incorporation of unsaturated fatty acids (UFAs) into membrane phospholipids as a function of decreasing temperature (68). UFAs adopt a more expanded conformation, pack less compactly, and possess lower melting temperatures than their saturated counterparts, allowing for their less orderly alignment within membrane phospholipids. This response presumably functions to offset the membrane gelling effects of reduced temperature, thereby maintaining biological membranes within a fluidity or phase optimized for growth.

Given the similar physical influences of decreased temperature and increased hydrostatic pressure on physical properties of membranes, it is pertinent to ask if pressure acclimation leads to similar membrane restructuring. Regulation of membrane fatty acid composition in response to pressure variation was first predicted and subsequently observed in membranes from various deep-sea fish (61). By measuring the fluidity of brain myelin samples from both shallow-water and deep-sea fish and correlating the increased proportion of UFAs in liver mitochondrial

membranes with capture depth, a role for homeoviscous pressure adaptation was noted. Subsequently, numerous piezophilic bacteria have been shown to regulate membrane fatty acid composition as a function of pressure. DeLong and Yayanos first observed this phenomenon by profiling the fatty acid changes in the piezophile CNPT3 as a function of varying pressure at constant temperature (23). A correlation was observed between growth pressure and the ratio of unsaturated-to-saturated membrane fatty acids. In particular, a marked increase in the amount of palmitoleic acid (16:1) and oleic acid (18:1) was observed as the growth pressure was increased above 17 MPa.

Among the numerous piezophilic species examined, increases in UFA content as a function of pressure appears to be a common trait (Figure 6). Such observations have led to the conclusion that pressure-dependent membrane restructuring may represent a fundamental adaptive mechanism of deep-sea inhabitants. This conclusion is supported by the finding that two non-piezophilic microorganisms, *Tetrahymena pyriformis* NT-1 (59) and *E. coli* (3), have been shown not to regulate membrane fatty acid structure as a function of pressure. Unlike temperature, pressure variation is a seldom encountered environmental parameter outside of the deep-sea. It could however be predicted that because high pressure exerts a physical change on membrane structure similar to that of a drop in temperature, most microbes would perceive high pressure as low temperature and respond accordingly to restore membrane homeostasis. The observed lack of homeoviscous pressure adaptation in *Tetrahymena* and *E. coli* demonstrates that the various enzymatic and genetic

mechanisms facilitating temperature-dependent membrane restructuring in non high-pressure adapted organisms are not preadapted to respond appropriately to high pressure. This result suggests fundamental differences between membrane-based adaptations of piezophilic and non-piezophilic species.

One particularly remarkable characteristic shared by many piezophilic and psychrophilic bacterial species is the production of the omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). Prior to the analyses of DeLong and Yayanos who first observed PUFA production in numerous deep-sea isolates (24), it was thought that bacteria only synthesized monounsaturated fatty acids (MUFAs), with the notable exception of a *Flexibacter* species (41). Subsequently, surveys of environmental isolates have shown there is a preponderance of PUFA-producing bacteria associated with cold (or permanently cold) and high pressure deep-sea environments compared to tropical and shallow-water environments (24, 76, 111, 112). Analyses reveal that only 1.5% of temperate marine isolates, approximately 14% of Antarctic isolates, and 27% of deep-sea isolates produce PUFAs. Moreover, studies investigating the intestinal microflora of deep-sea fish and shallow-sea poikilothermic animals showed that deep-sea inhabitants not only contained a higher proportion of PUFA producers but the percentage of PUFAs within these isolates were greater than those of shallow-water animals. Like other UFAs, PUFA production undergoes pressure (in piezophilic species) and temperature dependent regulation. By virtue of their extremely low melting temperatures (eg. EPA T_m = -54°C), PUFAs would be expected to exert a

disproportionately large effect on the phase and fluidity properties of membranes, effectively reducing the melting temperature and increasing the melting pressure of a lipid bilayer. Consequently, PUFA production and their temperature- and pressure-dependent regulation by deep-sea bacteria have been considered important adaptations to the deep-sea environment (24, 31, 75, 86, 100, 101).

FUTURE PROSPECTS

There is a need for more research into the biodiversity and distribution of piezophiles as well as for detailed dissection of functions required for life at high pressure. Such studies should include isolating novel microorganisms using a greater variety of culture media and exploring new environments such as those of deep-sea brines and deep-subsurface habitats. Genome sequence information together with functional genomic studies hold the promise of unparalleled advances in the understanding of these and other extremophiles. Isolating and characterizing additional proteins required for piezophily should be coupled with more detailed investigation of previously identified proteins such as selected cytochromes, CydD, ToxR, RseC, and RecD. It will also be important to examine protein structure-function relationships and the possible role of extrinsic factors in minimizing pressure inhibitory effects on macromolecule stability and activity. Finally, it should be emphasized that the search for microbes from the deep that produce novel secondary products or other products of biotechnological interest has only just begun.

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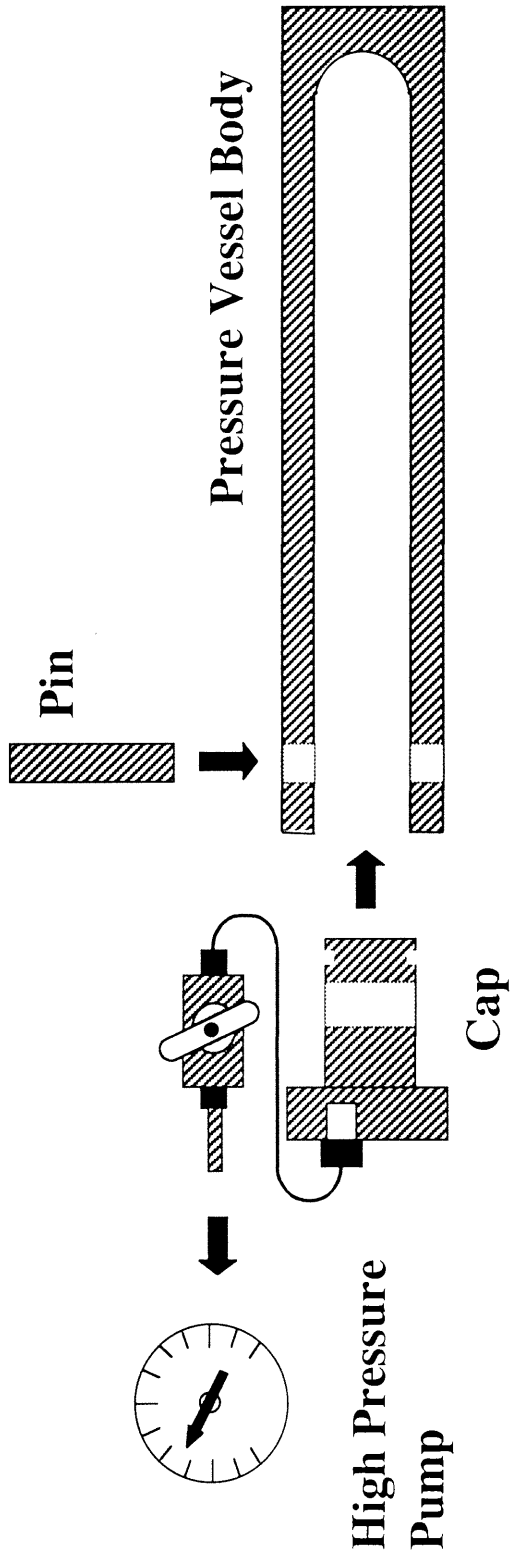
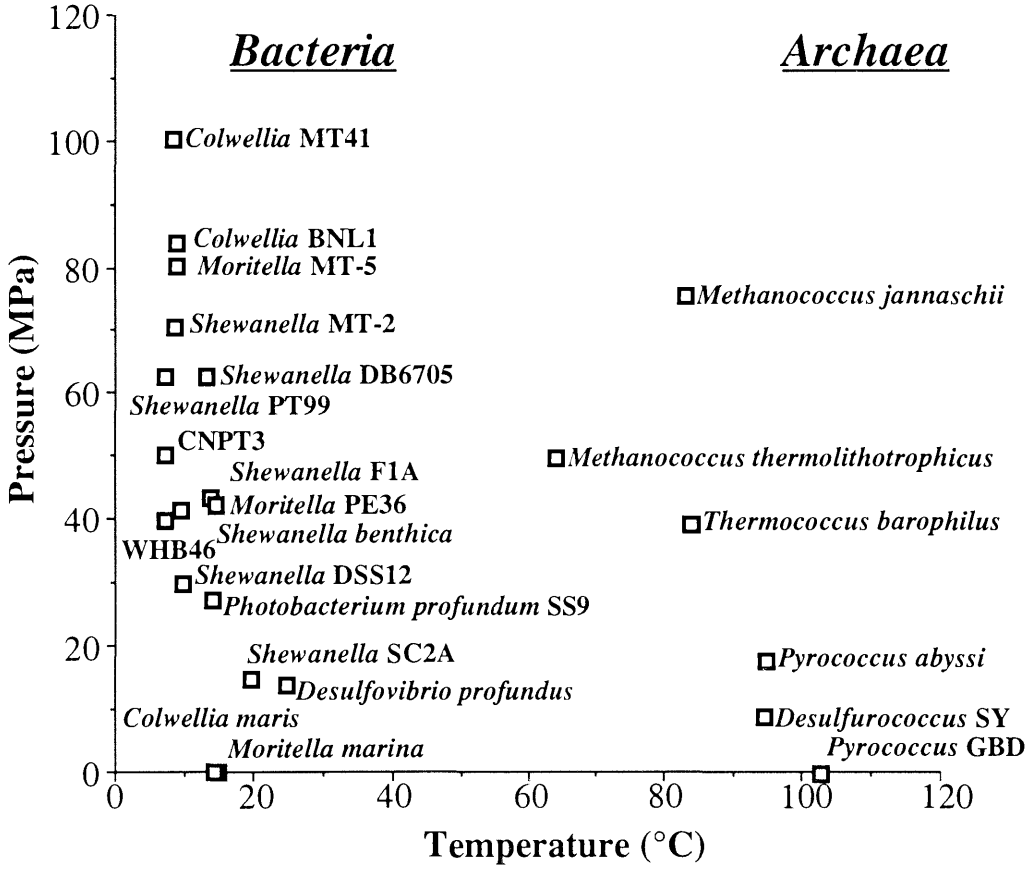
The text of Chapter I, in full, has been accepted for publication in the UNESCO Encyclopedia of Life Support Systems (EOLSS), 2002, (C. Gerday, ed.), EOLSS Publishers Co. Ltd. The dissertation author was the primary author of this chapter.

Table 1. High pressure microbiological environments on Earth and their documented approximate upper pressures

Environment	Approximate Pressure
Deep-sea water column/surface sediments	112 MPa
Deep-sea invertebrates	108 MPa
Deep-sea fish	63 MPa
Deep-sea brines	15 MPa
Hydrothermal vents	41 MPa
Whale falls	41 MPa
Lake Baikal, Siberia	16 MPa
Lake Vostok, Antarctica[a]	41 MPa
Deep marine sediments	14 MPa
Deep basaltic rock	67 MPa
Deep granitic rock	55 MPa
Deep oil reservoirs	31 MPa

[a]No microbiological studies have yet been done on samples from this deep freshwater environment.


Pressure Vessel Body
Pin
Cap
High Pressure
Pump


Bacteria
Archaea
Colwellia MT41
Colwellia BNL1
Moritella MT-5
Shewanella MT-2
Shewanella DB6705
Shewanella PT99
CNPT3
Shewanella F1A
Moritella PE36
Shewanella benthica
WHB46
Shewanella DSS12
Photobacterium profundum SS9
Shewanella SC2A
Desulfovibrio profundus
Colwellia maris
Moritella marina
Methanococcus jannaschii
Methanococcus thermolithotrophicus
Thermococcus barophilus
Pyrococcus abyssi
Desulfurococcus SY
Pyrococcus GBD
Pressure (MPa)
Temperature (°C)
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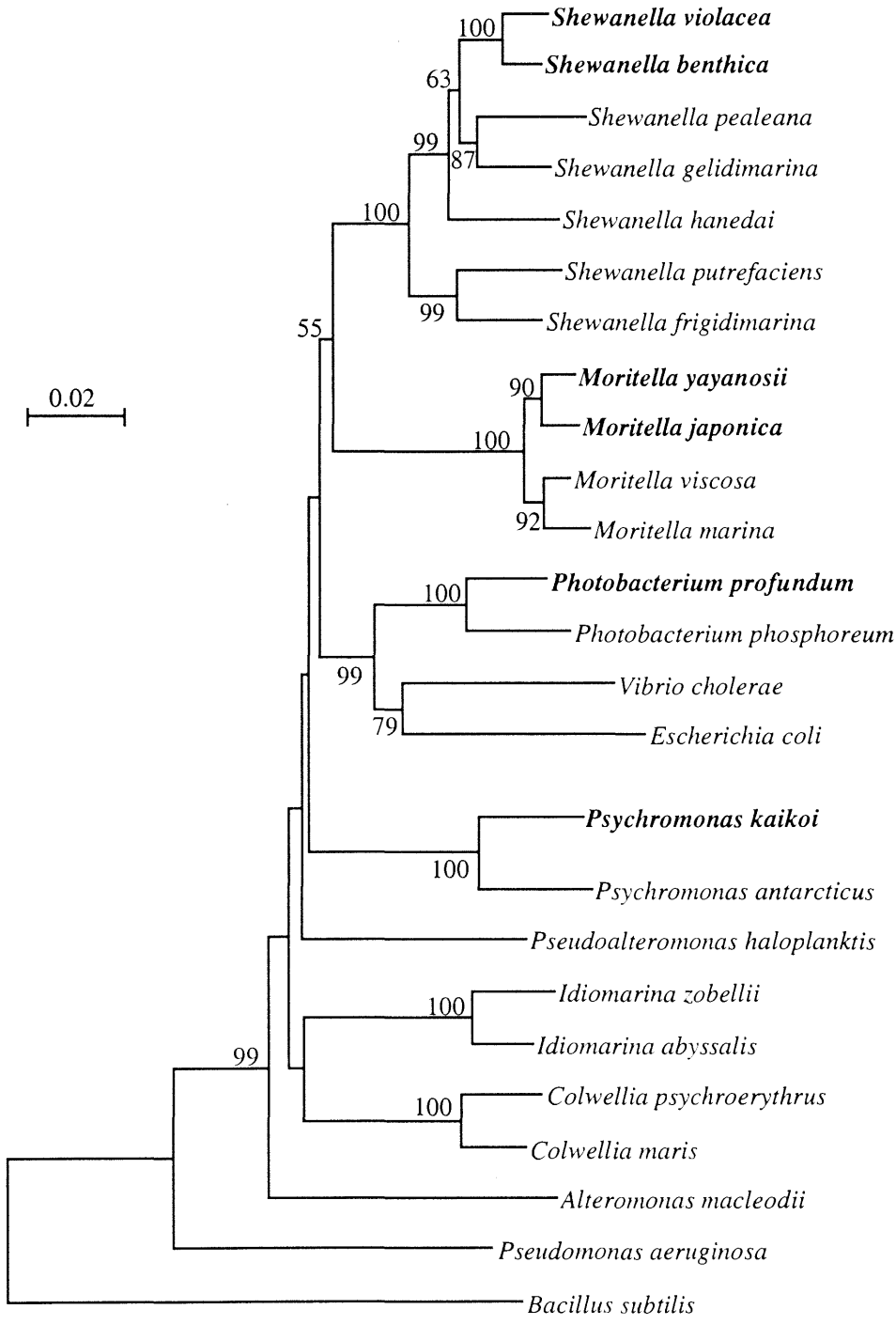
40
60
80
100
120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Shewanella pealeana
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Shewanella gelidimarina
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Shewanella hanedai
Shewanella putrefaciens
99
Shewanella frigidimarina
55
90
Moritella yayanosii
Moritella japonica
100
Moritella viscosa
92
Moritella marina
100
Photobacterium profundum
Photobacterium phosphoreum
99
Vibrio cholerae
79
Escherichia coli
Psychromonas kaikoi
100
Psychromonas antarcticus
Pseudoalteromonas haloplanktis
100
Idiomarina zobellii
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100
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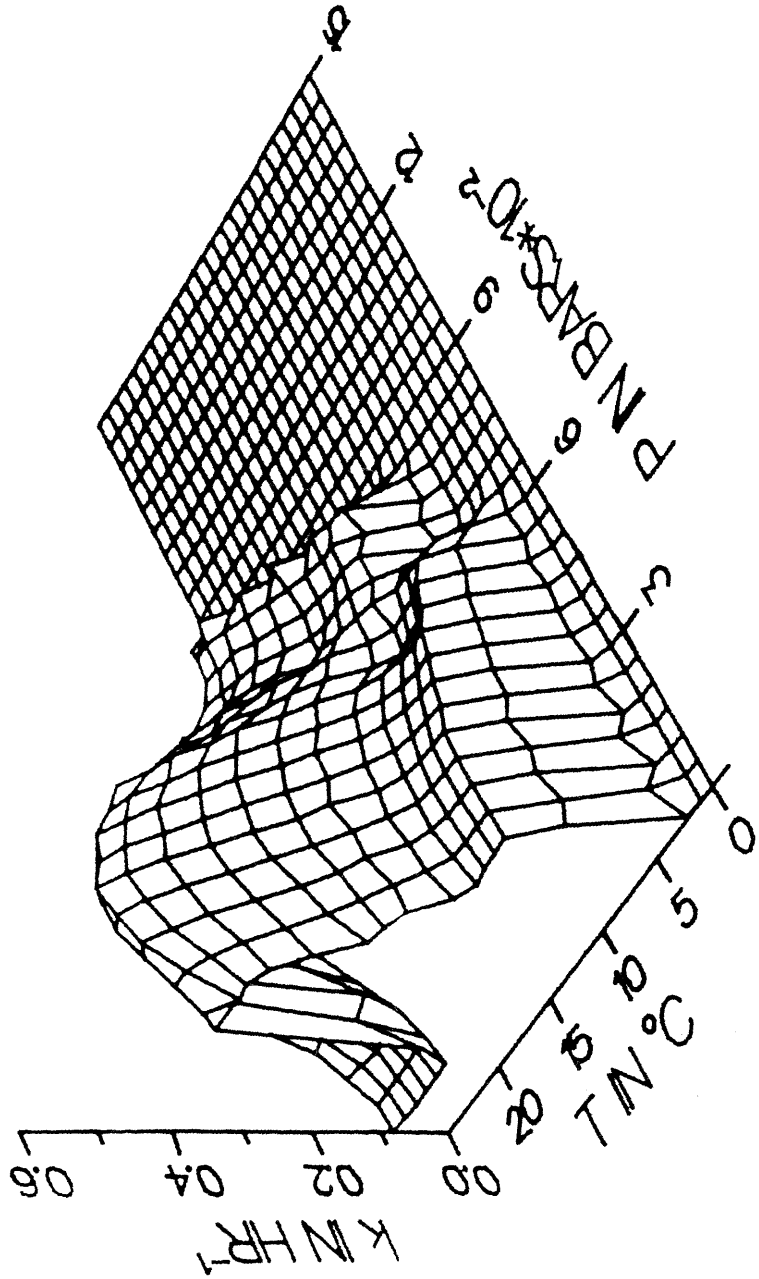
Alteromonas macleodii
Pseudomonas aeruginosa
Bacillus subtilis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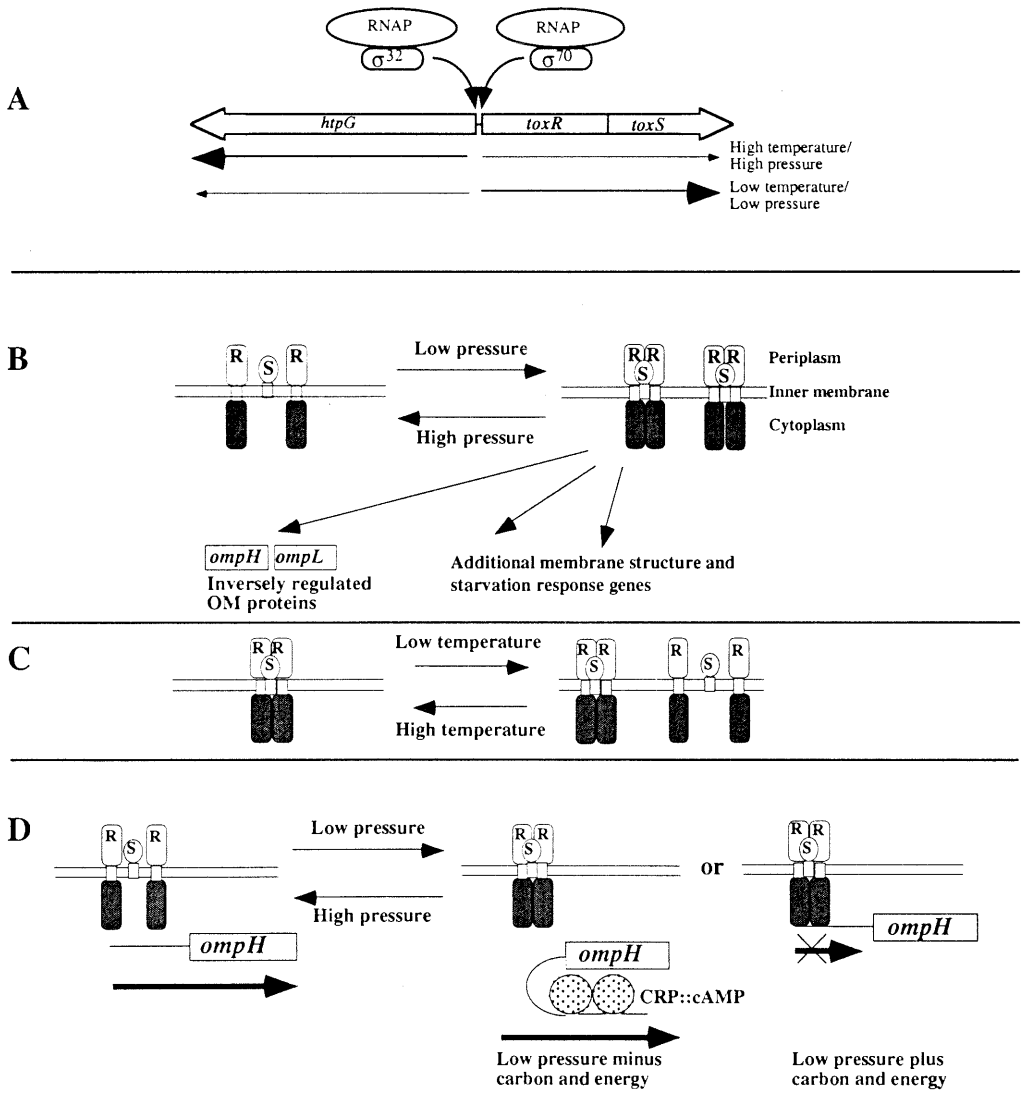
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Periplasm
Inner membrane
Cytoplasm
ompH
ompL
Inversely regulated
OM proteins
Additional membrane structure and
starvation response genes
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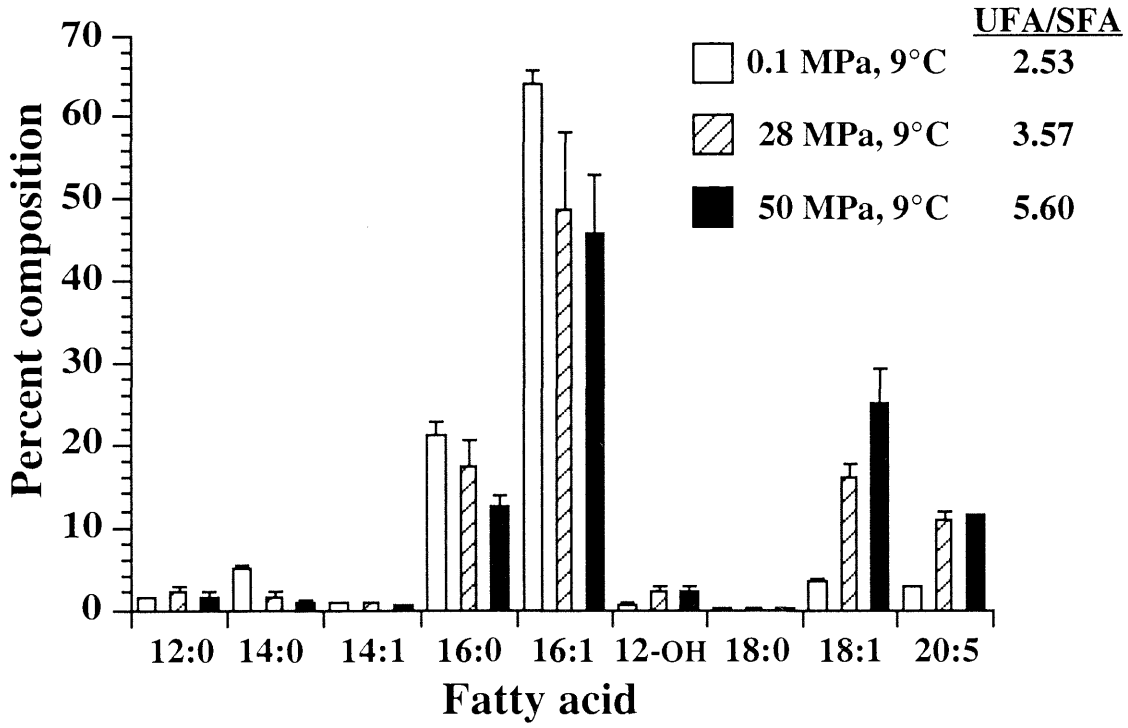
CRP::cAMP
ompH
Low pressure minus
carbon and energy
Low pressure plus
carbon and energy


UFA/SFA
0.1 MPa, 9°C 2.53
28 MPa, 9°C 3.57
50 MPa, 9°C 5.60
Percent composition
70
60
50
40
30
20
10
0
12:0 14:0 14:1 16:0 16:1 12-OH 18:0 18:1 20:5
Fatty acid

CHAPTER II

Role of Unsaturated Fatty Acids in Growth at High Pressure and Low Temperature in the Deep-Sea Bacterium *Photobacterium profundum* strain SS9

Role of Unsaturated Fatty Acids in Growth at High Pressure and Low Temperature in the Deep-Sea Bacterium *Photobacterium* Species Strain SS9

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INTRODUCTION

The study of barophilic (high pressure adapted) bacteria is providing clues to the adaptations of life in the largest portion of the known biosphere, the deep sea. Among the charactertistics believed to be critical for such life at high pressure and low temperature is the maintenance of appropriate membrane order or fluidity. It is presumably for this purpose that many barophiles display a high ratio of unsaturated to saturated fatty acids in their membrane phospholipids, which in many instances increases with growth pressure (1-4). High pressure promotes tighter packing of fatty acid chains (5). Because acyl chains with one or more double bonds align in a less orderly fashion than their saturated counterparts increases in unsaturation may be important for the homeostatic maintenance of appropriate membrane physical structure. Increases in fatty acid unsaturation with depth of occurrence of marine fishes has also been noted (6), suggesting that such membrane alterations are fundamental to the adaptations of many types of deep-sea organisms.

Additional interest in membrane structure has arisen from studies of pressure sensing. The deep-sea bacterium *Photobacterium* species strain SS9 is a genetically tractable model system for understanding both high pressure adaptation and pressure-sensing (7, 8). At 0.1 MPa it produces high levels of the protein OmpL in its outer membrane, while at its pressure optimum, 28 MPa, OmpL levels are reduced and those of another outer membrane protein, OmpH, are elevated. A cytoplasmic membrane spanning protein designated ToxR is responsible for inverse pressure regulation of the *ompL* and *ompH* genes (9). However, membrane structure also

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appears to be an integral part of *omp* gene pressure regulation. As shown in Fig. 1 the presence of membrane fluidizing local anesthetics prevent ToxR repression of *ompL* and induction of *ompH* at high pressure. This result is particularly noteworthy in view of the well documented antagonism between pressure and anesthetics in central nervous system function in vertebrates (10).

In order to begin to assess the role of membrane structure in vivo on the above processes, the effect of decreasing unsaturated fatty acid (UFA) levels on the growth properties of SS9 has been analyzed.

METHODS

SS9 strain DB110 was cultured at low and high pressure as previously described (8). Mutant EA5 was routinely grown with 0.16 mM oleic acid along with 0.5% Tween 40 as a solublizing agent in solid media or in the presence of 0.05% polyoxyethylene-sorbitan-monooleate (Tween 80) alone in liquid media. For the purpose of preparing oleic acid containing medium without detergent a modified medium was prepared according to the recipe for 2216 Marine Medium (Difco Laboratories) except for the omission of all divalent cation containing salts.

Nitrosoguanidine (NTG) mutagenesis and steptozotocin mutant enrichment were performed similarly to that described by Miller (11) and Lengeler (12), respectively. SS9 strain DB110 was exposed to 100 μg/ml NTG in 0.1 M citrate buffer (pH 7.2) supplemented with 3.2% Sea Salts (Sigma Chemical Co.) at 15°C under conditions resulting in 50% survival. Cells were then washed in Sea Salts and resuspended in 2216 Marine Medium containing the antibiotic (50 μg/ml) to kill growing cells. Every thirty minutes for two hours cells were diluted into 2216 Marine Medium and plated onto antibiotic free medium containing oleic acid. Colonies were then screened by replica plating for oleic acid auxotrophy. In this manner 5/6,345 colonies were identified as oleic acid requiring mutants.

Cerulenin was purchased from Sigma Chemical Company and used at a concentration of 12 μg/ml.

Fatty acid methyl esters were prepared from whole cell methanolysates according to Bligh and Dyer (13) and separated and analyzed on a Hewlett Packard 5890 gas chromatograph equipped with a carbowax 0.25 mm by 30 m column connected to a Hewlett Packard 55988A mass spectrometer.

RESULTS

As a first step in assessing the role of unsaturated fatty acids on the growth of SS9 the effect of the antibiotic cerulenin was tested. Cerulenin irreversibly inhibits fatty acid biosynthesis condensing enzymes in a variety of bacteria and fungi, particularly perturbing the formation of unsaturated fatty acids (UFAs) (14). As shown in Fig. 2

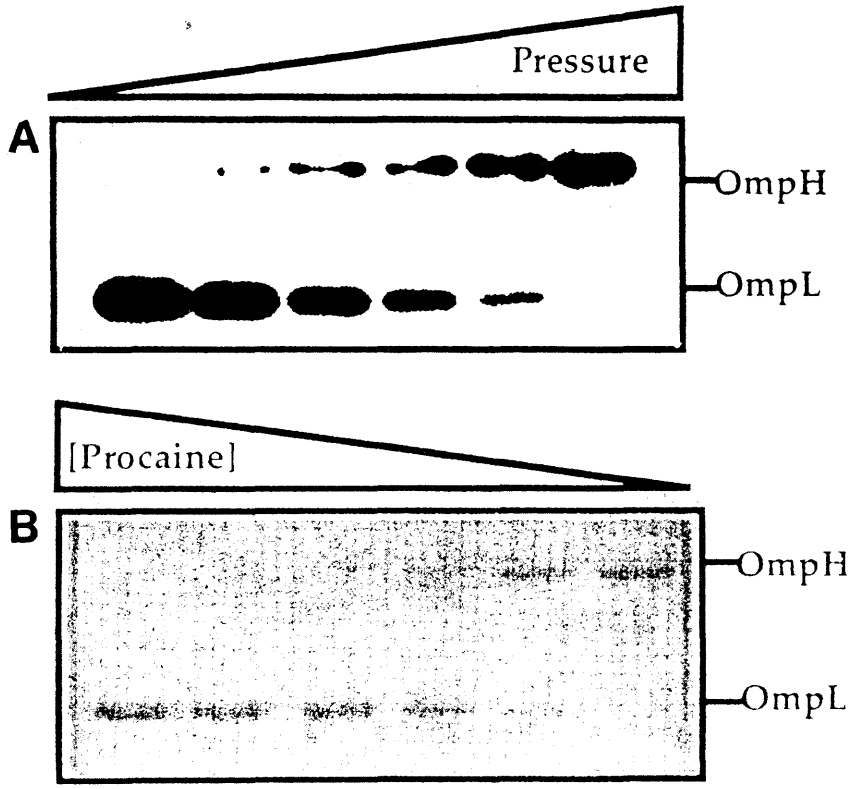

Figure 1. Anesthetic modulation of ToxR-mediated high pressure signaling. Panel A; Western blot analysis showing the abundance of the OmpH and OmpL proteins from SS9 cultures grown to mid-log phase at various pressures (taken from Welch and Bartlett, 1996). From left to right cultivation pressure was increased from 1 to 28 MPa in 4.7 MPa increments per lane. Panel B; Commassie Blue R stained SDS-PAGE of SS9 OMPs from cells grown to mid-log at 28 MPa and various concentrations of procaine (Welch and Bartlett; manuscript submitted). From left to right procaine concentration decreases from 3 mM to 0 mM in 0.6 mM changes per lane. Panel B SS9 Coomassie Blue R stained SDS-PAGE showing the abundance of the OmpH and OmpL proteins from SS9.

cerulenin was particularly effective at inhibiting the growth of SS9 at lower temperatures, 4°C versus 15°C, and higher pressures, 28 MPa versus 0.1 MPa.

To further study the role of UFAs in the temperature/pressure adaptation of SS9 mutants defective in UFA production were obtained as described in methods. All of these mutants were verified to be derivatives of SS9 based upon Coomassie Blue

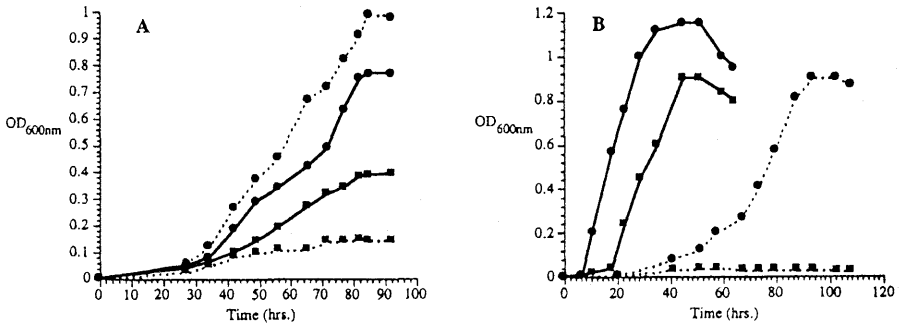

Figure 2. Effect of cerulenin on growth of SS9 at high pressure and low temperature. Panel A; untreated (circles) and cerulenin-treated DB110 (squares) grown at 28 MPa (dashed lines) and 0.1 MPa (solid lines) at 9°C in 2216-0.1M Hepes-0.4% Glucose. Panel B; untreated (circles) and cerulenin-treated DB110 (squares) grown at 4°C (dashed lines) and 15°C (solid lines) in 2216-0.1M Hepes-0.4% Glucose.

R staining of whole cell proteins after electrophoresis in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and comparison to DB110 proteins. Mutant EA5 was selected for further characterization. The oleic acid requirement of EA5 was verified both by its inability to grow in the presence of Tween detergents 20 and 40, which lack oleic acid, and by the presence of growth in modified Marine Medium containing 0.05% oleic acid sodium salt lacking any Tween detergent. Gas-chromatography-mass spectrometry analysis of the major fatty acid methyl esters prepared from 15°C aerobic stationary phase cultures revealed that EA5 produced approximately 50% less palmitoleic acid (16:1) accompanied by increased levels of lauric acid (12:0) and myristic acid (14:0). EA5's requirement for oleic acid prevented quantitation of endogenously produced 18:1 fatty acid levels.

The growth of DB110 and EA5 in the presence of 0.005% Tween 80 at low and high pressure and low and high temperature is shown in Fig. 3. At the temperature optimum for SS9, 15°C, EA5 exhibited a similar growth rate to that of the parental strain but growth yield was reduced by approximately 25%. In contrast, at 4°C, while DB110 grew at only a slightly reduced rate following a 50 hour lag, the growth of EA5 was markedly diminished, displaying a dramatically reduced growth rate at 4°C versus 15°C, and achieving less than one third the growth yield at 4°C versus 15°C even after nearly 200 hours of incubation. Cultivation of DB110 near the high pressure optimum for SS9, 28 MPa (9°C), revealed as expected a better growth rate and yield compared with that at 0.1 MPa. However, EA5 grew poorly at both low and high pressure, with 28 MPa growth being most severely reduced. The poor growth of EA5 at both low and high pressure is probably the result of the relatively low temperature, 9°C, used for the pressure cultivation experiments.

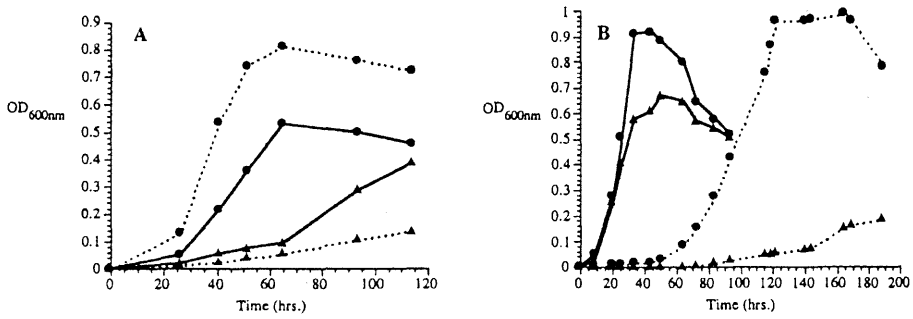

Figure 3. Growth of EA5 at high pressure and low temperature. Panel A; DB110 (circles) and EA5 (triangles) grown at 28 MPa (dashed lines) and 0.1 MPa (solid lines) at 9°C in 2216-0.005% Tween 80-0.1M Hepes-0.4% Glucose. Panel B; DB110 (circles) and EA5 (triangles) grown at 4°C (dashed lines) and 15°C (solid lines) in 2216-0.005% Tween 80.

DISCUSSION

The effect of decreased temperature and increased pressure on ordering membrane structure in vitro is well documented (15). However, much less is known about the role of membrane architecture on biological function in vivo. Indeed, while low temperature-sensitive fatty acid unsaturation mutants have previously been reported for the cyanobacterium *Synechocystis* PCC6803 (16), this is the first genetic report linking fatty acid unsaturation to growth ability at high pressure. Future studies will be needed to characterize the specific genetic alteration in EA5, to follow the environmental regulation of UFA synthesis, and to correlate UFA levels and membrane fluidity to growth and ToxR activity as a function of pressure and temperature.

CONCLUSION

Unsaturated fatty acids appear to be critical to the ability of the deep-sea bacterium *Photobacterium* species strain SS9 to grow at elevated pressure or decreased temperature.

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The text of Chapter II, in full, is a reprint of the material as it appears in High Pressure Biology and Medicine, 1998, (P. B. Bennett, I. Demchenko, and R. E. Marquis, ed.), University of Rochester Press, Rochester, N. Y. The dissertation author was the primary researcher pertaining to fatty acid work which forms the basis for this chapter.

CHAPTER III

Monounsaturated but Not Polyunsaturated Fatty Acids Are Required for Growth of the Deep-Sea Bacterium *Photobacterium profundum* strain SS9 at High Pressure and Low Temperature

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Monounsaturated but Not Polyunsaturated Fatty Acids Are Required for Growth of the Deep-Sea Bacterium *Photobacterium profundum* SS9 at High Pressure and Low Temperature

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There is considerable evidence correlating the production of increased proportions of membrane unsaturated fatty acids (UFAs) with bacterial growth at low temperatures or high pressures. In order to assess the importance of UFAs to microbial growth under these conditions, the effects of conditions altering UFA levels in the psychrotolerant piezophilic deep-sea bacterium *Photobacterium profundum* SS9 were investigated. The fatty acids produced by *P. profundum* SS9 grown at various temperatures and pressures were characterized, and differences in fatty acid composition as a function of phase growth, and between inner and outer membranes, were noted. *P. profundum* SS9 was found to exhibit enhanced proportions of both monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids when grown at a decreased temperature or elevated pressure. Treatment of cells with cerulenin inhibited MUFA but not PUFA synthesis and led to a decreased growth rate and yield at low temperature and high pressure. In addition, oleic acid-auxotrophic mutants were isolated. One of these mutants, strain EA3, was deficient in the production of MUFAs and was both low-temperature sensitive and high-pressure sensitive in the absence of exogenous 18:1 fatty acid. Another mutant, strain EA2, produced little MUFA but elevated levels of the PUFA species eicosapentaenoic acid (EPA; 20:5*n*-3). This mutant grew slowly but was not low-temperature sensitive or high-pressure sensitive. Finally, reverse genetics was employed to construct a mutant unable to produce EPA. This mutant, strain EA10, was also not low-temperature sensitive or high-pressure sensitive. The significance of these results to the understanding of the role of UFAs in growth under low-temperature or high-pressure conditions is discussed.

One of the characteristics believed critical for life at low temperatures or high pressures is the maintenance of appropriate membrane fluidity or phase. Reduced temperature and increased hydrostatic pressure exert profound physical influences on biological membranes, resulting in supraoptimal membrane viscosity or phase transition caused primarily by the tighter packing of the fatty acyl chains (9, 17, 26). Thus, at elevated pressures and/or low temperatures, acyl chains assume a closely packed, ordered array in which molecular motion is highly restricted. Such membrane gelling effects are predicted to provide a strong inducement for adaptive membrane restructuring in order to circumvent the deleterious consequences of altered membrane function. The maintenance of biological membranes in a narrow range of viscosity (homeoviscous response) (49) or within a liquid-crystalline phase (homeophasic response) (28) may be key to an organism's growth ability and survival. Membrane transport, intracellular signaling and gene regulation, membrane protein dispersion and protein-protein interactions within the lipid bilayer, and metabolic electron transport are reliant on an appropriate membrane physical structure (20).

Perhaps the most pervasive cellular response to a temperature change entails the retailoring of the membrane's fatty acid composition. It is well documented that the biological response of numerous bacteria to decreases in temperature results in substantial increases in the proportion of unsaturated fatty acids (UFAs) within membrane phospholipids (30). Likewise, many deep-sea bacteria display a high ratio of UFAs to saturated fatty acids (SFAs) in their membrane phospholipids, which in many instances increases with increasing growth pressure (11, 12, 21, 54). Fatty acyl chains containing one or more double bonds adopt a more expanded conformation, pack less compactly, and possess lower melting temperatures than their saturated counterparts, allowing for their less orderly alignment within the membrane phospholipids (17). Consequently, increases in membrane unsaturation may be important for the homeostatic maintenance of an appropriate physical structure of the membrane in response to environmental variables which elicit membrane gelling effects, such as reduced temperature and elevated pressure.

One particularly remarkable characteristic shared by many low-temperature-adapted (psychrophilic or psychrotolerant) and high-pressure-adapted (piezophilic or piezotolerant, previously termed barophilic and barotolerant [56]) bacteria is the production of the omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA; 20:5) and docosahexanenoic acid (DHA; 22:6). There is a preponderance of PUFA-producing bacteria associated with cold (or permanently cold) and high-pressure environments compared to tropical and shallow-water environments (12, 37, 60, 61). By virtue of their extremely low melting temperatures, such PUFAs would be expected to exert disproportionately large effects on membrane structure, effectively reducing the melting temperature and increasing the melting pressure of a lipid

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bilayer. Consequently, PUFA production and their temperature- and pressure-dependent regulation by deep-sea bacteria have been considered potentially important adaptations to the low temperatures and high hydrostatic pressures of the deep sea (12, 15, 38, 54, 55). The deep sea is characterized by a temperature of 2°C in most habitats and by hydrostatic pressures ranging from 10 MPa at a depth of 1,000 m to approximately 110 MPa in the Challenger Deep of the Mariana Trench at 10,898 m.

Whereas correlations have been found to exist between the degree of membrane unsaturation and growth under low-temperature and high-pressure conditions, confirmatory evidence that UFAs are indeed required for growth under these conditions is lacking. Prior studies have remained nearly exclusively descriptive and phenomenological, reporting the phenotypic changes which ensue in response to temperature and pressure variables. In the present study, we have addressed the relative importance of UFAs for growth of the psychrotolerant piezophilic deep-sea bacterium *Photobacterium profundum* SS9 at low temperatures and high pressures. *P. profundum* SS9 is a genetically tractable model system for studies of low-temperature and high-pressure adaptation (1). Isolated from an amphipod homogenate enrichment in the Sulu Sea at a depth of 2,551 m and an ambient temperature of approximately 9°C, *P. profundum* SS9 is capable of growth at temperatures of less than 2°C to greater than 20°C (optimal temperature, 15°C) and from 0.1 MPa (0.101 MPa = 1 atm = 1.01 bar) to nearly 70 MPa (optimal pressure, 28 MPa) (10). In addition, *P. profundum* SS9 produces the PUFA EPA (39). To begin assessment of the importance of fatty acid composition in vivo, the effects of altered UFA levels on the growth properties of *P. profundum* SS9 have been analyzed through UFA inhibitor analyses, the generation of mutants exhibiting altered fatty acid profiles, and the engineering of a *P. profundum* SS9 mutant defective in EPA production.

MATERIALS AND METHODS

Strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. profundum* strains were routinely cultured at 15°C in 2216 marine medium (28 g/liter; Difco Laboratories, Detroit, Mich.). All temperature experiments (15, 9, and 4°C) were conducted aerobically in 2216 medium. For solid media, agar (Difco Laboratories) was added at 17 g/liter. The antibiotics kanamycin (50 μg/ml for *Escherichia coli*; 200 μg/ml for *P. profundum* strains), rifampin (100 μg/ml), and ampicillin (100 μg/ml) were added to the medium when required. The antibiotic cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide) was added at 12 μg/ml when used in inhibition studies. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). When required, media were supplemented with oleic acid (18:1) in the form of Tween 80 (polyoxyethylenesorbitan-monooleate) at a final concentration of 0.025%. Tween 20 (polyoxyethylenesorbitan-monolaurate), Tween 40 (polyoxyethylenesorbitan-monopalmitate), and Tween 80 were obtained from Sigma Chemical Co.

High-pressure growth studies. High-pressure cultivation of *P. profundum* strains for growth studies or fatty acid analysis was conducted as previously described (6). Each culture was grown to stationary phase in 2216 marine medium at 1 atm (1 atm = 0.101 MPa) and 15°C. Stationary-phase cultures were diluted 1/200 into 2216 medium buffered with HEPES (100 mM, pH 7.5; Sigma Chemical Co.) containing 22 mM glucose (Sigma Chemical Co.). The diluted culture was used to fill 4.5- or 15-ml polyethylene transfer pipettes (Samco, San Fernando, Calif.). The pipettes were filled completely and then heat sealed with a hand-held heat sealing clamp (Nalgene, Rochester, N.Y.). Cells were incubated at a hydrostatic pressure of 0.1, 28, or 50 MPa (1, 280, or 500 atm, respectively) at 9°C (unless otherwise stated) in stainless steel vessels which could be pressurized by using water and a hydraulic pump and which were equipped with quick-connect fittings for rapid decompression and recompression as described by Yayanos and Van Boxtel (57).

Chemical mutagenesis. *P. profundum* DB110, a Lac$^-$, rifampin-resistant derivative of wild-type *P. profundum* SS9 (6), served as the parental strain for mutagenesis experiments. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Sigma Chemical Co.) mutagenesis experiments were performed according to the protocols of Miller (31). Streptozotocin (Sigma Chemical Co.) enrichment and selection were performed similarly to the procedures of Jacobson et al. (18). Prior to mutagenesis, kill curves were obtained for strain DB110, using various concentrations of the mutagen NG and the antibiotic streptozotocin in order to determine the effectiveness of killing. The conditions used here represent concentrations and times of exposure that resulted in 50% killing. Strain DB110 was grown in 25-ml batches in 2216 marine medium supplemented with 0.4% *N*-acetylglucosamine (NAG) (Sigma Chemical Co.) at 15°C in an environmental shaker to an optical density at 600 nm of 1.0, corresponding to a density of approximately 10^8 cells ml^{-1}. NAG supplementation was performed to preinduce cells for uptake of the antibiotic streptozotocin for later antibiotic selection. Streptozotocin is an analogue of NAG which is transported into the cell by the same phosphotransferase system proteins (23). Hence, preinduction with NAG facilitates uptake of streptozotocin. The 25-ml batches were divided into 5 equal volumes and centrifuged at 5,000 × *g* for 5 min. The cell pellets were washed once in 0.1 M citrate buffer (pH 7.2) supplemented with sea salts (32 g/liter; Sigma Chemical Co.) and resuspended in an equal volume of citrate buffer. NG was added to the cultures to a final concentration of 100 μg/ml. The cultures were allowed to incubate at 15°C for 4 h without shaking. After 4 h, the cultures were washed twice in 0.1 M phosphate buffer to remove the NG and resuspended in an equal volume of 2216 marine medium. The cultures were then allowed a 6-h outgrowth recovery period at 15°C. To enrich for fatty acid-requiring auxotrophs, the antibiotic streptozotocin was then added to each culture to a final concentration of 50 μg/ml and the cultures were allowed to incubate at 15°C with shaking for an additional 2 h. At 30-min intervals, 1 ml from each culture was removed and 100 μl was plated onto each of five antibiotic-free 2216 agar plates containing 0.005% oleic acid-Na$^+$ salt and 0.05% Tween 40 (Sigma Chemical Co.) as a solubilizing agent for the oleic acid. The plates were incubated at 15°C in the dark for approximately 5 to 7 days to allow growth of mutagenized cells. Colonies were then replica plated onto 2216 agar supplemented with oleic acid, as well as onto unsupplemented 2216 agar. Of the 6,345 colonies screened, 5 displayed an auxotrophic requirement for oleic acid, showing no growth on unsupplemented 2216 agar. These oleic acid auxotrophs were designated *P. profundum* EA1 to EA5.

Due to the extreme insolubility of the oleic acid-Na$^+$ salt alone in a marine medium, a result of the presence of a high concentration of divalent cations, a variety of liquid media were prepared for growth of these mutants. 2216 marine medium was first supplemented with 0.005% (wt/vol) oleic acid-Na$^+$ salt and 0.05% (vol/vol) Tween 40 (as a solubilizing agent); however, this was not an ideal medium due to the presence of large amounts of insoluble oleic acid. Alternatively, a modified medium was prepared according to the recipe for 2216 marine medium (Difco Laboratories), except for the omission of all divalent-cation salts, and supplemented with 0.005% oleic acid-Na$^+$ salt. This medium provided moderate growth of strain DB110 and the mutant strains. Finally, 2216 marine medium supplemented with Tween 80 (18:1) as a source of oleic acid was prepared. Tween 80 is an oleate ester of sorbitol and its anhydrides copolymer-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
P. profundum		
SS9	Wild type	10
DB110	Lac$^-$ Rif^r SS9 derivative	6
EA2	Oleic acid-auxotrophic mutant; DB110 derivative	This study
EA3	Oleic acid-auxotrophic mutant; DB110 derivative	This study
EA10	EPA-deficient mutant; DB110 derivative	This study
E. coli		
DH5α	*recA* strain used in maintaining plasmids	16
ED8654	Strain in which pRK2073 is maintained	33
Plasmids		
pRK2073	Helper plasmid carrying the *tra* genes necessary for conjugal transfer	3
pAMP1	Plasmid containing an 885-bp internal fragment of SS9 EPA ORF3/4; Amp^r	This study
pMUT100	Mobilizable suicide plasmid; Km^r	5
pEA30	710-bp internal fragment of SS9 ORF3/4 in pMUT100; Km^r	This study

TABLE 2. Fatty acid compositions of *P. profundum* strains as a function of growth pressure and temperature[a]

Strain	Culture conditions[b]	Mean % (by wt) of fatty acid ± SD										Unsaturation index[c]	UFA/SFA ratio[d]
		12:0	14:0	14:1	iso-16:0	16:0	16:1	12-OH	18:0	18:1	20:5		
DB110													
Untreated	15°C	1.8 ± 0.9	4.9 ± 1.4	1.2 ± 0.8	9.8 ± 3.1	21.7 ± 2.8	41.3 ± 8.3	3.0 ± 1.2	1.1 ± 0.4	9.9 ± 2.6	5.3 ± 1.1	79 ± 5.1	1.95
	4°C	2.7 ± 1.0	3.3 ± 0.8	2.5 ± 0.5	0.2 ± 0.1	20.2 ± 3.8	48.8 ± 6.8	3.4 ± 1.3	0.2 ± 0.1	10.3 ± 1.7	8.4 ± 1.1	104 ± 6.6	2.65
	0.1 MPa	1.5 ± 0.1	5.0 ± 0.3	0.8 ± 0.1		21.4 ± 1.5	64.1 ± 1.5	0.6 ± 0.1	0.2 ± 0.0	3.6 ± 0.1	2.7 ± 0.1	82 ± 2.4	2.53
	28 MPa	2.2 ± 0.6	1.6 ± 0.7	0.7 ± 0.1		17.5 ± 3.2	48.7 ± 9.6	2.0 ± 0.8	0.1 ± 0.1	16.2 ± 1.6	11.0 ± 0.8	121 ± 3.3	3.57
	50 MPa	1.4 ± 0.8	0.8 ± 0.5	0.5 ± 0.1		12.5 ± 1.2	45.9 ± 7.0	2.3 ± 0.5	0.1 ± 0.1	25.1 ± 4.1	11.4 ± 0.2	129 ± 7.2	5.60
Cerulenin treated[e]	15°C	4.9 ± 0.8	21.7 ± 2.7	4.5 ± 1.6	0.3 ± 0.3	28.3 ± 4.3	27.3 ± 4.2	3.8 ± 2.1	0.2 ± 0.1	0.3 ± 0.2	8.7 ± 2.6	76 ± 8.9	0.79
	4°C	9.3 ± 0.0	17.9 ± 0.8	9.8 ± 2.3		19.0 ± 0.4	22.4 ± 0.6	8,3 ± 0.1		0.3 ± 0.1	13.0 ± 0.9	98 ± 2.7	0.98
	0.1 MPa	9.5 ± 0.3	24.8 ± 3.2	5.6 ± 1.0		30.0 ± 2.9	10.8 ± 0.8	6.2 ± 0.7	0.2 ± 0.0	0.3 ± 0.0	12.6 ± 1.1	80 ± 1.9	0.45
	28 MPa	16.1 ± 2.3	35.1 ± 3.7	5.3 ± 0.9		17.2 ± 2.0	7.1 ± 1.1	6.3 ± 0.9			12.9 ± 1.2	77 ± 1.7	0.37
EA3													
	15°C	8.2 ± 1.3	19.7 ± 2.3	11.7 ± 2.8		21.5 ± 1.4	27.9 ± 2.9	5.0 ± 2.6	3.5 ± 0.5	0.9 ± 0.5	1.6 ± 0.3	49 ± 6.6	0.80
	4°C	9.2 ± 1.2	24.0 ± 0.6	10.9 ± 1.2		22.0 ± 0.7	20.9 ± 1.9	4.3 ± 1.6	0.3 ± 0.1	0.1 ± 0.0	8.3 ± 0.6	73 ± 3.9	0.72
	0.1 MPa	3.9 ± 0.2	19.3 ± 1.7	3.6 ± 0.2		53.4 ± 0.5	11.9 ± 1.1	3.5 ± 0.2	0.8 ± 0.1	0.5 ± 0.1	3.1 ± 0.2	32 ± 2.6	0.25
	28 MPa	3.6 ± 0.1	17.9 ± 0.4	3.1 ± 0.1		53.2 ± 0.1	15.3 ± 0.6	2.7 ± 0.3	1.2 ± 0.1	0.7 ± 0.1	2.3 ± 0.2	31 ± 0.5	0.28
EA2													
	15°C	3.5 ± 0.3	16.5 ± 1.2	1.1 ± 0.1		35.9 ± 0.5	10.1 ± 0.3	3.7 ± 2.0	2.6 ± 0.7	0.7 ± 0.1	25.8 ± 1.2	141 ± 6.0	0.64
	4°C	4.9 ± 0.2	24.4 ± 3.2	1.7 ± 0.6		26.9 ± 1.0	7.1 ± 0.5	4.7 ± 1.5	0.4 ± 0.1	0.2 ± 0.1	29.5 ± 1.9	157 ± 3.6	0.68
	0.1 MPa	4.5 ± 0.1	25.2 ± 0.1	0.5 ± 0.5		35.8 ± 2.2	3.9 ± 0.1	4.5 ± 1.1	0.4 ± 0.2	0.2 ± 0.1	24.8 ± 0.9	129 ± 4.5	0.45
	28 MPa	4.3 ± 0.6	15.5 ± 3.1	1.8 ± 0.3		29.7 ± 7.0	9.1 ± 0.1	3.9 ± 0.9	0.2 ± 0.1	0.8 ± 0.4	32.7 ± 4.5	176 ± 21.0	0.89
	50 MPa	4.0 ± 0.1	5.0 ± 0.1	0.4 ± 0.1		35.6 ± 1.3	18.3 ± 1.0	5.1 ± 0.6	0.3 ± 0.0	2.8 ± 0.1	28.3 ± 2.7	163 ± 12.4	1.11
EA10													
	15°C	2.5 ± 1.0	6.5 ± 2.2	4.7 ± 0.5	5.0 ± 1.5	16.7 ± 2.8	46.3 ± 3.8	3.3 ± 0.7	1.0 ± 0.2	14.0 ± 1.4		65 ± 3.0	2.43
	4°C	3.0 ± 0.2	3.2 ± 0.7	4.1 ± 0.7	2.1 ± 1.0	10.6 ± 0.9	55.2 ± 3.9	3.9 ± 0.2	0.2 ± 0.1	17.7 ± 3.3		77 ± 1.3	4.53
	0.1 MPa	3.1 ± 0.1	3.7 ± 0.3	2.2 ± 0.3		20.4 ± 0.5	52.9 ± 1.0	4.6 ± 0.4	0.4 ± 0.1	12.7 ± 0.1		68 ± 0.8	2.46
	28 MPa	2.8 ± 0.2	1.4 ± 0.2	1.6 ± 0.0		12.2 ± 2.0	52.3 ± 0.3	4.2 ± 0.1	0.1 ± 0.1	25.4 ± 2.6		79 ± 2.4	4.81
	50 MPa	2.6 ± 1.0	1.6 ± 0.3	1.1 ± 0.2		13.5 ± 0.3	51.6 ± 3.6	5.5 ± 1.2	0.1 ± 0.1	24.0 ± 3.8		77 ± 0.2	4.31

[a] Data represent values derived from triplicate samples harvested in the late exponential phase of growth.
[b] 15 and 4°C cultures were grown aerobically in 2216 marine medium at 0.1 MPa; 0.1-, 28-, and 50-MPa cultures were grown at 9°C in 2216 marine medium containing 22 mM glucose buffered with 100 mM HEPES.
[c] Unsaturation index was calculated as the sum of the mean percentages (by weight) of the UFA species multiplied by the number of double bonds (12).
[d] UFAs, 14:1, 16:1, 18:1, and 20:5; SFAs, 12:0, 14:0, 16:0, and 18:0.
[e] Cerulenin was used at 12 μg/ml.

ized with approximately 20 mol of ethylene oxide per mol of sorbitol and its anhydrides. Such Tween products are highly soluble in water due to their hydrophilic character supplied by the free hydroxyl and oxyethylene groups, while the lipophile portion is found in the esterified fatty acid chains. This medium provided excellent growth of *P. profundum* strains and was used for routine culturing of the mutants.

Gene disruption mutagenesis. Construction of an EPA-deficient strain of *P. profundum* SS9 was performed via gene disruption mutagenesis with the mobilizable suicide plasmid pMUT100 (encoding kanamycin resistance) (5). An 885-bp internal fragment of an SS9 EPA biosynthetic open reading frame (designated ORF3/4) was PCR amplified from strain DB110 genomic DNA with the following primers, designed from the known EPA ORF3/4 gene sequence of *Shewanella* sp. strain SCRC-2738 (59): 5′-CUACUACUACUAACAGCGAAA TGCTTATCAAG-3′ (primer 1) and 5′-CAUCAUCAUCAUGCCACCAAAA CCAAATGAGCTAATAC-3′ (primer 2). The PCR product was first cloned into pAMP1 by using a Gibco BRL CloneAmp system (Life Technologies, Gaithersburg, Md.) and subsequently subcloned into pMUT100 as an *Eco*RI-*Bam*HI fragment, yielding pEA30. Due to an internal *Eco*RI site within the SS9 ORF3/4 PCR product, pEA30 contains a 710-bp fragment. Bacterial conjugations were used to transfer plasmid pEA30 from *E. coli* into *P. profundum* DB110 as described by Chi and Bartlett (6). Kan^r exconjugants arose from integration of the pMUT100 plasmid into the genome of *P. profundum* SS9. These experiments yielded the EPA-deficient strain *P. profundum* EA10, containing a disruption in EPA ORF3/4 which was confirmed by Southern blot analysis (45). Genomic DNA from *P. profundum* EA10 and DB110 was digested with restriction enzyme *Pst*I, *Hin*dIII, *Bgl*II, *Hpa*I, or *Kpn*I and probed with the internal fragment of ORF3/4 harbored on pEA30, labelled with [α-^{32}P]dCTP by random priming (Life Technologies). Evidence for gene disruption was revealed by the replacement of discrete fragments in the strain DB110 genome by fragments 6.2 kb larger in the case of strain EA10. Killing experiments were conducted at an extremely high pressure (100 MPa) and an extremely low temperature (−20°C) to assess the susceptibility of strain EA10 to such extremes compared to the parental strain, DB110. Stationary-phase cultures of strains EA10 and DB110 were diluted to a density of approximately 10^7 cells ml^{-1} and incubated either at 100 MPa (9°C) or −20°C (0.1 MPa) for up to 6 h. Every 30 min for 100-MPa incubations and every 45 min for −20°C incubations, CFU of both strains per milliliter were determined at 15°C (0.1 MPa) following culture dilution and plating onto 2216 marine agar.

Fatty acid analyses and lipid extracts. Cells grown at various hydrostatic pressures or temperatures were harvested in late exponential phase via centrifugation at 5,000 × *g*, washed in an equal volume of 50% artificial seawater (16 g of Sigma sea salts per liter; Sigma Chemical Co.), frozen at −70°C, and lyophilized prior to fatty acid methyl ester (FAME) derivatization. Whole-cell methanolysates were used throughout the study for FAME preparation and analysis. FAMEs were prepared by reacting 10 mg (dry weight) of a lyophilized cell sample with 5% H_2SO_4 in anhydrous methanol at 90°C for 90 min in 1.5-ml sample vials with Teflon-lined caps (Wheaton, Millville, N.J.). Samples were allowed to cool, and FAMEs were extracted twice with hexane and nonesterified fatty acids saponified with 10% NaCl. Following FAME derivatization, the hexane layer was removed and evaporated completely under a gentle stream of N_2. The FAME residue was then redissolved in 25 μl (final volume) of hexane containing a known concentration of 19:0 methyl ester as an internal standard and stored at −70°C until analysis.

Analyses of the FAME preparations were performed with a Hewlett-Packard model 5890 gas chromatograph equipped with an Econo-Cap EC-Wax (Carbowax) capillary column (30 m by 0.25 mm [internal diameter] by 0.25 μm; Alltech Associates Inc., Deerfield, Ill.) connected to a Hewlett-Packard model 5988A mass spectrometer (MS). Samples were injected in the split mode with a split ratio of 25:1. After 1 min at 165°C, the oven was temperature programmed to increase from 165 to 260°C at a rate of 6°C min^{-1}. Helium was used as the carrier gas, and the injector was maintained at 250°C. Peak areas were quantified, and mass spectra were acquired and processed with Hewlett-Packard G1034C MS ChemStation software operated in the scan acquisition mode. MS operating conditions were as follows: electron multiplier, 1,800 V; transfer line, 250°C; electron impact energy, 70 eV; scan threshold, 50; 1.3 scans s^{-1} with a mass range of 50 to 550 atomic mass units; and solvent delay, 2.35 min. Compounds were identified by comparison of their retention times with those of known standards, and sample mass spectra data were compared to the mass spectra data of 75,000 compounds in the Chemstation NBS75K library. EPA production was confirmed by comparison of mass spectra data of the EPA methyl ester standard (Sigma Chemical Co.) with that of the corresponding peak from *P. profundum* SS9. Fatty acids are denoted as ratios of the number of carbon atoms to the number of double bonds.

IM and OM separation and analysis. Isolations of inner (IM) and outer (OM) membrane fractions were performed with *P. profundum* DB110 grown at 15°C in 2216 marine medium at atmospheric pressure according to the methods of Schnaitman (47). Cells (500 ml) were harvested in late exponential phase at a density of approximately 10^8 ml^{-1} by centrifugation at 5,000 × *g* for 10 min. Cell pellets were weighed and resuspended in a volume of ice-cold sucrose buffer (200 mM Tris [pH 7.8], 5 mM EDTA, 0.25 M sucrose, and 0.5 mg of lysozyme ml^{-1}) such that 30% of the total volume was cells. The cells were then transferred to an Erlenmeyer flask and lysed by being subjected to six cycles of alternating freezing (in a dry ice-ethanol bath) and thawing (in a room-temperature water bath). The cell lysate was then poured into 20 volumes of cold 200 mM Tris, pH

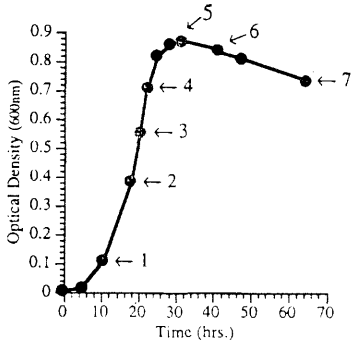

FIG. 1. Dependence of cellular fatty acid composition in *P. profundum* DB110 on phase of growth. Shown is the growth curve for DB110 at 15°C and 0.1 MPa in 2216 marine medium. Arrows denote times at which cells were harvested for fatty acid analysis (see Table 3 for corresponding fatty acid profiles).

TABLE 3. Fatty acid profiles corresponding to harvest times indicated on growth curve in Fig. 1

Time point	Mean % (by wt) of fatty acid species										Unsaturation index[a]	UFA/SFA ratio[b]
	12:0	14:0	14:1	iso-16	16:0	16:1	12-OH	18:0	18:1	20:5		
1	3.6	12.9	7.1		21.3	46.9	5.6		2.1	0.5	58.1	1.5
2	3.1	7.3	3.9		19.5	45.3	4.5	1.7	9.3	5.4	85.5	1.8
3	3.0	5.4	2.7		21.0	47.0	3.8	1.5	10.4	5.2	86.1	2.1
4	2.8	5.0	2.1	1.9	22.0	44.8	3.9	1.5	10.2	5.8	86.1	2.0
5	2.6	5.2	2.2	4.9	22.5	42.9	4.8	1.4	8.9	4.6	77.0	1.8
6	2.8	4.7	2.0	7.3	19.3	44.1	5.3	1.3	8.6	4.6	77.7	2.1
7	2.5	5.0	1.6	10.9	19.5	43.7	4.8	1.5	7.5	3.0	67.8	1.9

[a] Unsaturation index was calculated as the sum of the mean percentages (by weight) of the UFAs multiplied by the number of double bonds (12).
[b] UFAs, 14:1, 16:1, 18:1, and 20:5; SFAs, 12:0, 14:0, 16:0, and 18:0.

TABLE 4. Fatty acid compositions of *P. profundum* SS9 IM and OM fractions

Fatty acid species	Mean % (by wt) ± SD (n = 3)	
	IM	OM
12:0	2.9 ± 0.1	7.6 ± 0.3
14:0	5.1 ± 0.8	7.2 ± 0.6
14:1	2.5 ± 0.6	2.5 ± 0.6
iso-16:0	4.3 ± 1.9	2.1 ± 1.6
16:0	25.0 ± 1.5	20.1 ± 1.2
16:1	31.7 ± 5.0	31.1 ± 0.7
12-OH	3.2 ± 2.1	12.3 ± 1.3
18:0	6.6 ± 2.8	2.6 ± 1.0
18:1	12.1 ± 3.7	9.7 ± 1.1
20:5	6.5 ± 0.2	4.7 ± 1.2

7.8, containing 0.5 mM $MgCl_2$ and 0.1 mg of DNase I (Calbiochem, La Jolla, Calif.) ml^{-1}. This material was then passed through an 18-gauge needle for 10 min to shear the DNA and disperse the cell debris. Unbroken cells were removed by centrifugation twice at 7,000 × *g*, with the supernatant being decanted between clearing spins. The envelope material, consisting of IM and OM, was recovered by centrifugation at 27,000 × *g* for 30 min. The membrane pellet was dried under vacuum without heat and resuspended in 1 ml of 25% (wt/wt) sucrose containing 5 mM EDTA. Sucrose gradients, each containing 5 mM EDTA, were prepared as follows in SW41 ultracentrifuge tubes (Beckman Instruments, Fullerton, Calif.): 0.5 ml of 55% (wt/wt) sucrose, 2.1 ml of 50% sucrose, 2.1 ml of 45% sucrose, 2.1 ml of 40% sucrose, 2.1 ml of 35% sucrose, and 2.1 ml of 30% sucrose. The 1-ml membrane suspension in 25% sucrose was layered onto the gradient, and the gradient was spun for 14 h, in an SW41 ultracentrifuge rotor at 92,000 × *g* and 4°C. The OM appeared as a pair of similar opalescent bands at about 50% (wt/wt) sucrose. The IM was a translucent, yellowish band at about 36% (wt/wt) sucrose. Fractions were removed from the gradients and analyzed for purity of composition by (i) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and (ii) enzyme assays for both D-lactate dehydrogenase activity and succinate dehydrogenase activity. SDS-PAGE analysis was used for comparison of the OM fractions to OM proteins isolated by a Triton X-114 detergent extraction method (4) modified as described by Chi and Bartlett (6). OM fractions appeared nearly identical to the Triton X-114 extraction OM preparations. Bicinchoninic acid protein assays (Pierce, Rockford, Ill.) were performed to quantitate the total protein present in the membrane fractions, and D-lactate and succinate dehydrogenase activity assays were used as markers for the cytoplasmic membrane fractions as described by Osborn et al. (42). Fractions identified as IM had D-lactate and succinate dehydrogenase specific activities (in micromoles per minute per milligram of protein) of 1.2 and 0.7, respectively. Fractions identified as OM had D-lactate and succinate dehydrogenase activities of 0.09 and 0.01, respectively, indicating that the level of contamination of the OM with IM was very low. Once membrane fractions were confirmed and their purity was assessed, fatty acid analysis was performed as described above.

RESULTS

Fatty acid composition of *P. profundum* SS9. The major fatty acids produced by *P. profundum* SS9 include (shown as the systematic name followed by the common name) 12:0 (dodecanoic acid; lauric acid), 14:0 (tetradecanoic acid; myristic acid), 14:1 (*cis*-7-tetradecanoic acid; myrisoleic acid), iso-16:0 (14-methyl-pentadecanoic acid), 16:0 (hexadecanoic acid; palmitic acid), 16:1 (*cis*-9-hexadecanoic acid; palmitoleic acid), 12-OH (3-hydroxydodecanoic acid; β-hydroxylauric acid), 18:0 (octadecanoic acid; stearic acid), 18:1 (*cis*-11-octadecanoic acid; *cis*-vaccenic acid), and 20:5 (all-*cis*-5, 8, 11, 14, 17-eicosapentaenoic acid; EPA). The fatty acid profile of *P. profundum* SS9 cultivated aerobically at 15°C (0.1 MPa) was similar to that recently reported by Nogi et al. (39) under similar conditions. Included in Tables 2 and 3 and Fig. 1 are the unsaturation index values (sums of the percentages [by weight] of UFA species multiplied by the numbers of double bonds) (12) and the UFA/SFA ratios for the various strains and conditions used in this study in order to display the relative degree of membrane unsaturation.

Dependence of fatty acid composition on growth phase. The percentages of the major fatty acid species were found to vary depending on the phase of growth. This was characterized for *P. profundum* DB110 grown aerobically at 15°C and 0.1 MPa (Fig. 1). Across the spectrum of time periods examined, the most dramatic regulation involved the progressive increase in the branched-chain fatty acid iso-16:0 as a function of culture age. iso-16:0 levels were consistently undetected until late exponential phase and then increased throughout stationary phase, reaching nearly 11% of the total fatty acid (by weight) in advanced stationary-phase cultures. 18:1 and EPA levels increased from early to late log phase, whereas 14:0 decreased from early log phase to later growth stages. The unsaturation index values and UFA/SFA ratios were lowest during early exponential phase and highest during late log phase. In light of these results, care was taken to analyze the fatty acid contents of all *P. profundum* strains within a particular growth phase, specifically late exponential phase (corresponding to an optical density at 600 nm of approximately 0.7), so that the data would not be skewed as a result of growth phase differences.

Analysis of IM and OM composition. Fatty acid composition differences as a function of cellular location were also investigated (Table 4). IM and OM were isolated by density gradient centrifugation from strain DB110 grown at 15°C. Compared to the IM fraction, the OM fraction was enriched with the hydroxylated fatty acid 12-OH (3.2% versus 12.3%, respectively). The specific enrichment of the OM with 12-OH is likely to

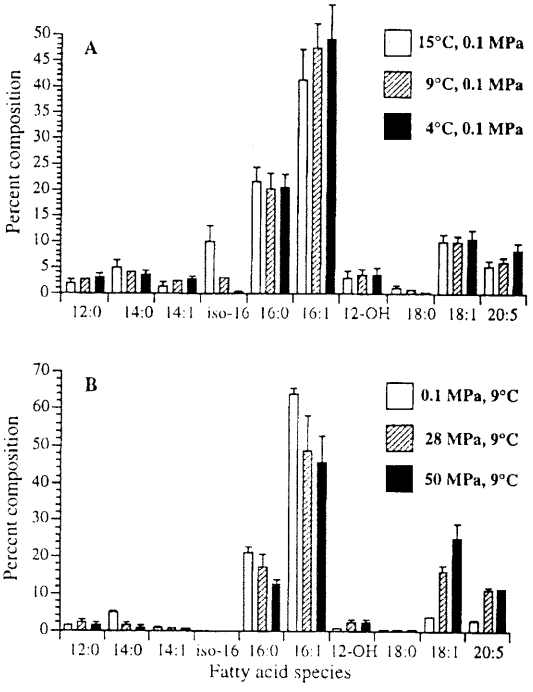

FIG. 2. Effect of growth temperature (A) and growth pressure (B) on cellular fatty acid composition in *P. profundum* DB110 (1 MPa = 10 bar ≈ 9.87 atm). Data represents mean percentages (by weight) of fatty acid species, ± standard deviations, derived from triplicate samples harvested in the late exponential phase of growth. See Materials and Methods for cultivation conditions.

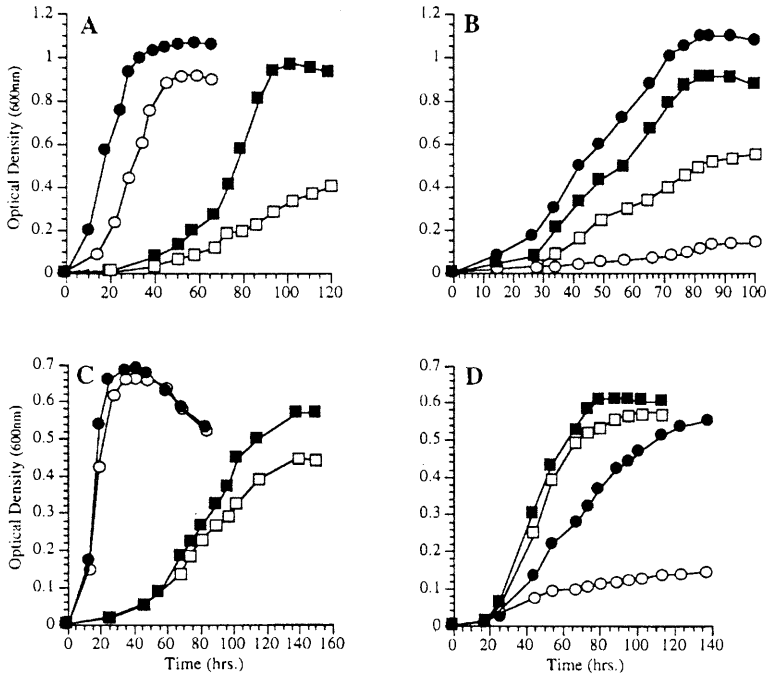

FIG. 3. Effect of cerulenin (12 μg/ml) on the growth of *P. profundum* DB110 at various temperatures (A and C) and pressures (B and D) with or without exogenous 18:1 in the form of 0.025% Tween 80. (A) ●, 15°C, without cerulenin; ○, 15°C, with cerulenin; ■, 4°C, without cerulenin; □, 4°C, with cerulenin. (B) ●, 28 MPa, without cerulenin; ○, 28 MPa, with cerulenin; ■, 0.1 MPa, without cerulenin; □, 0.1 MPa, with cerulenin. (C) ●, 15°C, with cerulenin and 18:1; ○, 15°C, with cerulenin but without 18:1; ■, 4°C, with cerulenin and 18:1; □, 4°C, with cerulenin but without 18:1. (D) ●, 28 MPa, with cerulenin and 18:1; ○, 28 MPa, with cerulenin but without 18:1; ■, 0.1 MPa, with cerulenin and 18:1; □, 0.1 MPa, with cerulenin but without 18:1.

derive from the lipid A component of the lipopolysaccharide layer (44). In addition, the OM contained a higher percentage of the shorter-chain SFAs, specifically 12:0 (7.6% in OM versus 2.9% in IM) and, to a lesser extent, 14:0 (7.2% in OM versus 5.1% in IM). This higher percentage of SFAs in the OM is consistent with findings in *E. coli* (24). UFA types exhibited no dramatic differential localization between the membrane fractions.

Temperature- and pressure-dependent regulation of fatty acid composition. Comparison of the fatty acid profiles of *P. profundum* DB110 grown under different conditions indicated that low temperature and high pressure both enhanced UFA levels, but to different extents and with different selective effects (Fig. 2). It should be noted that the 15, 9, and 4°C cultures were grown aerobically, whereas all experiments in pressurizable bulbs necessitated growth under fermentative conditions with glucose and buffer added to the cultures. With decreased temperature, moderate to slight increases in the proportions of 16:1 and 18:1 occurred along with a significant increase in EPA and a dramatic reduction in the proportion of iso-16:0. Changes in pressure resulted in an even more dramatic effect on the fatty acid composition. The proportion of 18:1 increased from 3.6 to 16.2% of total fatty acids as cells were pressurized from 0.1 MPa (9°C) to 28 MPa (9°C), with an additional increase to 25.1% upon progression from 28 MPa (9°C) to 50 MPa (9°C). Over the pressure range tested, the proportion of EPA also increased, from 2.7% at 0.1 MPa (9°C) to 11.4% at 50 MPa (9°C). No iso-16:0 was produced in any of the cultures grown in the pressurizable bulbs regardless of the growth phase. Akin to observations made for other piezophilic species (12, 21), the relative degree of fatty acid unsaturation in *P. profundum* SS9 increased in response to decreased temperature or increased hydrostatic pressure, as indicated by the general increase in unsaturation index values and UFA/SFA ratios as a function of decreased temperature or increased pressure (Table 2).

Effect of cerulenin treatment on growth at high pressures and low temperatures. As a first step in assessing the role of UFAs in the growth of *P. profundum* SS9, the effect of the antibiotic cerulenin was tested. Cerulenin irreversibly inhibits fatty acid biosynthesis enzymes β-ketoacyl-acyl carrier protein synthases I and II in a variety of bacteria and fungi, perturbing the formation of UFAs (41). Cerulenin treatment allowed us to specifically determine the effect of diminished monounsaturated fatty acid (MUFA) levels (specifically 16:1 and 18:1) on growth of *P. profundum* SS9. As shown in Table 2, analysis of the fatty acid composition of cerulenin-treated strain DB110 under the various temperature and pressure growth conditions revealed a nearly complete inhibition of 18:1 production and moderate reductions in 16:1. In addition, cerulenin elicited dramatic increases in the proportions of 12:0, 14:0, and 14:1 as well as moderate increases in EPA. The fact that EPA levels rose while 18:1 values declined is consistent with separate pathways directing the biosynthesis of the two UFAs, with the pathway responsible for 18:1 production being far more sensitive to the antibiotic than that of the EPA pathway.

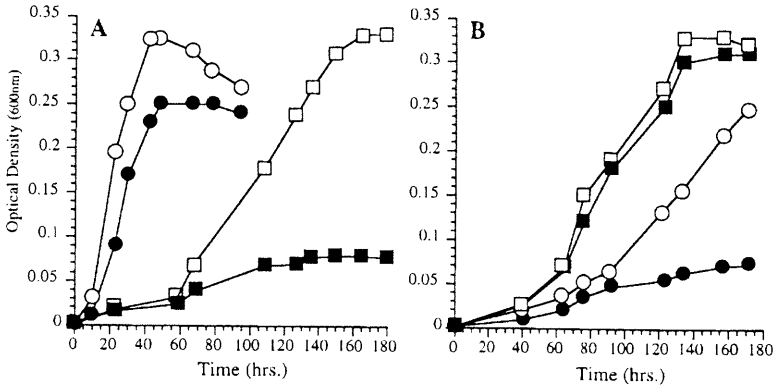

FIG. 4. Growth characteristics of *P. profundum* SS9 oleic acid-auxotrophic chemical mutant EA3 at various temperatures (A) and pressures (B) in the absence or presence of exogenous 18:1 in the form of 0.025% Tween 80. (A) ●, 15°C, without 18:1; ○, 15°C, with 18:1; ■, 4°C, without 18:1; □, 4°C, with 18:1. (B) ●, 28 MPa, without 18:1; ○, 28 MPa, with 18:1; ■, 0.1 MPa, without 18:1; □, 0.1 MPa, with 18:1.

The effect of cerulenin treatment on growth was particularly inhibitory at low temperature and at high pressure (Fig. 3A and B). However, if cerulenin-treated cultures are supplemented with exogenous 18:1, in the form of 0.025% Tween 80, a reversal of the cerulenin inhibition was observed to various degrees (Fig. 3C and D). Cells grown in the presence of both cerulenin and 18:1 exhibited a modest enhancement of growth at low temperature and a marked enhancement of growth at high pressure (28 MPa) compared to cerulenin-treated cells grown in the absence of the added fatty acid supplement. These results suggest that 18:1 is important for low-temperature and high-pressure growth.

Growth characteristics and fatty acid analyses of oleic acid-auxotrophic mutants. A second line of experimentation which was performed to address the importance of particular UFAs for growth at low temperature or high pressure involved the generation of a collection of chemical mutants exhibiting an auxotrophic requirement for oleic acid (18:1). We predicted that many of these mutants would be altered in the production of certain UFAs. Of the 6,345 NG-mutagenized colonies screened, 5 displayed a requirement for oleic acid, exhibiting negligible or complete lack of growth on unsupplemented 2216 agar. These mutant strains were designated *P. profundum* EA1 to EA5. All of these mutants were verified to be derivatives of *P. profundum* SS9 based on Coomassie blue R staining of whole-cell proteins and SDS-PAGE analysis with comparison to strain DB110 proteins. All of these mutants exhibited a specific enhancement of growth in the presence of UFAs, since SFAs such as palmitic acid (16:0) and lauric acid (12:0) failed to compensate for their growth defects. Only mutant EA5 exhibited an absolute requirement for oleic acid for growth.

The fatty acid profiles of mutants EA1 to EA4 were obtained. However, mutant EA5 could not be accurately analyzed due to its obligate requirement for exogenous 18:1. Of the five mutants isolated, mutants EA1 and EA4 exhibited the least-stringent requirements for exogenous 18:1 and displayed wild-type levels of all fatty acids. However, mutants EA2 and EA3 exhibited markedly altered fatty acid profiles relative to strain DB110 and were therefore selected for further analysis.

The fatty acid compositions of strains EA2 and EA3 grown at various temperatures and pressures are listed in Table 2. Strain EA3 exhibited severely diminished 16:1, 18:1, and EPA levels as well as elevated proportions of 12:0, 14:0, and 14:1 under routine culture conditions of 15°C (0.1 MPa). At low temperature, strain EA3 produced fivefold-higher EPA levels, but little difference in fatty acid composition was evident in cells grown at low and high pressures. Figure 4 shows the growth characteristics of strain EA3 at various pressures and temperatures in the presence or absence of exogenous 18:1 in the form of Tween 80 at a concentration of 0.025%. Without 18:1 supplementation, strain EA3 exhibited dramatic low-temperature and high-pressure sensitivities, exhibiting virtually no growth at 4°C (0.1 MPa) or 28 MPa (9°C). However, strain EA3 displayed dramatically enhanced growth at a low temperature (4°C) or an elevated pressure (28 MPa) in the presence of Tween 80. These results are qualitatively consistent with the cerulenin studies, although the influence of exogenous 18:1 on growth at low temperature was substantially more dramatic for strain EA3 than for cerulenin-treated cells. The growth characteristics of strain EA3 were also similar to those previously reported for strain EA5 (2).

The fatty acid profile of strain EA2 was similar to that of strain EA3 in that it produced diminished levels of MUFAs

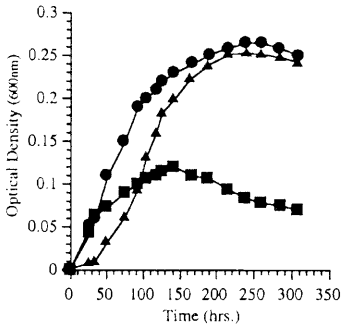

FIG. 5. Growth characteristics of *P. profundum* SS9 chemical mutant EA2 at various pressures. Cultures were grown at the corresponding pressure (9°C) without exogenous 18:1 supplementation. (Refer to Fig. 6 for comparison to DB110 under identical pressure conditions.) ▲, 50 MPa; ●, 28 MPa; ■, 0.1 MPa.

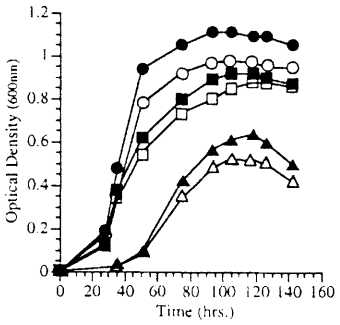

FIG. 6. Growth characteristics of EPA-deficient mutant EA10 versus strain DB110 at various pressures (9°C). ▲, DB110, 50 MPa; ●, DB110, 28 MPa; ■, DB110, 0.1 MPa; △, EA10, 50 MPa; ○, EA10, 28 MPa; □, EA10, 0.1 MPa.

16:1 and 18:1 and elevated levels of 14:0. However, strain EA2 produced substantially less 16:1, and it constitutively overproduced EPA to a level nearly fivefold higher than strain DB110 at 15°C (approximately 28% of the total fatty acids [Table 2]). Also unlike strain EA3, the growth of strain EA2 was not low-temperature or high-pressure sensitive. Although the growth rate and yield of strain EA2 were poor under all conditions, it was not particularly low-temperature or high-pressure sensitive whether it was grown with or without exogenous 18:1. The growth characteristics of strain EA2 at various pressures without 18:1 supplementation are shown in Fig. 5. Remarkably, strain EA2 grew at 50 MPa (9°C) nearly the same as it did at 28 MPa (9°C), and it exhibited a dramatic growth reduction at 0.1 MPa (9°C). Expressed as growth yield ratios, strain EA2 exhibited 50/0.1 and 28/0.1 MPa ratios of 2.2 and 2.3, respectively. In contrast, the corresponding growth yield ratios for strain DB110 under identical conditions were 0.64 (50/0.1 MPa) and 1.25 (28/0.1 MPa). Possibly the overproduction of EPA by strain EA2 enhanced its growth at a low temperature and high pressure and, conversely, inhibited its growth at an elevated temperature or decreased pressure.

Growth characteristics and fatty acid analysis of strain EA10, a *P. profundum* SS9 mutant defective in EPA production. The results presented above indicate a role for MUFAs in the growth of *P. profundum* SS9 at low temperatures and at elevated pressures (cerulenin and strain EA3 data), as well as a possible role for EPA under similar conditions, at least when the proportions of certain other fatty acids have been altered by mutation (strain EA2 data). To assess the role of EPA more directly, a reverse-genetics methodology was employed to construct a mutant unable to produce EPA. This first entailed the cloning of an internal fragment of a *P. profundum* SS9 EPA biosynthesis gene by making use of the previously published sequence of a cluster of genes required for EPA biosynthesis from *Shewanella* sp. strain SCRC-2738 (59) and sequence information obtained from *Vibrio marinus* of genes involved in the production of DHA (22:6*n*-3) (22). From these reports seven open reading frames (ORFs) were determined to be necessary for imparting the ability to produce PUFAs to recombinant *E. coli* strains.

An internal fragment of a homologue of *Shewanella* ORF3/4 from *P. profundum* SS9 was amplified and cloned by PCR with primers derived from SCRC-2738 ORF3/4 positions 542 to 561 and 1403 to 1428. This amplification yielded a product of the expected size whose sequence possessed a high degree of relatedness, 83 and 87% identity at the DNA and deduced protein levels, respectively, to ORF3/4 from *Shewanella* sp. strain SCRC-2738. Construction of a *P. profundum* SS9 EPA mutant, designated strain EA10, followed the introduction of the SS9 sequence into a suicide plasmid and its delivery into *P. profundum* DB110 by conjugal transfer (described in Materials and Methods). Kanamycin-resistant exconjugants were screened initially by examining their fatty acid profiles at 15°C (0.1 MPa) for the lack of EPA production. One of these mutants was subsequently confirmed by Southern blot analysis to be a gene disruption mutant and designated *P. profundum* EA10 (data not shown).

The fatty acid profile of strain EA10 grown under various conditions is listed in Table 2. By comparison with strain DB110, under identical conditions strain EA10 considerably upregulated its proportion of MUFAs (18:1, 16:1, and 14:1) while 16:0 levels tended to remain lower than those of strain DB110. At a low temperature (4°C), strain EA10 exhibited markedly reduced SFA content, with significant increases in 16:1 and 18:1, relative to the level obtained by a 15°C cultivation. The most dramatic effect of high pressure (28 MPa, 9°C) on strain EA10 was to increase the proportion of 18:1 fatty acid from 12.7 to 25.4%.

Surprisingly, when the growth of strain EA10 was examined at a low temperature and high pressure, no significant deviations from wild-type growth were evident, except for a modest reduction in growth yield under all conditions tested. The growth characteristics of strain EA10 versus strain DB110 at various pressures are shown in Fig. 6. Strains EA10 and DB110 exhibited nearly identical growth rates at 4 and 15°C (0.1 MPa) or at 0.1, 28, and 50 MPa (9°C). Moreover, the combined effects of increased pressure and decreased temperature (28 MPa, 4°C) resulted in nearly identical growth abilities for the two strains. Under these conditions (28 MPa, 4°C), strain EA10 displayed a UFA/SFA ratio of 8.38, with 18:1 and 16:1 comprising 27.2 and 58% of the cellular fatty acids, respectively. This is in comparison to strain DB110, which, under identical conditions, exhibited a UFA/SFA ratio of 3.1, with 18:1, 16:1, and EPA comprising 20, 37, and 14.8% of the total cellular fatty acids, respectively. These results indicate that under the laboratory conditions used in this study, EPA is not vital to the growth of *P. profundum* SS9 over the course of many generations, even under low-temperature or high-pressure conditions, situations in which its levels are typically upregulated. Likewise, no differences between the two strains with regard to survival at extremes of temperature (−20°C, 0.1 MPa) or pressure (100 MPa, 9°C) were identified (data not shown).

DISCUSSION

The present study has evaluated the types of fatty acids produced by the psychrotolerant, piezophile *P. profundum* SS9, their distribution between the IM and OM, and their regulation as a function of growth phase, temperature, and hydrostatic pressure. In addition, the significance of UFAs in growth at low temperatures and elevated pressures was explored through the use of a fatty acid synthesis inhibitor and by mutant analysis.

In general, the cellular distribution of fatty acids in *P. profundum* SS9 is similar to that reported for other gram-negative bacteria. For example, the high proportion of the hydroxylated fatty acid 12-OH, derived from the lipid A component of the lipopolysaccharide layer, and the elevated levels of shorter-chain SFAs present in the OM are consistent with findings for other gram-negative bacteria (24, 44). In addition, MUFAs

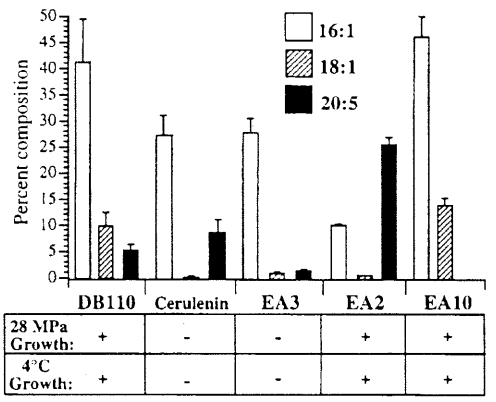

FIG. 7. 16:1, 18:1, and EPA (20:5) levels, ± standard deviations, of strain DB110, cerulenin-treated strain DB110 (12 μg/ml), strain EA3, strain EA2, and strain EA10 grown at 15°C (0.1 MPa) (n = 3) and their corresponding growth phenotypes at 28 MPa (9°C) and at 4°C (0.1 MPa).

and EPA are present in large amounts in both membranes, revealing no dramatic differential localization.

P. profundum SS9 was found to exhibit markedly increased levels of the branched-chain fatty acid iso-16:0 concomitant with entry into stationary phase. The nature of growth phase regulation of fatty acid production depends on the microorganism being studied (14, 25, 29, 36, 40). Our results are similar to those obtained in studies of another gram-negative marine isolate, which exhibited progressive increases in branched-chain fatty acids with culture age (40), and are also similar to the observed induction of cyclopropane fatty acids with the onset of stationary phase in *E. coli* (25, 29).

Modulation of membrane lipids by temperature or pressure is well established among many poikilothermic organisms. Such adjustments most notably include changes at the level of fatty acyl chain composition. The responses of bacteria to a reduced temperature or elevated pressure frequently entail the increased incorporation into membrane phospholipids of UFAs, which include PUFAs in those organisms capable of their production (11, 12, 15, 25, 35, 36, 54). Fatty acid profiling of *P. profundum* SS9 revealed a pronounced regulation of cellular fatty acid composition in response to changes in temperature or pressure, most notably a greater proportion of 16:1 and EPA at low temperatures and an increased proportion of 18:1 and EPA at elevated pressures.

Many deep-sea isolates exhibit substantial increases in MUFAs in response to increased cultivation pressure. The piezophilic bacterium CNPT3, which grows optimally at hydrostatic pressures of 30 to 50 MPa, does not produce PUFAs yet is capable of growth at pressures up to nearly 70 MPa (11). As a function of increasing pressure, CNPT3 exhibits higher proportions of 16:1 and 18:1 while the relative amounts of 14:1, 16:0, and 14:0 decrease. Likewise, it was suggested that the pressure-induced increases in the MUFA iso-17:1 contributed to the piezotolerance of the deep-sea bacterium RS103 (21).

Thus, a correlation between the degree of fatty acid unsaturation and the cultivation temperature or pressure has frequently been drawn. However, to address the possible adaptive role of UFAs in membrane function in vivo at low temperatures or high pressures, physiological and genetic experiments are required. Here the functional significance of UFAs was examined by using the β-ketoacyl-acyl carrier protein synthase I and II inhibitor cerulenin to preferentially inhibit MUFA synthesis and by obtaining mutants altered in UFA synthesis. Figure 7 summarizes the 16:1, 18:1, and EPA levels of the various *P. profundum* strains (DB110, EA3, EA2, and EA10) and cerulenin-treated strain DB110 at 15°C (0.1 MPa) along with their low-temperature (4°C) and high-pressure (28 MPa) growth phenotypes. Those strain-treatment combinations which resulted in reduced MUFA (18:1 and 16:1) levels without dramatic compensatory increases in the proportion of EPA (i.e., strain EA2) exhibited both low-temperature and elevated-pressure sensitivity (cerulenin treatment and strain EA3); conversely, those strain-treatment combinations which exhibited wild-type (or higher) 16:1 and 18:1 levels (strains DB110 and EA10) displayed low-temperature- and elevated-pressure-adapted growth. These results, in conjunction with the fact that 18:1 supplementation was able to complement the ability of strain EA3 to grow at low temperature and high pressure and when cells were treated with cerulenin, suggests that MUFAs are particularly important for growth of *P. profundum* SS9 under low-temperature or high-pressure conditions. Furthermore, the fact that a *P. profundum* SS9 mutant defective in EPA production retained both elevated-pressure- and reduced-temperature-adapted growth via modulations solely in MUFAs is consistent with the primary importance of MUFAs under these conditions.

A decrease in MUFA levels may be partially compensated for by EPA overproduction. Strain EA2 overproduces EPA while underproducing MUFAs. This strain is fascinating because of the shift in its growth ability at higher pressures. However, because the growth rate and yield of this strain are so reduced compared with those of wild-type *P. profundum* SS9, it would appear that EPA is a poor substitute for MUFAs.

What is the explanation for the need for MUFAs during growth at low temperatures and high pressures? Two potential hypotheses can be proposed. Foremost, it is possible that these fatty acids are required to maintain membrane fluidity or phase within an acceptable range for optimal growth. Studies of *E. coli* and *Acholeplasma laidlawii* (27, 43) have suggested that the ability of microorganisms which are unable to effectively regulate their membrane lipid fatty acid composition to grow at various temperatures may be determined by the phase state of their membrane lipids, as evidenced by the severe impairment of growth when cells exhibit more than about half of their lipids in the gel state (29). Indeed, it has been shown that membrane proteins of highly ordered gel-phase membranes are inactive or are excluded (50). To address this hypothesis, it will be necessary to directly measure physical properties of membranes of different *P. profundum* strains at various temperatures and pressures.

Alternatively, the critical nature of MUFAs could be based on a role in specific membrane protein interactions which, if disrupted, result in altered growth ability at reduced temperatures or elevated pressures. This suggests a need for MUFAs in local—as opposed to global—membrane processes. This latter hypothesis is consistent with the notion of a lipid annulus surrounding individual membrane proteins (34, 53). However, despite evidence that many membrane-associated proteins have an absolute lipid requirement for activity (46), we are unaware of any such proteins exhibiting a strict functional requirement for phospholipids with particular fatty acyl chains.

A major result of this work was the discovery that neither low-temperature- nor high-pressure-adapted growth mandates EPA production in *P. profundum* SS9, at least under the culture conditions employed. The ability of EPA-deficient strain EA10 to grow at a reduced temperature or an elevated pres-

sure (Fig. 6) was essentially the same as that of the parental strain. This result was surprising. The few previous studies which have examined phenotypic effects associated with altered PUFA production, the majority of which having been conducted in cyanobacteria, plants, and fungi, have implicated PUFA production as being a necessary component for growth at low temperatures (19, 32, 48, 51, 52).

The distribution of EPA-producing bacteria in the environment has also been taken as evidence of a need for EPA for growth at low temperatures or high pressures. The discovery by DeLong and Yayanos (12) that numerous deep-sea bacterial isolates contain substantial quantities of omega-3 PUFAs, namely, EPA and DHA (22:6), led to the speculation that such polyenoic fatty acids are specifically involved in the adaptation of piezophilic bacteria to the high-pressure, low-temperature conditions prevalent in the deep-sea environment. Since then, it has been confirmed that PUFA production occurs in numerous bacterial species isolated from Antarctic regions as well as temperate marine environments. Nichols et al. (37) have compiled data regarding the percentage of EPA producers isolated from various environments. Analyses revealed that only 1.5% of temperate marine isolates (60, 61), approximately 14% of Antarctic isolates (37), and 27% of deep-sea isolates (12) produce EPA. More recently, Yano et al. (55) investigated the distributions of bacteria containing PUFAs (both EPA and DHA) in the intestines of deep-sea fish and shallow-sea poikilothermic animals. Not only did the intestinal microflora of deep-sea fish contain a higher proportion of PUFA producers, but the percentage of PUFAs within these isolates was also greater than that of shallow-water animals. These results all suggest that there is a preponderance of PUFA producers associated with high-pressure and low-temperature environments.

What then is the explanation for EPA-deficient strain EA10's apparent lack of low-temperature or elevated-pressure sensitivity? Foremost, its increased MUFA content may be capable of compensating for the absence of EPA. Increased unsaturation of a membrane phospholipid-bound fatty acid does not result in a linear decrease in the membrane phase transition temperature. Biophysical studies employing synthetic mixed acid phosphotidylcholines (PC) have shown that the introduction of a double bond into 18:0/18:0-PC, yielding 18:0/18:1-PC, lowers the gel–to–liquid-crystalline phase transition temperature by nearly 50°C, whereas incorporation of the PUFA 20:4*n*-3 to yield 18:0/20:4-PC lowers the phase transition temperature by only an additional 19°C (8). Thus, in the case of strain EA10, the increased levels of 16:1 and 18:1 may provide adequate compensation for the loss of EPA. Alternatively, EPA may be required only under certain physiological conditions not evaluated in our work.

A final possibility is that EPA (and possibly DHA) is not required for psychrotolerant or piezotolerant bacterial growth but is needed as a nutritional source by higher organisms with which the EPA-producing microorganisms have established symbiotic associations. DeLong and Yayanos have previously suggested a specific role for in situ secondary production of PUFAs by piezophilic bacteria (12). Indeed, many of the PUFA-producing microorganisms that have been discovered have been isolated from vertebrate or invertebrate sources (10, 12, 13, 55, 58, 59).

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CHAPTER IV

FabF is Required for Piezoregulation of *cis*-Vaccenic Acid Levels and Piezophilic Growth of the Deep-Sea Bacterium *Photobacterium profundum* strain SS9

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FabF Is Required for Piezoregulation of *cis*-Vaccenic Acid Levels and Piezophilic Growth of the Deep-Sea Bacterium *Photobacterium profundum* Strain SS9

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To more fully explore the role of unsaturated fatty acids in high-pressure, low-temperature growth, the *fabF* gene from the psychrotolerant, piezophilic deep-sea bacterium *Photobacterium profundum* strain SS9 was characterized and its role and regulation were examined. An SS9 strain harboring a disruption in the *fabF* gene (strain EA40) displayed growth impairment at elevated hydrostatic pressure concomitant with diminished *cis*-vaccenic acid (18:1) production. However, growth ability at elevated pressure could be restored to wild-type levels by the addition of exogenous 18:1 to the growth medium. Transcript analysis did not indicate that the SS9 *fabF* gene is transcriptionally regulated, suggesting that the elevated 18:1 levels produced in response to pressure increase result from posttranscriptional changes. Unlike many pressure-adapted bacterial species such as SS9, the mesophile *Escherichia coli* did not regulate its fatty acid composition in an adaptive manner in response to changes in hydrostatic pressure. Moreover, an *E. coli fabF* strain was as susceptible to elevated pressure as wild-type cells. It is proposed that the SS9 *fabF* product, β-ketoacyl–acyl carrier protein synthase II has evolved novel pressure-responsive characteristics which facilitate SS9 growth at high pressure.

Increased hydrostatic pressure and reduced temperature elicit similar physical effects on the phase and fluidity properties of membrane lipids. As growth temperature is lowered or growth pressure is elevated, biological membranes undergo a reversible change from a fluid disordered state to a nonfluid ordered state (22, 29). Such changes would seem particularly problematic for life in deep ocean environments. Many poikilothermic organisms respond to decreased temperature and/or increased hydrostatic pressure by altering their membrane lipid composition, apparently to tailor the membrane with physical properties suited to prevailing environmental conditions. Such changes may include increases in fatty acyl chain unsaturation, decreases in mean chain length, increased methyl branching, *cis*/*trans* isomerization of unsaturated fatty acid double bonds, increases in the ratio of anteiso branching relative to iso branching, acyl chain shuffling between the phospholipid *sn*-1 and *sn*-2 positions, or phospholipid headgroup composition changes (22, 25, 36, 39). Among these changes, the most common change observed among deep-sea bacteria involves the incorporation into membrane phospholipids of increased proportions of unsaturated fatty acids (UFAs) (1, 11, 12, 47). UFAs adopt a more expanded conformation, pack less compactly, and possess lower melting temperatures than their saturated counterparts, allowing for their less orderly alignment within membrane phospholipids (22). This response presumably functions to offset the membrane gelling effects of increased pressure or decreased temperature, thereby maintaining biological membranes in a fluidity or phase optimized for growth. In addition to producing increased amounts of monounsaturated fatty acids (MUFAs) such as palmitoleic acid (16:1n-9) and *cis*-vaccenic acid (18:1n-11), many deep-sea bacteria also produce substantial quantities of omega-3 polyunsaturated fatty acids (PUFAs) at high pressure (1, 12, 47, 48).

The way in which a bacterial species modulates its membrane fatty acid unsaturation depends on its method of UFA synthesis and involves either an aerobic or anaerobic mechanism. In gram-positive bacteria and cyanobacteria, a double bond is introduced into a preexisting fatty acid chain by means of an oxygen-dependent desaturase system (13, 44). In contrast, gram-negative bacteria employ an anaerobic pathway, whereby at a discrete point in the elongation cycle of fatty acid biosynthesis a *cis* double bond is introduced (38). Some bacteria utilize both the anaerobic and aerobic desaturation pathways (34).

In *Escherichia coli*, where the mechanisms of anaerobic UFA synthesis have been well characterized, the response to temperature downshift entails the restructuring of membrane fatty acid composition by increasing the amount of 18:1 and decreasing the amount of palmitic acid (16:0) incorporated into membrane phospholipids (33). This regulation is an intrinsic property of the fatty acid biosynthetic enzyme β-ketoacyl-ACP (acyl carrier protein) synthase II (KAS II), product of the *fabF* gene (10, 14, 17). *E. coli* KAS II is one of three isozymes that catalyze the elongation of fatty acyl chains. Specifically, KAS II catalyzes the elongation of palmitoleoyl-ACP (16:1) to *cis*-vaccenoyl-ACP (18:1) in UFA synthesis. Neither mRNA nor protein synthesis is required for increased 18:1 production at reduced temperature, indicating that thermal modulation of fatty acid production is controlled at the level of KAS II activity (17). Indeed, the elongation activity of KAS II is temperature dependent, exhibiting decreased K_m for palmitoleoyl-ACP and increased relative V_{max} at reduced temperatures (16, 18). *E. coli fabF* mutants possess a deficiency in 18:1 synthesis as well as a loss of 18:1 thermal regulation (19).

Because of the critical role of *fabF* (KAS II) in thermal modulation of UFA production in *E. coli*, we predicted that a similar role exists for KAS II in the deep-sea bacterium *Pho-*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Photobacterium profundum		
SS9	Wild type	11
DB110	Lac$^-$ Rif^r; SS9 derivative	8
EA40	*fabF* disruption mutant; DB110 derivative	This study
E. coli		
SJ16	*panD2 zad-220*::Tn*10*	24
MR86	*fabF*::Km^r insertion; SJ16 derivative	31
MG1655	λ^-	21
1100	λ^- *glnV44 rfbD1 endA1 spoT1 thi-1*	15
Plasmids		
pCR2.1	Vector for cloning PCR products, Km^r	Invitrogen
pEA39	740-bp internal fragment of SS9 *fabF* in pCR2.1, Km^r	This study
pMUT100	Mobilizable suicide plasmid, Km^r	7
pEA40	740-bp internal fragment of SS9 *fabF* in pMUT100, Km^r	This study
pEA401	SS9 genomic library clone containing *fabF* and flanking DNA, Km^r	This study
pKT231	Broad-host-range vector, Km^r Sm^r	3
pEA44	SS9 *fabF* in pKT231, Sm^r	This study

tobacterium profundum strain SS9 and furthermore that SS9 KAS II is required for 18:1 piezoregulation and piezoadaptation. Such properties could distinguish the SS9 KAS II enzyme from its homologue in bacteria which have not evolved adaptations for substantially elevated pressures. Consistent with this hypothesis, recent studies in our lab using the fatty acid biosynthesis inhibitor cerulenin and mutants altered in the abundance of various UFAs indicated that MUFAs but not PUFAs are required for high-pressure and low-temperature adaptation in *P. profundum* strain SS9 (1). Here we report the cloning of the SS9 *fabF* gene, the engineering of an SS9 mutant harboring a disruption in *fabF*, and the growth characteristics and fatty acid analysis of this mutant. In addition, an *E. coli fabF* mutant and parental strain were compared with respect to fatty acid composition and growth ability as a function of pressure and temperature. Our results indicate that SS9 *fabF* has evolved novel characteristics critical to pressure sensing and high-pressure adaptation.

MATERIALS AND METHODS

Strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. profundum* strains were routinely cultured at 15°C, 1 atm (=0.101 MPa) in 2216 marine medium (28 g/liter; Difco Laboratories, Detroit, Mich.). All temperature experiments (15 and 4°C) were conducted aerobically in 2216 marine medium for *P. profundum* strains unless otherwise indicated. *E. coli* strains SJ16 and MR86 were graciously provided by John E. Cronan, Jr. *E. coli* strains were routinely cultured in Luria-Bertani (LB) media (30). For solid media, agar (Difco Laboratories) was added at 17 g/liter. The antibiotics kanamycin (50 μg/ml for *E. coli*; 200 μg/ml for *P. profundum* strains), streptomycin (50 μg/ml for *E. coli*; 150 μg/ml for *P. profundum* strains), rifampin (100 μg/ml), and tetracycline (12 μg/ml) were added to the media when required. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Exogenous supplementation of marine media with fatty acids (i.e., Na^+ salts) is not possible due to insolubility problems resulting from the presence of a high concentration of divalent cations. Tween compounds, however, are highly soluble in marine media and have been used for exogenous supplementations (1). Oleic acid (18:1) in the form of Tween 80 (polyoxyethylenesorbitan monooleate; Sigma) was added at a final concentration of 0.025% (vol/vol).

High-pressure growth studies. High-pressure cultivation of *P. profundum* strains for growth studies or fatty acid analysis were conducted as previously described (1). Cultivation of *E. coli* strains at elevated pressures was similarly performed. Each *E. coli* culture was grown to stationary phase in LB medium at 1 atm. Stationary-phase cultures were diluted 1/400 into LB medium buffered with HEPES (100 mM, pH 7.5; Sigma) containing 22 mM glucose (Sigma). The diluted culture was used to fill 4.5- or 15-ml polyethylene transfer pipettes (Sarneo, San Fernando, Calif.). Pipettes were filled completely and then heat sealed with a hand-held heat sealing clamp (Harwil, Oxnard, Calif.). Cells were incubated at 0.1 or 30 MPa (1 or 300 atm, respectively) of hydrostatic pressure at 37°C (unless otherwise stated) in stainless steel pressure vessels equipped with quick-connect fittings for rapid decompression and recompression as described by Yayanos and Van Boxtel (50).

DNA sequencing and analysis. Double-stranded DNA sequencing reactions were performed using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, Calif.) and run on an Applied Biosystems 373A DNA sequencer. Global similarity searches were performed using the BLAST network service (2). Multiple alignments were performed using ClustalW (23) in conjunction with GeneDoc software (35).

***fabF* insertional inactivation mutagenesis.** An internal fragment of the SS9 *fabF* gene was initially PCR amplified from *P. profundum* strain DB110 genomic DNA, using primers fabF1 5′-GTGTCCAAGCGTCGTGTAGTTGT-3′ and fabF4 5′-GCGTGTTCGTACTCTTCAAG-3′. These primers were created by analysis of conserved regions from alignment of the *E. coli* and *Vibrio harveyi fabF* gene sequences in GenBank. The resultant 740-bp PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.), generating pEA39, and sequenced using M13R and T7 primers to confirm the identity of the product. The PCR product was then subcloned into the mobilizable suicide plasmid pMUT100 (Kan^r) (7) as an *Eco*RI fragment yielding pEA40. Bacterial conjugations were used to transfer plasmid pEA40 from *E. coli* into *P. profundum* strain DB110 as described by Chi and Bartlett (8). Kan^r exconjugants arose from integration of plasmid pMUT100 into the chromosome of *P. profundum* strain DB110 in a single crossover event giving rise to two deleted copies of the gene, one copy with a 5′ deletion and the other with a 3′ deletion. These experiments yielded *P. profundum* strain EA40 containing a disruption in the *fabF* gene and was confirmed by Southern blot analysis (40). Genomic DNA from *P. profundum* strains EA40 and DB110 was digested with restriction enzymes *Bgl*II, *Hind*II, *Hpa*I, or *Pst*I and probed using the *fabF* internal fragment harbored on pEA39 labeled with [α-^{32}P]dCTP by random priming (Life Technologies, Gaithersburg, Md.).

Isolation of the SS9 *fabF* gene. A *P. profundum* SS9 genomic library (6) was screened using an internal fragment of SS9 *fabF* in order to identify recombinant library clones harboring the SS9 *fabF* gene. Colony hybridizations were performed according to standard protocols (40). Plasmid DNA was isolated from positively hybridizing clones and subsequently sequenced. The SS9 *fabF* gene was PCR amplified from strain DB110 genomic DNA, using primers fabF-F(5′-CT AGTAATGGCTCTTGAAGAAG-3′) and fabF-R(5′-AATTCTTCACGGCAA AATTA-3′). The PCR product was cloned into pCR2.1 and subsequently subcloned into pKT231 (3) as a *Hind*III-*Xho*I fragment, yielding pEA44.

Fatty acid analyses. Extraction and analysis of fatty acid methyl ester preparations via combined gas chromatography-mass spectrometry were performed as previously described (1). Compounds were identified by comparison of retention times with those of known standards (Sigma) as well as sample mass spectra data compared to the Hewlett-Packard G1034C MS ChemStation software NBS75K library containing mass spectra data of 75,000 known compounds. Fatty acids are denoted as number of carbon atoms:number of double bonds. Inner and outer membrane separation and fatty acid analysis on *P. profundum* strain EA40 grown in the presence of 0.025% Tween 80 at 28 MPa (9°C) were performed in order to show that the 18:1 from Tween 80 was incorporated into membrane phospholipids as previously described (1).

RNA isolation and Northern analyses. Total RNA was extracted from *P. profundum* strains grown at various temperatures and pressures using the RNAzol B method (Tel-Test, Inc., Friendswood, Tex.). Equivalent amounts of RNA (10 μg) were electrophoresed through 1.2% formaldehyde agarose, blotted onto a Magnacharge nylon transfer membrane (MSI, Westboro, Mass.), and subjected to Northern analysis using the PCR product contained within pEA39 as the hybridization probe labeled with [α-^{32}P]dCTP by random priming (Life Technologies). Hybridizations were conducted using QuikHyb hybridization solution (Stratagene, La Jolla, Calif.) at a temperature of 64°C.

Nucleotide sequence accession number. The sequence of *P. profundum* strain SS9 *fabF*, along with the partial sequences of *acpP* and *pabC*, is deposited in the GenBank database under accession no. AF188707.

RESULTS

Isolation and analysis of the *P. profundum* strain SS9 *fabF* gene. Using PCR primers designed from conserved portions of known *fabF* sequences (*E. coli* and *V. harveyi*), a 740-bp product was amplified from *P. profundum* strain DB110 genomic DNA and subcloned into pCR2.1 (Invitrogen) to generate pEA39, and its nucleotide sequence was determined. Global similarity searches using gapped-BLAST (2) indicated that the insert DNA present on pEA39 contained an open reading frame with a high degree of similarity to FabF proteins from *V. harveyi* (E value of 5E-107) and from *E. coli* (E value of 2E-101). The entire SS9 *fabF* gene was then isolated following colony blot hybridization of a SS9 genomic library (6), using the insert on pEA39 as a hybridization probe. From all of the three positively hybridizing clones obtained in this way, sequence analysis indicated the presence of *fabF* DNA. One of the three plasmids chosen for further study, pEA401, contained a 7.5-kb insert. Sequence analysis of pEA401 revealed a gene organization flanking *fabF* identical to that present in *E. coli*, *V. harveyi*, and *Pseudomonas aeruginosa* (27, 31, 41); in particular it contained the 3′ end of *acpP*, followed by *fabF* and *pabC*. Further sequence downstream of SS9 *pabC* contained on pEA401 was not obtained.

The SS9 *fabF* gene was found to display a high degree of similarity and identity to both the *E. coli* and *V. harveyi fabF* genes at both nucleotide and deduced amino acid sequence levels. At the DNA level, the SS9 *fabF* sequence was 73 and 69% identical to *fabF* sequences of *V. harveyi* and *E. coli*, respectively. In addition, possible Rho-independent terminator sequences are present within the SS9 *acpP-fabF* intergenic region (ending 70 bp upstream of the *fabF* GTG start) and the *fabF-pabC* intergenic region (ending 67 bp upstream of the *pabC* ATG start). These structures are similar in location to putative terminators identified upstream of *fabF* in *V. harveyi*, *P. aeruginosa*, and *E. coli* and downstream of *fabF* in *V. harveyi* and *P. aeruginosa*. The predicted amino acid sequence of SS9 KAS II (FabF) was 79 (90) and 76% (88%) identical (similar) to KAS II sequences of *V. harveyi* and *E. coli*, respectively. No dramatic differences in pI value or amino acid composition were observed between the KAS II enzymes analyzed.

***fabF* transcript analysis.** To identify and determine the sizes of SS9 *fabF* transcripts, Northern blotting was performed (Fig. 1). Northern blot analysis of strain DB110 revealed two *fabF* probe-specific transcripts, one major transcript at approximately 1.9 kb and one minor transcript of approximately 1.5 kb. Zhang and Cronan (52) examined the expression of the *E. coli fabF* gene and identified two transcripts identical in size to those produced by SS9. Their analysis revealed that the 1.5-kb transcript was a *fabF*-specific mRNA, whereas the 1.9-kb transcript was most likely the product of cotranscription of *fabF* and the upstream *acpP* gene. In SS9, neither of these transcripts exhibited differential abundance at decreased temperature or elevated pressure.

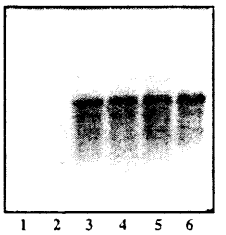


FIG. 1. Northern analysis of *fabF* expression in *P. profundum* strains EA40 (*fabF*) and DB110 (wild type). Cells were grown to late exponential phase for total RNA isolation and probed using internal fragment of SS9 *fabF* as described in Materials and Methods. Lanes: 1, EA40 at 4°C; 2, EA40 at 15°C; 3, DB110 at 4°C; 4, DB110 at 15°C; 5, DB110 at 28 MPa (9°C); 6, DB110 at 0.1 MPa (9°C).

Isolation of an SS9 *fabF* mutant. To explore the in vivo regulation and function of KAS II in SS9, a *fabF* mutant was constructed. The isolation of an SS9 *fabF* mutant followed the introduction of an internal fragment of the SS9 *fabF* gene, harbored on pEA40, into strain DB110 by conjugal transfer. The ColE1 replicon on this plasmid is mobilizable but replication impaired in SS9 and thus serves as a suicide plasmid allowing selection of plasmid integrants into targeted regions of the chromosome, i.e., those cloned on the plasmid. Kan^r exconjugants were screened initially by examining their fatty acid profiles at 4°C compared to 15°C. One exconjugant displaying greatly reduced levels of 18:1 at 4°C was designated EA40 and saved for further study. The creation of a *fabF* insertion mutation in EA40 was verified by both Southern and Northern blotting. Southern analysis revealed the replacement of specific restriction endonuclease fragments in strain DB110 by DNA approximately 6.3 kb larger in the case of strain EA40 (data not shown). In addition, no *fabF*-specific transcripts were detected in total RNA extracted from mutant EA40 grown under various temperature conditions (Fig. 1), providing additional verification of the insertional inactivation of *fabF* in this strain.

Characterization of the *fabF* mutant as a function of temperature. The percentages and types of fatty acids produced by strains DB110 and EA40 under different growth conditions are listed in Table 2. When examined at a temperature of 15°C, mutant EA40 exhibited greatly diminished 18:1 levels relative to the parental strain DB110 (4 versus 9.9%, respectively). Moreover, when examined at the reduced temperature of 4°C, mutant EA40 displayed further reductions in 18:1 content, contrary to that observed in strain DB110. At 4°C strain, DB110 produced approximately 10.3% 18:1, whereas the *fabF* mutant produced only 0.7% 18:1.

In concert with the reduction of 18:1, the *fabF* mutant exhibited elevated levels of the saturated fatty acid 14:0 and the MUFA 14:1 and also upregulated the abundance of the omega-3 PUFA all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA; 20:5). Specifically at 4°C, mutant EA40 also displayed dramatically reduced 16:0 and 16:1 levels relative to parental strain DB110. We have previously noted that chemical mutants of SS9 or drug treatments which reduce the amount of MUFAs produced by SS9 result in increased 14:0 and EPA levels (1). We do not yet understand how it is that a *fabF* mutation induces such pleiotropic effects on fatty acid composition. As previously reported for *P. profundum* strain SS9, differences in iso-16:0 content reflect differences in phase of growth at time of harvesting (1). Overall, the ratios of unsaturated to saturated fatty acids (UFA/SFA) of mutant EA40 were compara-

TABLE 2. Fatty acid composition of *P. profundum* strains as a function of varying temperature and pressure[a]

Fatty acid species	Mean wt% fatty acid species ± SD							
	Wild type				*fabF*			
	Temp (°C)		Pressure (MPa)		Temp (°C)		Pressure (MPa)	
	15	4	0.1 MPa	28	15	4	0.1 MPa	28
12:0	1.8 ± 0.9	2.7 ± 1.0	1.5 ± 0.1	2.2 ± 0.6	3.2 ± 0.5	4.3 ± 1.4	4.0 ± 1.3	3.4 ± 0.6
14:0	4.9 ± 1.4	3.3 ± 0.8	5.0 ± 0.3	1.6 ± 0.7	13.2 ± 1.7	18.6 ± 3.2	19.6 ± 1.3	14.0 ± 1.8
14:1	1.2 ± 0.8	2.5 ± 0.5	0.8 ± 0.1	0.7 ± 0.1	4.6 ± 1.1	6.3 ± 0.8	4.8 ± 0.9	3.1 ± 0.8
Iso-16:0	9.8 ± 3.1	0.2 ± 0.1			1.2 ± 1.3			
16:0	21.7 ± 2.8	20.2 ± 3.8	21.4 ± 1.5	17.5 ± 3.2	19.2 ± 2.6	10.5 ± 2.2	19.7 ± 1.3	16.2 ± 1.2
16:1	41.3 ± 8.3	48.8 ± 6.8	64.1 ± 1.5	48.7 ± 9.6	41.5 ± 4.8	39.3 ± 6.8	31.4 ± 1.1	44.9 ± 0.7
12-OH	3.0 ± 1.2	3.4 ± 1.3	0.6 ± 0.1	2.0 ± 0.8	3.8 ± 1.0	4.1 ± 1.2	3.4 ± 1.2	5.2 ± 0.1
18:0	1.1 ± 0.4	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	1.9 ± 0.4	0.1 ± 0.0	0.7 ± 0.1	0.4 ± 0.1
18:1	9.9 ± 2.6	10.3 ± 1.7	3.6 ± 0.1	16.2 ± 1.6	4.0 ± 0.8	0.7 ± 0.1	1.1 ± 0.3	1.1 ± 0.3
20:5	5.3 ± 1.1	8.4 ± 1.1	2.7 ± 0.1	11.0 ± 0.8	7.1 ± 1.7	15.9 ± 2.1	15.1 ± 3.8	11.4 ± 1.5
UFA/SFA ratio[b]	1.95	2.65	2.53	3.57	1.53	1.91	1.20	1.77

[a] Data represent values derived from triplicate samples harvested in late-exponential-phase growth; 15 and 4°C cultures were grown aerobically at 0.1 MPa; 0.1- and 28-MPa cultures were grown at 9°C in 2216 marine medium containing 22 mM glucose buffered with 100 mM HEPES.
[b] UFAs, 14:1, 16:1, 18:1, and 20:5; SFAs, 12:0, 14:0, 16:0, and 18:0.

ble to those for strain DB110 under various temperature conditions (Table 2).

The growth characteristics of the *fabF* mutant at two temperatures are shown in Fig. 2A. At both 15 and 4°C, mutant EA40 displayed growth rates and yields essentially identical to those for parental strain DB110. These results are consistent with findings in *E. coli*, where no growth defect at reduced temperatures have been ascribed to *fabF* strains (43).

Characterization of the *fabF* mutant as a function of pressure. Table 2 displays the fatty acid profiles of mutant EA40 and strain DB110 at 0.1 MPa (9°C) and 28 MPa (9°C). At 0.1 MPa (9°C), mutant EA40 exhibited markedly reduced 18:1 and 16:1 levels and substantially increased 14:0, 14:1, and EPA levels compared to strain DB110. Similarly, at elevated pressure the most dramatic alterations in fatty acid content of mutant EA40 relative to strain DB110 included severe reduction in 18:1 content (1.1 versus 16.2%, respectively) and elevated 14:0 content. These high-pressure EA40 values are similar to those of the mutant grown at 4°C (0.1 MPa). However, at the elevated pressure of 28 MPa (9°C), mutant EA40 did not upregulate EPA production as it did at 4°C. EPA content actually decreased upon a shift in growth pressure from 0.1 MPa (9°C) to 28 MPa (9°C) in mutant EA40 (15.1 versus 11.4%, respectively). This is in contrast to strain DB110, wherein EPA content increased from 2.7 to 11% upon pressurization to 28 MPa. Moreover, the UFA/SFA ratios of mutant EA40 at 28 and 0.1 MPa (1.77 and 1.20, respectively) were substantially lower than those for strain DB110 (3.57 and 2.53, respectively).

The growth characteristics of mutant EA40 at two pressures are shown in Fig. 2B. The effect of *fabF* disruption on cell growth was detrimental at elevated pressure. At 0.1 MPa (9°C), the mutant and parental strains exhibited essentially identical growth abilities. However, at an elevated pressure of 28 MPa (9°C), mutant EA40 displayed an extended lag phase, decreased growth rate, and reduced overall yield in comparison to strain DB110. These results represent the first identification of a growth phenotype associated with sole disruption or mutation of the *fabF* gene.

It is conceivable that the difference between temperature and pressure growth and fatty acid regulation observed in strain EA40 is the result of an anaerobic effect (which pressure cultivation necessitates). In other words, high-pressure cultivation conditions alone may be responsible for the observed

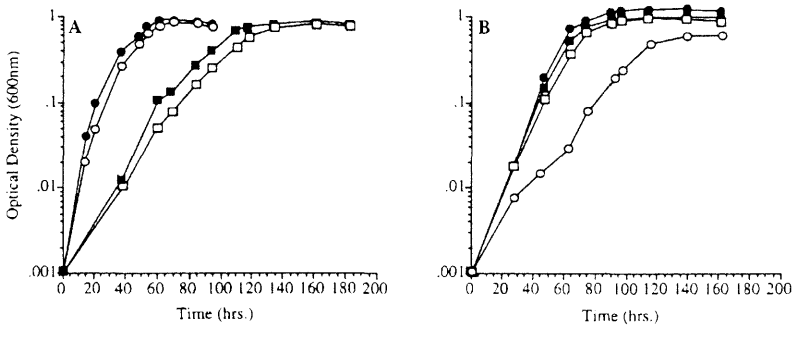


FIG. 2. Growth characteristics of *fabF* disruption mutant *P. profundum* strain EA40 and strain DB110 as a function of temperature (A) and pressure (B). (A) ●, DB110 at 15°C; ○, EA40 at 15°C; ■, DB110 at 4°C; □, EA40 at 4°C. (B) ●, DB110 at 28 MPa; ○, EA40 at 28 MPa; ■, DB110 at 0.1 MPa; □, EA40 at 0.1 MPa.

DHA. Such isolates have been found to be particularly prevalent in high-pressure, low-temperature deep-sea habitats and permanently cold marine environments (DeLong & Yayanos, 1986; Nichols *et al.*, 1993; Yano *et al.*, 1997). The enrichment of PUFA-producing strains from these environments has led to speculation that PUFA synthesis is an important adaptation for countering the effects of elevated hydrostatic pressure and low temperature on membrane fluidity or phase. In strains which have been analysed, PUFA synthesis undergoes temperature-dependent and, for deep-sea isolates, pressure-dependent regulation. Typically, as cultivation temperature is decreased, and/or pressure increased, PUFA incorporation into membrane phospholipids is enhanced. This modulation is thought to maintain appropriate membrane physical structure (Russell & Nichols, 1999). However, for at least one psychrotolerant piezophilic (high-pressure-adapted) deep-sea bacterium, *Photobacterium profundum* strain SS9, growth at high pressure and low temperature does not depend upon PUFA synthesis (Allen *et al.*, 1999).

A variety of bacterial fatty acid biosynthetic mechanisms exist which vary with taxonomic identity and class of fatty acid product (Cronan & Rock, 1996; Fujii & Fulco, 1977; Rawlings, 1998). Some reports have suggested bacterial omega-3 PUFA production to be mediated by undefined desaturases (Russell & Nichols, 1999; Tanaka *et al.*, 1999; Watanabe *et al.*, 1997). However, sequence studies of bacterial genes required for PUFA biosynthesis have gradually led to a reappraisal of this view. Initial insight into the genetics of bacterial PUFA synthesis was gained by the cloning and analysis of a 38 kbp genomic fragment from the EPA producer *Shewanella* sp. strain SCRC-2738 (Yazawa, 1996). Five *Shewanella* genes, designated ORFs 2, 5, 6, 7 and 8, were shown to be necessary for recombinant EPA synthesis in *Escherichia coli* and in the marine cyanobacterium *Synechococcus* sp. (Takeyama *et al.*, 1997; Yazawa, 1996). A subsequent analysis of the predicted amino acid sequences of the products of these genes indicated that they are most related to microbial polyketide synthase (PKS) complexes and fatty acid synthase (FAS) enzymes (Metz *et al.*, 2001). PKS enzymes catalyse the synthesis of a wide array of complex natural products by the repetitive condensation and processing of simple monomeric substrates in a process resembling fatty acid synthesis (Hopwood & Sherman, 1990). In addition to the *Shewanella* sp. SCRC-2738 sequences, related genes partially responsible for PUFA production have been analysed from the DHA-producing bacterium *Moritella marina* strain MP-1 (formerly *Vibrio marinus*) (Tanaka *et al.*, 1999) and from a DHA-producing thraustochytrid marine protist belonging to the genus *Schizochytrium* (Metz *et al.*, 2001).

Recently, Metz *et al.* (2001) reported biochemical analyses of PUFA production in *E. coli* strains harbouring *Shewanella* sp. SCRC-2738 DNA and in the *Schizochytrium* species. Consistent with the examination of enzyme domains, isotopic labelling studies provided compelling support for a PKS-like pathway of PUFA synthesis in both systems studied (Metz *et al.*, 2001). However, whereas considerable advances have been made towards a mechanistic understanding of microbial PUFA production, very little is known about the regulation of PUFA synthesis. The present study reports the cloning and molecular analysis of genes responsible for EPA synthesis, herein referred to as *pfa* (polyunsaturated fatty acid) genes, from the deep-sea bacterium *P. profundum* strain SS9. Transcriptional regulation of the SS9 *pfaA–D* genes was analysed as a function of varying temperature and hydrostatic pressure, and SS9 mutants containing polar insertions in two *pfa* genes were used to verify gene function and to help delineate the transcriptional organization of the *pfa* operon. Furthermore, an SS9 mutant that overproduces EPA was characterized and found to upregulate *pfa* gene transcription.

METHODS

Strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. *P. profundum* strains were routinely cultured at 15 °C, 1 atmosphere (1 atm = 0·101 MPa) in 2216 Marine Medium (28 g litre; Difco). All temperature experiments (15 and 4 °C) were conducted aerobically in 2216 Marine Medium. For solid media, agar (Difco) was added at 17 g l^{-1}. Antibiotics kanamycin (50 μg ml^{-1} for *E. coli*, 200 μg ml^{-1} for *P. profundum* strains), rifampicin (100 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}) were added to media when required. All antibiotics were obtained from Sigma. High-pressure cultivation of *P. profundum* strains for growth studies, fatty acid analyses and extraction of RNA was as previously described (Allen *et al.*, 1999).

Construction of an SS9 genomic fosmid library. Preparation of a genomic library of *P. profundum* strain DB110 in the pFOS1 vector (Kim *et al.*, 1992) was performed as described by Stein *et al.* (1996). Briefly, high-molecular-mass genomic DNA was isolated from strain DB110 by lysis and extraction in agarose plugs and subsequently digested with *Sau*3A. DNA fragments between 35 and 45 kbp were purified following gel electrophoresis using the GeneClean Spin Kit (Bio 101). Size-selected DNA was ligated into the *Bam*HI site of pFOS1 vector arms, *in vitro* packaged using Gigapack III XL packaging extracts (Stratagene), and transfected to *E. coli* DH10B. Approximately 960 fosmid clones were individually picked into 96-well microtitre dishes containing LB medium plus 20 μg chloramphenicol ml^{-1} and 10% (v/v) glycerol and stored at −80 °C until further analysis.

DNA hybridizations and fosmid clone manipulations. Fosmid library clones were replicated onto MagnaCharge (MSI, Westboro, MA, USA) nylon filters and hybridized to DNA probes obtained from internal fragments of SS9 *pfa* genes using standard protocols (Sambrook *et al.*, 1989). Previously we reported the cloning of an 885 bp internal fragment of the SS9 *pfaA* gene (designated ORF 3/4 in the previous report) making use of arbitrary primers derived from the *Shewanella* sp. SCRC-2738 EPA gene sequence (GenBank accession no. U73935) (Allen *et al.*, 1999). An internal fragment of SS9 *pfaD* was subsequently obtained using primers ORF9-3 (5′-CGTTGAAGCATCAGCTTTCTT-3′) and ORF9-2 (5′-TACGCCCATCTCGAACATATC-3′) derived from SCRC-2738 EPA gene sequence. The resultant 571 bp PCR product contained a high degree of similarity, 79% identity at the DNA level, to the SCRC-2738 *pfaD* homologue (designated ORF7; Yazawa,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Photobacterium profundum		
SS9	EPA$^+$; wild-type strain	DeLong *et al.* (1997)
DB110	Lac$^-$ Rif^r; SS9 derivative	Chi & Bartlett (1993)
EA2	EPA-overproducing chemical mutant	Allen *et al.* (1999)
EA10	*pfaA* insertion mutant (EPA$^-$)	Allen *et al.* (1999)
EA50	*pfaD* insertion mutant (EPA$^-$)	This study
Shewanella sp.		
SC2A	EPA$^+$	DeLong *et al.* (1997)
Plasmids		
pFOS1	Fosmid cloning vector	Kim *et al.* (1992)
pFOS8E1	Fosmid clone containing SS9 *pfaA–D*	This study
pMUT100	Mobilizable suicide plasmid, Km^r	Brahamsha (1996)
pCR2.1	PCR cloning vector, Km^r	Invitrogen
pDP18	*In vitro* transcription vector	Ambion

1996)). Filters were initially probed using the internal fragment of SS9 *pfaA*, stripped of bound probe, and reprobed with the *pfaD* probe. Of the 42 clones to which both probes hybridized, fosmid 8E1 (the clone with the smallest insert size of 33·1 kbp) was selected for further analysis and sequencing. Fosmid DNA was purified using the Qiagen Plasmid Midi kit and digested with *Not*I to excise the cloned insert. A subclone library of fosmid 8E1 was prepared by digesting the *Not*I insert with *Sau*3A, size selecting for 1–2 kbp fragments, and ligation into *Bam*HI-digested pUC18. Plasmid minipreparations of the fosmid 8E1:pUC18 subclones were prepared and sequenced using pUC18-specific primers flanking the cloned inserts.

DNA sequencing and analysis. Double-stranded DNA sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and run on an Applied Biosystems model 373 DNA sequencing system. Initial sequence analysis and contig assembly was performed using Sequencher 3.1 software (Gene Codes Corp.). Additional sequence needed to fill in contig gaps was obtained using sequence-specific oligonucleotide primers and sequencing of PCR products. Global similarity searches were performed using the BLAST network service (Altschul *et al.*, 1997). Multiple alignments were performed using ClustalW (Higgins & Sharp, 1988) in conjunction with GeneDoc software (Nicholas & Nicholas, 1997). Domain arrangement analyses of the predicted amino acid sequences of the *pfa* genes were conducted using the ProDom database of protein domain families (Corpet *et al.*, 1999), the Conserved Domain Database with Reverse Position Specific BLAST (Altschul *et al.*, 1997) and the ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/hits_motifscan).

Insertional inactivation mutagenesis. Following previously published procedures (Allen & Bartlett, 2000; Allen *et al.*, 1999), insertional inactivation mutagenesis was performed targeting the SS9 *pfaD* gene. Briefly, an internal fragment of *pfaD* was PCR-amplified using primers ORF9-3 and ORF9-2 (sequences listed above; amplified region corresponding to 23708–24278 of GenBank accession no. AF409100), cloned into the pCR2.1-TOPO vector (Invitrogen) and subcloned into the mobilizable suicide vector pMUT100 (Brahamsha, 1996). The *pfaD*:pMUT100 construct was introduced into SS9 from *E. coli* by conjugal transfer as described by Chi & Bartlett (1993). Kanamycin-resistant exconjugants arose from plasmid integration into the SS9 chromosome in a single crossover event yielding strain EA50, with *pfaD* insertionally inactivated. The site of plasmid insertion was verified by PCR amplification of a portion of the *pfaD* gene using primers located upstream of the insertion site together with pMUT100-specific primers.

Cloning of phosphopantetheinyl transferase (PPTase) genes. The *Bacillus subtilis sfp* gene (GenBank accession no. X63158) was isolated from *B. subtilis* by PCR amplification of the complete gene using primers 5′-TGCTGAATTATGCTGTGGCAAGGC-3′ and 5′-GCTTCTCGAAATGATGTTCCCCGG-3′. In attempts to isolate PUFA synthase PPTase gene sequences, degenerate PCR primers were designed to conserved PPTase motifs found by alignment of known PPTase protein sequences including the PUFA synthase PPTase of *Shewanella* sp. strain SCRC-2738 (ORF2; GenBank accession no. U73935). Template DNA isolated from a variety of EPA-producing bacterial strains was employed; however, only DNA from the psychrotolerant, moderate piezophile *Shewanella* sp. strain SC2A (DeLong *et al.*, 1997) yielded an amplification product of the expected size relative to the SCRC-2738 sequence. Using forward primer 5′-GGCGATAAAGGYAARCCK-3′ and reverse primer 5′-CAACGHTCRATRTCWCCACC-3′ a 212 bp product was sequenced whose deduced amino acid sequence showed 48% identity and 61% similarity over 72 amino acids to the SCRC-2738 ORF2 product. In order to obtain flanking DNA sequence, primers internal to the SC2A PPTase sequence were designed for inverse PCR (Ochman *et al.*, 1990) and an SC2A cosmid library (Chilukuri & Bartlett, 1997) was screened using the SC2A PPTase internal fragment. Colony hybridizations were performed according to standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was isolated from positively hybridizing clones and sequenced. The complete sequence of the *Shewanella* sp. strain SC2A PPTase is deposited under GenBank accession no. AF467805.

Fatty acid analyses. Extraction and analysis of fatty acid methyl ester preparations via combined gas chromatography-mass spectroscopy were performed as previously described (Allen *et al.*, 1999). Fatty acids are denoted as number of carbon atoms:number of double bonds.

RNA isolation and ribonuclease protection assay (RPA) analyses. Total RNA was extracted from mid-exponential-phase *P. profundum* strains grown at various temperatures and pressures using the RNAzol B method (Tel-Test, Friendswood, TX, USA). [α-^{32}P]UTP-labelled RNA probes were synthesized using the T7 RNA polymerase MAXIscript *in vitro* transcription kit (Ambion, Austin, TX) and RPAs were performed using the RPA III kit according to the manufacturer's protocols (Ambion). Probe template preparations involved the PCR amplification of fragments of SS9 *pfa* genes, cloning of PCR products into the pCR2.1-TOPO vector and subsequent subcloning of inserts into the pDP18 transcription vector (Ambion). The sizes of full-length probes and protected fragments were as follows (the positions of *pfa* RPA probes are indicated with reference to GenBank accession no. AF409100): *pfaA* 377/268 bp (9422–9689), *pfaB* 465/322 bp (15332–15653), *pfaC* 477/344 bp (19133–19476), *pfaD* 477/344 bp (23385–23728), *pfaA/B* 520/264 bp (15020–15283), *pfaB/C* 516/260 bp (17129–17388), *pfaC/D* 511/255 bp (23052–23306). RNA probes were purified from denaturing acrylamide continuous gels, co-precipitated with 10 µg total RNA and hybridized overnight at 45 °C. Following RNase treatment, protected fragments were separated on denaturing acrylamide gels (5% acrylamide/8 M urea) against undigested probe and appropriate controls. For detection of probes and protected fragments, gels were transferred to filter paper and exposed to X-ray film overnight. [α-^{32}P]UTP-labelled RNA Century Markers (Ambion) were used as size standards.

Primer extension analysis. Primer extension analysis of the SS9 *pfaA–D* genes was performed using the Primer Extension System-AMV Reverse Transcriptase kit (Promega). RNA was isolated from SS9 strain DB110 using the RNAzol B method. Multiple *pfa* extension primers were tested; however, only *pfaA* primers 5′-GCCATGCCAACAATCGCAAT-3′ (position 7529–7548) and 5′-GTTGCGATTAGGCAACTGGTGA-3′ (position 7379–7400) yielded extension products. Primers were end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labelled primers were annealed to 40 µg SS9 RNA and extended using AMV-RT. For DNA sequencing, *pfaA* plasmid templates were constructed using DNA amplified with primers 5′-AACCTCTTGCTCCAGTGATTG-3′ and 5′-TATCACGGTCGTATGTTTCCG-3′ (amplified fragment position 7067–7856), cloned into pCR2.1, and sequenced using the labelled primers used for primer extension in conjunction with the *fmol* DNA Cycle Sequencing System Kit (Promega). DNA fragments were resolved on 8 M urea/8% polyacrylamide gels.

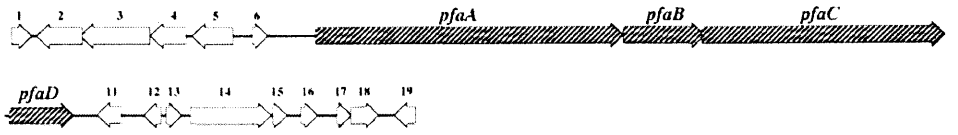
RESULTS

Isolation and sequence analysis of the *P. profundum* strain SS9 *pfa* gene cluster

The isolation of SS9 genes required for EPA synthesis (termed *pfaA–D*; *p*olyunsaturated *f*atty *a*cid) first involved the generation of a large insert fosmid library of SS9 genomic DNA and the subsequent probing of this library using a partial fragment of the SS9 *pfaA* gene previously isolated (Allen *et al.*, 1999). Based on omega-3 PUFA synthase sequence information from *Shewanella* sp. SCRC-2738 (GenBank accession no. U73935) and *Moritella marina* (GenBank accession no. AB025342), the size of the SS9 gene cluster was predicted to be approximately 18 kbp. In order to optimize identification of library clones containing the complete EPA gene cluster from SS9, clones hybridizing positively with the proximal *pfaA* gene fragment were subsequently probed using an internal fragment of the distal SS9 *pfaD* gene. Fosmid 8E1 (insert size of 33100 bp) was found to positively hybridize to both probes and was selected for sequencing. Fig. 1 shows the genetic organization of the 19 predicted ORFs identified within this sequence as well as the size and similarity of these ORFs to other sequences present in GenBank based on BLAST searches. All SS9 sequences have been deposited in GenBank under accession number AF409100.

The SS9 *pfaA–D* genes span a region of 17347 bp (*pfaA* 7722 bp, *pfaB* 2100 bp, *pfaC* 5877 bp, *pfaD* 1635 bp). The deduced amino acid sequence of the SS9 *pfa* genes had a high degree of similarity and identity to *pfa* homologues in *Shewanella* and *Moritella* (% identity/similarity): SS9 *pfaA* – 62%/72% *Shewanella*, 46%/61% *Moritella*; SS9 *pfaB* – 44%/58% *Shewanella*, 18%/33% *Moritella*; SS9 *pfaC* – 67%/77% *Shewanella*, 45%/60% *Moritella*; SS9 *pfaD* – 82%/88% *Shewanella*, 62%/76% *Moritella*. Phylogenetically, SS9 is closely related to both *Shewanella* and *Moritella*, and the high degree of similarity of the SS9 and *Shewanella pfa* gene products likely reflects functional relatedness of the products (i.e. EPA vs DHA). In addition, the predicted proteins possess strong similarity to PKSs from *Streptomyces*, *Mycobacterium* and *Bacillus*, as well as cyanobacterial heterocyst glycolipid synthases and eukaryotic FAS enzymes. Comparison of ORFs in SS9, *Shewanella* and *Moritella* flanking their respective *pfa* gene clusters showed no sequence conservation with the exception of SS9 ORF6, which showed a high degree of identity (85%) to an ORF also located immediately upstream of *pfaA* in *Moritella* (ORF7; GenBank accession no. AB025342). The function of this gene is not known but it possesses homology to a hydroxyacyl-CoA thioesterase (product of the *fcbC* gene) of *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000).

Of the 19 ORFs identified, seven did not possess any significant homology to GenBank sequences and were noted as hypothetical proteins. Upstream of the SS9 *pfa* genes is a putative methyl-accepting chemotaxis protein (similar to *Vibrio cholerae* protein VCA093) and a cluster of four genes which appear to be arranged in an operon structure. Included in this cluster is a putative *fabH* paralogue. We denote this gene as a putative paralogue because a homologue of *fabH* has previously been cloned and sequenced from SS9, located within a distinct cluster of genes involved in saturated and monounsaturated fatty acid synthesis, an organization found in numerous γ-proteobacteria (our unpublished results). In addition, putative haloalkane dehalogenase, acyl-CoA ligase, and steroid dehydrogenase genes were identified within this upstream cluster. Based on BLAST searches, each of these four genes displays the highest similarity to genes in the plant pathogen *Xylella*



ORF	Size (bp)	Similarity
1	513 partial	putative methyl-accepting chemotaxis protein (*Vibrio cholerae* protein VCA0093)
2	1116	putative steroid dehydrogenase (*Xylella fastidiosa* protein XF1970)
3	1755	putative acyl-CoA ligase (*Xylella fastidiosa* protein XF2276)
4	915	putative haloalkane dehalogenase (*Xylella fastidiosa* protein XF1965)
5	1071	putative *fabH* paralog; 3-oxoacyl-ACP synthase III (*Xylella fastidiosa* protein XF1970)
6	402	ORF6; *Moritella marina* ORF7 homolog; possible hydroxyacyl-CoA thioesterase
7	**7722**	***pfaA*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase A**
8	**2100**	***pfaB*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase B**
9	**5877**	***pfaC*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase C**
10	**1635**	***pfaD*; Eicosapentaenoic acid (20:5n-3) fatty acid synthase D**
11	630	ORF11; hypothetical protein
12	414	ORF12; hypothetical protein
13	243	ORF13; hypothetical protein
14	2046	alkaline serine protease (*Vibrio metschnikovii vapT*)
15	393	ORF15; hypothetical protein
16	477	ORF16; hypothetical protein
17	384	ORF17; hypothetical protein
18	762	ORF18; hypothetical protein
19	556 partial	formyltetrahydrofolate deformylase; purine biosynthesis; (*Pseudomonas aeruginosa purU*)

Fig. 1. *P. profundum* strain SS9 *pfa* gene cluster and flanking DNA. Graphic map showing the organization of the 19 ORFs identified from the sequencing of the 33 100 bp genomic insert of fosmid 8E1 together with the size of the identified ORFs and their similarity to sequences present in GenBank based on BLAST searches. All SS9 sequences have been deposited under GenBank accession no. AF409100.

fastidiosa (GenBank accession no. AE003849). Unlike SS9, however, none of these genes are linked in the *Xylella* genome. Downstream of the SS9 *pfa* cluster numerous ORFs of unknown function were identified as well as an alkaline serine protease (homologue of *Vibrio metschnikovii vapT*) and a formyltetrahydrofolate deformylase gene (homologue of *Pseudomonas aeruginosa purU*) involved in purine biosynthesis.

Analysis of SS9 EPA biosynthetic enzymes

Similarity searches of the SS9 *pfa* gene products revealed significant matches to numerous multifunctional enzyme complexes involved in such processes as polyketide antibiotic synthesis (Hopwood & Sherman, 1990; Pfeifer & Khosla, 2001), eukaryotic fatty acid synthesis (Beaudoin *et al.*, 2000; Parker-Barnes *et al.*, 2000) and heterocyst glycolipid synthesis (Campbell *et al.*, 1997). Domain analyses within individual *pfa* gene products also revealed numerous enzyme domains characteristic of functions present in bacterial type II fatty acid synthesis. This type of organization is similar to type I PKSs, multifunctional enzymes containing sets of FAS-related activities for successive rounds of polyketide chain elongation and derivatization (Rawlings, 2001). Fig. 2 shows the domain organization of the SS9, *Shewanella* and *Moritella pfaA–D* deduced amino acid sequences. Seven enzyme domains were identified within the *pfa* products: β-ketoacyl-ACP synthase (KS), acyl CoA-ACP transacylase (AT), acyl carrier protein (ACP), β-ketoacyl-ACP reductase (KR), chain length factor (CLF; possible malonyl-ACP decarboxylase activity – Bisang *et al.*, 1999), β-hydroxyacyl-ACP dehydratase/isomerase (DH/I) and enoyl reductase (ER).

The domain organization of the SS9 and *Shewanella pfa* products was identical with the exception of one additional ACP domain found in *Shewanella* PfaA (six ACPs compared to five in SS9 PfaA). Pfa domains of the DHA-producing *Moritella* differed from the EPA-producing SS9 and *Shewanella* products by the inclusion of one additional KS (or CLF) domain in PfaB. CLF domains are homologous to KS domains but the KS active site has a conserved cysteine residue whereas in CLF a glutamine residue exists (Bisang *et al.*, 1999). In *Moritella*, PfaB has a region homologous to the C-terminal domain of KS but lacks an active-site sequence. Similarly, the second KS-like domain present in *Moritella* PfaC lacks both a KS active-site motif and the diagnostic glutamine residue of CLF.

Interestingly, growth of SS9 in the presence of the fungal antibiotic cerulenin, a potent irreversible inhibitor of fatty acid biosynthetic condensing enzymes such as KAS I and KAS II (Omura, 1981), has no effect on EPA production (Allen *et al.*, 1999). This resistance could result from blocked access of cerulenin to the Pfa KS active sites, reflecting structural differences between the Pfa KS domains and type II KAS enzymes.

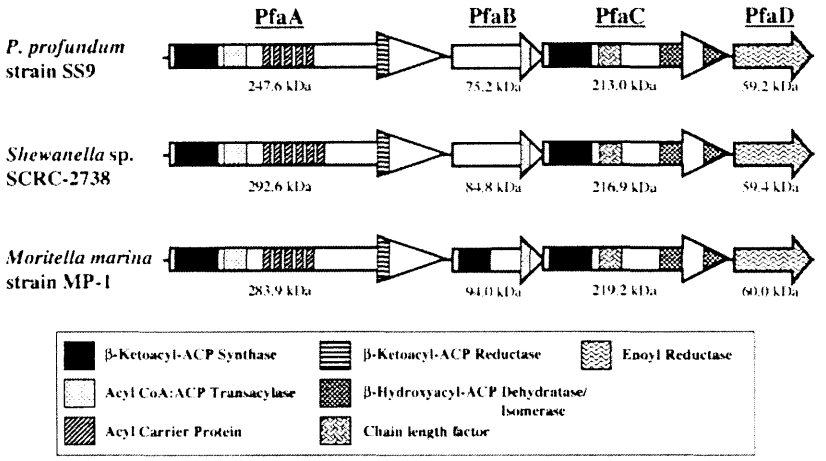


Fig. 2. Comparison of enzyme domains identified within bacterial *pfaA–D* gene products. *P. profundum* strain SS9 and *Shewanella* sp. strain SCRC-2738 produce EPA whereas *M. marina* strain MP-1 produces DHA. Enzyme domains (represented as filled regions) were identified within individual gene products by conserved motif database searches as described in Methods. The predicted sizes of deduced amino acid sequences are noted beneath each product.

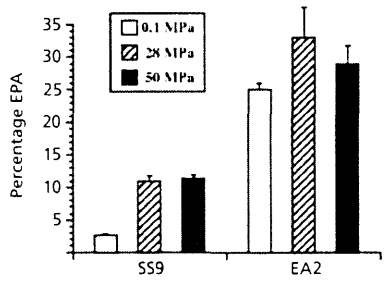


Fig. 3. Effect of varying hydrostatic pressure on EPA percentage composition in wild-type SS9 and chemical mutant strain EA2. Fatty acids were recovered and analysed from mid-exponential-phase cells cultivated at the corresponding pressure (9 °C) as described in Methods. Data represent mean percentage composition (by weight) ± standard deviation ($n = 3$).

EPA synthesis in SS9 and overproducing strain EA2

The percentage composition of EPA present in SS9 membranes undergoes temperature- and pressure-dependent modulation (Allen *et al.*, 1999). An increase in cultivation pressure from 0·1 MPa to 28 MPa [0·1 MPa = 1 atm = 1 bar] results in nearly a fourfold increase in EPA percentage composition (Fig. 3). Similarly, EPA percentage composition undergoes moderate increase in response to reduced cultivation temperature, i.e. 15 °C to 4 °C (Allen *et al.*, 1999). Fig. 3 shows the percentage composition of EPA as a function of varying hydrostatic pressure in wild-type SS9 and an SS9 mutant strain found to overproduce EPA. This strain, designated EA2, was isolated as an oleic acid (18:1*n*-9)-requiring auxotrophic chemical mutant (Allen *et al.*, 1999). Strain EA2 constitutively produces EPA at a level nearly fivefold that of wild-type SS9 grown at atmospheric pressure (Fig. 3).

Transcripitonal analyses of the SS9 *pfa* operon: ribonuclease protection assays and primer extension

RPAs were performed on SS9 *pfa* genes with RNA isolated from strains cultivated at various hydrostatic pressures and temperatures (Fig. 4). RPA analyses were chosen due to the potential large size of transcripts and indication of relatively weak expression of the *pfa* genes from prior Northern blot analysis attempts. No differences in the relative transcript abundance of any of the four *pfa* genes were detected as a function of varying temperature (15 °C vs 4 °C, Fig. 4B, lanes 3 vs 4). RNA extracted from cells cultivated at varying hydrostatic pressure (0·1 MPa vs 28 MPa) revealed different results. When cells were grown at elevated pressure, transcript abundance of all *pfa* genes was considerably less than when cells were grown at atmospheric pressure (Fig. 4B, lanes 5 vs 6), a confounding result given the observed increase in EPA percentage composition as a function of increased hydrostatic pressure (Fig. 3). RPA analyses of *pfa* gene expression were also performed for the EPA-overproducing strain EA2 (Fig. 4B, lanes 7). Results for EA2 revealed dramatic upregulation of *pfa* gene transcription compared to wild-type SS9 cultivated under identical conditions (15 °C, 0·1 MPa).

In order to delineate the transcriptional organization of the SS9 *pfa* gene cluster, RPA analyses were performed on RNA extracted from SS9 grown at 15 °C, 0·1 MPa,

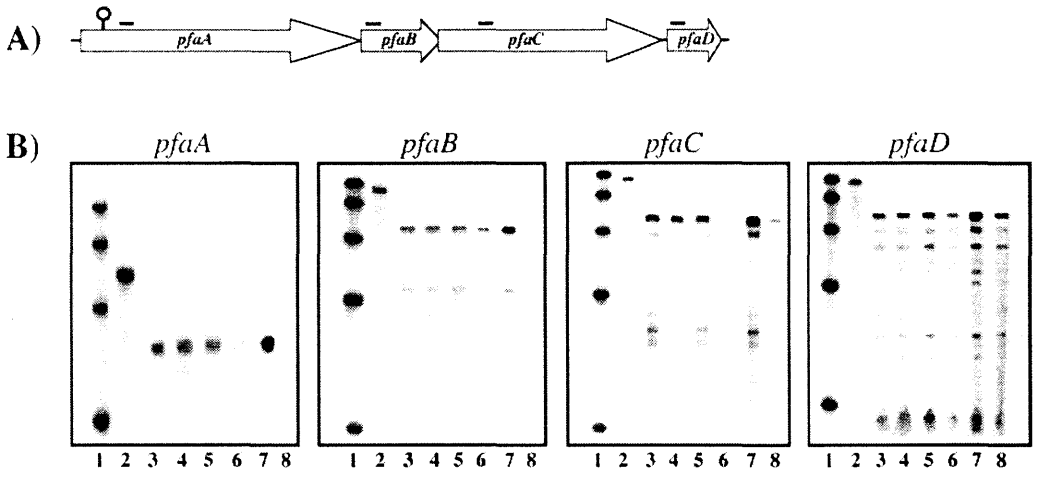


Fig. 4. RPA analyses of SS9 *pfaA–D* gene transcription. (A) Schematic representation of *pfa* operon showing location of RPA probes (lines) and site of *pfaA* insertion in EPA defective mutant strain EA10 (lollipop). (B) RPA results of *pfaA–D* expression. RPA analyses were performed as described in Methods with 10 μg total RNA using ^{32}P-labelled riboprobes. Lanes: 1, RNA Century size markers (500, 400, 300, 200, 100 bases); 2, unprotected full-length probes; 3, SS9 at 15 °C; 4, SS9 at 4 °C; 5, SS9 at 0·1 MPa (9 °C); 6, SS9 at 28 MPa (9 °C); 7, EA2 (EPA-overproducing chemical mutant) at 15 °C; 8, EA10 (*pfaA* insertion strain) at 15 °C.

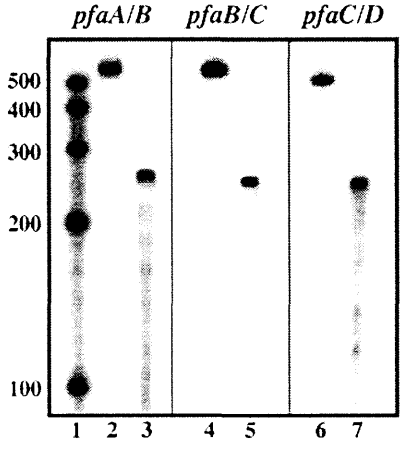


Fig. 5. RPA analysis of SS9 *pfa* gene linkage: *pfaA/B*, *pfaB/C*, *pfaC/D*. RPA probes were designed spanning the intergenic regions of adjacent *pfa* genes. RNA was extracted from SS9 cultivated at 15 °C. Lanes: 1, RNA Century size maker; 2, 4 and 6, unprotected full-length probes; 3, 5 and 7, protected fragments.

using probes spanning the putative intergenic regions of the *pfa* genes (i.e. *pfaA/B*, *pfaB/C*, *pfaC/D*; Fig. 5). Linkage RPAs showed protected fragments of the expected size, indicating that adjacent *pfa* genes are co-transcribed or that promoters for downstream genes are present upstream of the probe-binding sites. Furthermore, transcript analyses were performed using RNA extracted from the *pfaA* insertion strain EA10. This strain contains a pMUT100 (Brahamsha, 1996) insertion in the 5′ end of *pfaA*. EA10 RPA analyses were used to determine whether there was transcriptional linkage between *pfaA* and any of the downstream *pfa* genes. Using RNA from EA10 no RNA protection was detected with the *pfaA* or *pfaB* probes. These results indicate that transcription upstream of *pfaA* drives *pfaA* and *pfaB* co-transcription. Analysis of *pfaC* expression in this strain showed a faint protected fragment suggesting the presence of a weak promoter located in the region spanning the sites of the *pfaB* and *pfaC* probes. Finally, a *pfaD* product of comparable intensity was present using RNA from wild-type SS9 or EA10, indicating the likelihood of a promoter between the region of the *pfaC* and *pfaD* probes. RPA analyses thus revealed two major promoter regions and one minor promoter region driving expression of the SS9 *pfa* gene cluster, giving rise to *pfaA–C*, *pfaC* and *pfaD* transcripts.

In order to localize these promoter regions, primer extension reactions were performed at multiple sites within each of the *pfa* genes. All attempts to determine transcriptional start sites within *pfaB*, *pfaC* and *pfaD* failed to reveal extension products even with RNA concentrations up to 100 μg total RNA. The lack of detection of *pfaC* and *pfaD* transcription initiation sites, despite the evidence for *pfaC* and *pfaD* promoters, presumably stems from low transcript abundance or stability. However, the transcriptional start upstream of

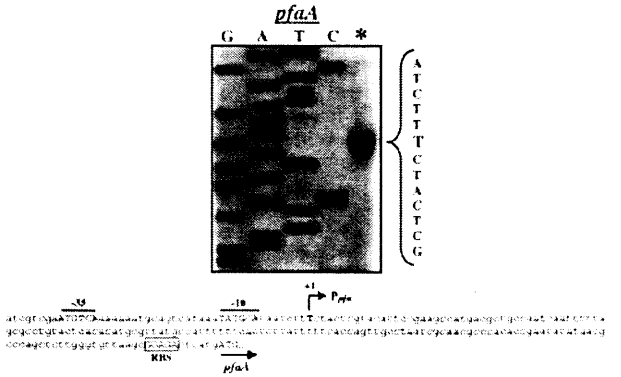


Fig. 6. Determination of SS9 *pfaA* transcriptional start site by primer extension analysis. The *pfaA* extension product (lane denoted by an asterisk) was detected using the ^{32}P-labelled primer located at position −75 to −54 relative to the translational start of *pfaA*. Dideoxy DNA sequencing reaction mixtures using the same primer were electrophoresed in parallel (lanes G, A, T and C). The nucleotide sequence of the transcribed strand immediately flanking the transcriptional start T is bracketed to the right of the sequencing ladder. The nucleotide sequence and promoter features of the upstream *pfaA* region are shown below the primer extension analysis results.

pfaA was readily mapped (Fig. 6). *pfaA* primer extensions were performed with primers corresponding to positions +75 to +94 and −75 to −54 with respect to the 5′ end of *pfaA*. Both primers provided identical results indicating transcription initiation 167 bp upstream of the *pfaA* ATG translational start (Fig. 6 shows results obtained with the −75 to −54 primer). Putative −35 (ATGTCA) and −10 (TATGCA) regions were identified as well as the putative ribosome-binding site (GGAGA) located at position −11 to −7.

Insertional inactivation mutagenesis of the SS9 *pfaD* gene

In an analogous manner to the isolation of *pfaA* insertion mutant EA10, a mutant containing an insertionally inactivated *pfaD* was constructed, resulting in strain EA50. This *pfaD* mutant, like the *pfaA* insertion strain, did not produce EPA under any culture conditions, nor did it accumulate any fatty acids that could serve as intermediates in the EPA biosynthetic pathway (data not shown). Thus, both *pfa* operons are required for EPA biosynthesis. Mutant EA50 also shared with EA10 the phenotype of no growth defect when cultivated at elevated hydrostatic pressures or reduced temperatures despite its gross alteration in fatty acid composition.

Introduction of PPTase genes into *E. coli* harbouring SS9 *pfaA–D*

In an attempt to achieve recombinant EPA production in *E. coli* we sought to obtain a gene encoding the last remaining enzyme activity needed: PPTase activity. PPTase genes were recovered from both the EPA-producing deep-sea *Shewanella* sp. strain SC2A (DeLong *et al.*, 1997) and *B. subtilis*. Based on BLAST analyses, the SC2A putative PPTase shares 55% identity (89/161 amino acids) with the *Shewanella* sp. strain SCRC-2738 PUFA synthase PPTase (ORF2) shown to be required for recombinant EPA production in *E. coli* host strains (Metz *et al.*, 2001). Introduction of either the SC2A PPTase gene or the *sfp* gene, the surfactin-synthetase-activating PPTase of *B. subtilis* (Nakano *et al.*, 1992), into *E. coli* strains expressing SS9 *pfaA–D* from pFOS8E1 consistently failed to yield recombinant EPA synthesis.

DISCUSSION

Progress towards understanding microbial PUFA production has benefited from the recent identification and characterization of genes involved in their synthesis (Metz *et al.*, 2001; Tanaka *et al.*, 1999; Yazawa, 1996). These analyses have provided clues to the mode of synthesis, although biochemical investigations will be needed to resolve many aspects of the pathway. The organization of microbial PUFA synthases is in stark contrast to known eukaryotic PUFA synthesis mechanisms involving elongases and position-specific, oxygen-dependent desaturases (Napier & Michaelson, 2001; Parker-Barnes *et al.*, 2000). Furthermore, numerous PUFA-producing bacterial strains, including SS9, are capable of producing PUFAs under strictly anaerobic conditions, thus precluding the involvement of an oxygen-dependent mechanism (our unpublished results; Metz *et al.*, 2001; Nichols *et al.*, 1992). Based on the identification of multiple enzyme domains within *pfa* gene products and ^{13}C-labelling studies (Metz *et al.*, 2001) it is now evident that PUFA production shares many features with polyketide synthesis. Analysis of the four SS9 *pfa* gene products has revealed 10 regions possessing enzyme activities akin to those found in PKS and FAS systems (Fig. 2). The activities of these integrated domains include condensation reactions (KS domains), acyl CoA:ACP transfer reactions (AT), multiple acyl carrier protein domains (ACP), ketoacyl reduction reactions (KR), chain length factor domains (CL) presumably involved in decarboxylation reactions, dehydratase/isomerase reactions (DH/I), and enoyl reduction reactions (ER). These domains presumably catalyse the reiterative steps in the processing of the growing acyl chain, where each condensation reaction is followed by complete or abbreviated reductive reaction cycles of ketoreduction, dehydration/isomerization, and enoyl reduction.

The ability to introduce multiple double bonds into a single acyl chain in the absence of desaturation reactions likely arises from the activities of the DH/I domains present in the microbial PUFA synthases (bacterial PfaC homologues and *Schizochytrium* ORF C). Such dehydration/isomerization reactions would be analogous to those catalysed by FabA (β-hydroxydecanoyl-ACP dehydratase) in bacterial monounsaturated fatty acid synthesis (Cronan & Rock, 1996). By dehydration of the β-hydroxyacyl-ACP substrate, product of β-ketoacyl-ACP synthase condensation and subsequent reduction of the β-ketoester by β-ketoacyl-ACP reductase, a *trans* double bond is introduced into the growing acyl chain. In selective rounds of acyl chain elongation these double bonds are either preserved by isomerization to the *cis* form to form an unsaturated acyl-ACP or reduced by an enoyl reductase to a saturated acyl-ACP. Metz *et al.* (2001) propose a hypothetical pathway for EPA synthesis in *Shewanella* sp. SCRC-2738 wherein position-specific isomerases (*trans*-2,*cis*-3 and *trans*-2,*cis*-2) are involved in *trans*/*cis* double bond isomerization. Such a mechanism may be consistent with two DH/I domains being present in PfaC homologues (Fig. 2). Alternatively, the two DH/I domains could be analogous to the FabA and FabZ β-hydroxyacyl-ACP dehydratase isozymes found in *E. coli* which differ in reactivity and specificity (Heath & Rock, 1996).

Unique to microbial PUFA synthases is the presence of clustered repetitive ACP domains (Fig. 2): SS9 *pfaA* possesses five ACP domains, *Shewanella* sp. has six, *Moritella* has five, and *Schizochytrium* has nine (Metz *et al.*, 2001; Tanaka *et al.*, 1999). Intermediates in the biosynthetic process are presumably bound to these ACP domains as thioesters with AT domains being required for the loading of the starter and extender units. The significance of the disparity in the number of ACP repeats among the PUFA synthase systems is unknown.

Currently, we have been unable to achieve recombinant EPA synthesis in *E. coli* with the introduced SS9 *pfaA–D* genes. This shortcoming arises from the need for an additional gene whose product is required for the post-translational modification of the constituent ACP domains present in PfaA. This activity is achieved by a PPTase which converts apo-ACP to its active holo-form by transfer of a 4′-phosphopantetheinyl moiety from coenzyme A to ACP (Lambalot *et al.*, 1996). In *Shewanella* sp. SCRC-2738 a fifth gene, designated ORF2, required for recombinant EPA synthesis in *E. coli* has been identified as a PPTase and is located within close proximity of the *pfaA–D* operons (Metz *et al.*, 2001). Unlike SCRC-2738, the PUFA synthase PPTase is unlinked from the other *pfa* genes in SS9 and *Moritella*. We have been unable to clone the ORF2 homologue from either SS9 or *Moritella*. During attempts to obtain recombinant EPA synthesis, we introduced two PPTase genes into *E. coli* harbouring SS9 *pfaA–D*, *B. subtilis sfp* and a *Shewanella* ORF2 homologue. The *B. subtilis sfp* gene, involved in surfactin biosynthesis, encodes a PPTase with a broad substrate recognition spectrum (Nakano *et al.*, 1992; Reuter *et al.*, 1999). In addition, a PPTase homologue which contained a high degree of identity to *Shewanella* sp. SCRC-2738 ORF2 was cloned and sequenced from the EPA producer *Shewanella* sp. strain SC2A. Expression of either of these genes in *E. coli* harbouring SS9 *pfaA–D* failed to yield recombinant EPA synthesis, suggesting a high degree of specificity of individual PPTases to their cognate ACPs.

While substantial progress has been made towards a mechanistic understanding of microbial PUFA synthesis, very little information exists regarding the regulation of bacterial PUFA production. In those organisms that have been studied, modulation of PUFA percentage composition occurs during changes in cultivation temperature or pressure. For example, growth of SS9 at a hydrostatic pressure of 28 MPa results in an approximately fourfold increase in EPA percentage composition relative to growth at 0·1 MPa (Fig. 3). At the outset of our studies, one possibility was that this modulation was the result of transcriptional regulation of the EPA biosynthetic genes.

RPA analyses performed on each of the SS9 *pfa* genes using RNA extracted from SS9 cells cultivated at various temperatures and pressures revealed that the *pfa* genes are not transcriptionally regulated in an adaptive manner in response to these parameters (Fig. 4). The observed reduction in *pfa* transcript abundance at elevated pressure is confounding and could result from diminished transcription initiation or increased transcript turnover at high pressure. Numerous prokaryotic species regulate percentage composition of particular membrane fatty acids in response to cultivation parameters. In *E. coli* increased *cis*-vaccenic acid (18:1*n*-11) composition at low temperature is an intrinsic property of the fatty acid biosynthetic enzyme KAS II (β-ketoacyl-ACP synthase II), product of the *fabF* gene, and a similar regulatory mechanism may account for increased *cis*-vaccenic acid composition at high pressure in SS9 (Allen & Bartlett, 2000; Cronan & Rock, 1996). In both bacteria, *fabF* is not transcriptionally regulated and, at least for the *E. coli* enzyme, it is the relative activity of the enzyme at different temperatures that is responsible for the increased production of 18:1 at low temperature. Hence, the possibility exists that PUFA synthases exhibit temperature/pressure-responsive characteristics.

Transcriptional analyses indicate that the *pfa* gene cluster is organized into two operons, *pfaA–C* and *pfaD* (Figs 4 and 5). Evidence in support of this conclusion includes the presence of overlapping start/stop codons of adjacent genes, RPA results with probes spanning intergenic regions, and transcript analyses of a strain containing a polar insertion within *pfaA*. The transcriptional start of *pfaA* has been mapped to 169 bp upstream of the translational start (Fig. 6).

Results from SS9 suggest that the pathway for PUFA synthesis is separate and distinct from the type II FAS producing monounsaturated and saturated fatty acids (Allen & Bartlett, 2000; Allen *et al.*, 1999). Many of the type II FAS genes have been cloned and sequenced from

SS9 and *Moritella* (Allen & Bartlett, 2000; Tanaka *et al.*, 1999). Metz *et al.* (2001) reported a probable PUFA synthetic mechanism reliant on malonyl-CoA derived from acetate as would be expected for the type II FAS system. An interesting question is the 'cross-talk' that exists between the two systems with regard to coordinated expression and lipid incorporation. Some initial insight into this interplay has been provided by *pfa* transcript analysis of an SS9 mutant strain, designated EA2, originally isolated as an oleic acid auxotrophic chemical mutant that overproduces EPA nearly fivefold compared to wild-type SS9 (Fig. 3). In addition, this strain greatly underproduces monounsaturated fatty acids (MUFAs) (Allen *et al.*, 1999). *pfa* transcript analyses in this strain reveal substantial *pfaA–D* overexpression relative to wild-type SS9 (Fig. 4). While the nature of the mutation in this strain has yet to be resolved, two opposing hypotheses can be proposed. Either this strain harbours a lesion resulting in decreased MUFA production which results in compensatory increases in *pfa* transcription and EPA synthesis, or the mutation results in overexpression of both *pfa* operons and the cellular response is decreased MUFA synthesis. Both models require the presence of a transcription factor that modulates *pfa* gene expression.

The high degree of sequence similarity between the bacterial (*Shewanella* sp SCRC-2738, *M. marina* and SS9) and the eukaryotic microbe *Schizochytrium pfa* genes suggests the possible involvement of horizontal gene transfer in the acquisition of the *pfa* gene clusters in the marine environment. However, among the three bacterial strains whose *pfa* gene clusters have been cloned and sequenced no sequence conservation flanking the *pfa* clusters is observed with the exception of a single undefined ORF located upstream of *pfaA* in SS9 and *Moritella*. Furthermore, there is no apparent GC bias among the *pfaA–D* genes nor is there indication of flanking genes possessing functions which could facilitate horizontal transfer.

Located upstream of the SS9 *pfa* cluster resides an intriguing cluster of four genes which appear to be organized into a possible operon (Fig. 1). Included in this cluster is a putative *fabH* (3-oxoacyl-ACP synthase III; KAS III) paralogue. This *fabH* paralogue is distinct from the *fab* cluster *fabH* homologue, involved in type II fatty acid biosynthesis initiation, which we have cloned and sequenced from SS9 (our unpublished results). Multiple *fabH*-like sequences have been identified in a few bacterial species including *B. subtilis* (*yjaX*, GenBank accession no. F69842, and *yhfB*, Y14083) and *V. cholerae* (GenBank accession nos A82423 and H82128). Within this cluster also reside a putative haloalkane dehalogenase, a probable acyl-CoA ligase, and a putative steroid dehydrogenase/isomerase. Proteins of the hydroxysteroid dehydrogenase/isomerase family (Labrie *et al.*, 1992) are unusual in prokaryotic organisms, with only a single other bacterial homologue having been identified in the plant pathogen *Xylella fastidiosa* (GenBank accesssion no. AE004004). The sequence and possible operon structure of this gene cluster suggest that their products could function in a common metabolic process including some aspect of fatty acid physiology. Curiously, upstream of the *M. marina pfa* gene cluster lie two genes presumably involved in fatty acid metabolism as well, a 3-ketoacyl-CoA thiolase/acetyl-CoA acetyltransferase and a probable lipid A acyltransferase (ORFs 1 and 3, GenBank accession no. AB025342).

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CHAPTER VI

Conclusions and Suggestions for Future Research

The observation that bacteria alter the fatty acid composition of membrane phospholipids in response to temperature variation has been recognized for more than 40 years (4). Perhaps the most pervasive fatty acid compositional change observed in response to temperature decrease is the introduction of increased proportions of unsaturated fatty acids (UFAs) into membrane phospholipids. Such changes act to offset the membrane altering physical influences of low temperature. Broadly, the maintenance of a similar membrane structure at different growth temperatures has been termed homeoviscous or homeophasic adaptation (5, 9). Extension of the theories of homeoviscous and homeophasic adaptation to growth at varying hydrostatic pressures followed the discovery of pressure-induced alterations in fatty acid composition in a high pressure adapted (piezophilic) bacterial isolate (1). Subsequent analyses of have shown increased UFA levels at elevated pressure to be a common phenomenon in numerous high pressure adapted bacteria.

Whereas correlations have been found to exist between the degree of membrane unsaturation and growth under high pressure conditions, confirmatory evidence that UFAs are indeed required for growth under these conditions has not been available. This dissertation has attempted to make progress towards this end by employing molecular genetic techniques to address fundamental questions regarding the role and regulation of UFA production employing the piezophilic deep-sea bacterium *Photobacterium profundum* strain SS9. SS9 has served as an ideal model system for high pressure adaptation studies owing to its amenability to genetics, relatively rapid doubling time, and growth ability over wide temperature and pressure ranges.

To assess the importance of fatty acid composition for growth at high pressure and low temperature *in vivo*, the effects of altered UFA levels on the growth properties of SS9 were analyzed through UFA synthesis inhibitor studies, the random generation of mutants exhibiting altered fatty acid profiles, and the direct engineering of SS9 mutant strains defective in monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) production. As growth pressure increases from 0.1 MPa to 50 MPa, SS9 cells dramatically increase 18:1 (*cis*-vaccenic acid) and EPA levels. As a function of decreasing temperature, 16:1, 18:1, and EPA percent composition increase modestly. Treatment of cells with the antibiotic cerulenin (2,3-epoxy-4oxo-7,10-dododecadienamide) resulted in specific growth impairment at high pressure and low temperature concomitant with decreased levels of 18:1 and 16:1 fatty acids. Cerulenin irreversibly inhibits fatty acid biosynthetic enzymes β-ketoacyl-acyl carrier protein synthases I and II, perturbing the formation of UFAs. Supplementation of cultures with exogenous 18:1 in the presence of cerulenin however reversed the growth defects. The generation of SS9 chemical mutants which displayed altered fatty acid profiles led to similar conclusions as the cerulenin results. Strains that exhibited altered MUFA synthesis capabilities showed specific growth defects at high pressure and reduced temperatures which could also be remedied by exogenous supplementation with MUFAs. These results provided the first evidence that pressure-dependent increases in UFAs, specifically 18:1, are important adaptive acclimation mechanisms for growth under varying pressure regimes.

Indication that 18:1 may be particularly important for growth at high pressure led to further investigations targeting a key enzyme involved in 18:1 synthesis from SS9. In *E. coli*, where the mechanisms of UFA synthesis have been well characterized, the response to temperature decrease entails the restructuring of membrane fatty acid composition by increasing the amount of 18:1 and decreasing the amount of 16:1 incorporated into membrane phospholipids. This regulation is an intrinsic property of the fatty acid biosynthetic enzyme β-ketoacyl-ACP synthase II (KAS II), product of the *fabF* gene. Specifically, KAS II catalyzes the elongation of 16:1-to-18:1 in UFA synthesis. Neither mRNA nor protein synthesis is required for increased 18:1 at reduced temperature suggesting that thermal modulation of fatty acid production is controlled at the level of enzyme activity. Indeed, the elongation activity of KAS II has been shown to be temperature dependent, exhibiting decreased K_m for 16:1 and increased relative V_{max} at reduced temperatures. *E. coli fabF* mutants possess a deficiency in 18:1 synthesis as well as a loss of 18:1 thermal modulation.

An SS9 strain harboring an insertionally inactivated *fabF* gene displayed specific growth impairment at elevated hydrostatic pressure concomitant with diminished 18:1 production. However, growth ability could be restored to wild-type levels by the addition of exogenous 18:1 to the growth medium. This observation served to underscore the specific importance of 18:1 in growth at elevated hydrostatic pressure. SS9 *fabF* transcript analysis did not reveal that the *fabF* gene is transcriptionally regulated at high pressure suggesting that the elevated 18:1 levels produced result from posttranscritional changes. Just as the *E. coli* enzyme is "temperature activated",

it is predicted that the SS9 enzyme has evolved novel pressure-responsive characteristics which facilitate growth at high pressure. The possible pressure enhanced activity of SS9 KAS II suggests this fatty acid enzyme may be an effective pressure sensor in piezophilic microorganisms. Experiments are currently underway to kinetically characterize the SS9 KAS II enzyme as a function of pressure and temperature.

In this study a comparative analysis of *E. coli fabF* was performed at high pressure. Fatty acid profiling of a variety of *E. coli* strains at high and low pressures showed no significant changes in fatty acid percent composition. This suggests the capacity for thermal regulation of UFAs does not predispose microorganisms to respond in a similar fashion to pressure change. Unlike SS9, an *E. coli fabF* mutant did not exhibit enhanced pressure sensitivity despite producing approximately 21-fold less 18:1 than an isogenic wild-type strain under elevated pressure conditions. This suggests that something other than phospholipid fatty acid composition likely limits growth of this mesophile at high pressure.

At the predicted amino acid level, SS9 KAS II is 76% identical and 88% similar to *E. coli* KAS II. Subtle modifications between the two enzymes are likely to account for the profound differences observed with regard to pressure-responsive activity. If the SS9 enzyme is found to possess significant kinetic adaptation to elevated pressure it will be of interest to model SS9 KAS II relative to the *E. coli* enzyme for which the crystal structure has been solved. Foreseeable future experiments could entail site-

directed mutagenesis of *E. coli* KAS II and assay for potential pressure-responsive activity of the recombinant enzyme.

In order to explain the critical role of MUFAs in growth at high pressure and low temperature two hypotheses can be proposed. Foremost, it is possible that these fatty acids are required to maintain membrane fluidity or phase within an acceptable range. Studies with mesophilic species have suggested that the ability of microorganisms which are unable to regulate their membrane lipid fatty acid composition to grow at various temperatures may be determined by the phase state of their membranes, as evidenced by the severe growth impairment when cells exhibit more than about half of their lipids in the gel state (5). Alternatively, the critical nature of MUFAs may be based on a role in specific membrane protein interactions which, if disrupted, result in altered growth ability under membrane gelling conditions such as high pressure or low temperature. This latter hypothesis suggests a need for MUFAs in local as opposed to global membrane processes. Clearly, further work is required to delineate the precise mechanism of fatty acid dependent growth impairment at elevated pressure and low temperature.

The inability to modulate membrane structure under high pressure conditions likely perturbs both the barrier and catalytic functions of the membrane. Analysis of membrane integrity at high pressure in mutant strains exhibiting altered UFA profiles could be assessed for example by ion flux permeability. If isolated or whole cell membranes were found to possess significant increases in H^+ permeability this would indicate a global role for UFAs in growth at elevated pressure. To study membrane

transport processes and pH homeostasis a combination of microelectrode ion-selective techniques in conjunction with fluorescent ratio imaging microscopy could be employed as described by Shabala *et al.* (8). Dissolution of a proton motive force across the membrane would possibly implicate aspects of electron transport impairment at high pressure. Furthermore, it would be interesting to investigate more fully the role of electron transport components such as cytochromes in growth of SS9 at high pressure. Analysis of electron transport mechanisms in piezophilic *Shewanella* strains have revealed modified bioenergetic modes under varying pressure conditions (3). Specifically, the cytochrome *bd* complex has been observed only in cells grown at high pressure in *Shewanella* sp. strain DSS12 (10) and two types of *c*-type cytochromes , one cytoplasmic and one membrane bound, have been shown to produced by the piezophile *Shewanella benthica* strain DB-127F with only the membrane bound variant occurring under high pressure conditions (6). Furthermore, at high pressure a *ccb*-type quinol oxidase undergoes increased abundance in strain DB-127F (7).

In order to correlate growth ability and membrane fatty acid composition with membrane physical structure, electron paramagnetic resonance spectroscopy (EPR) of spin-labeled lipid dispersions could be performed. This would involve the spin-labeled preparation of lipids from various SS9 UFA mutant strains cultivated under varying pressure and temperature regimes with which EPR measurements could be performed at varying pressures and temperatures. Spin labels are sensitive and informative monitors of molecular organization and dynamics in biomembranes (2).

Perturbation of the spin label within lipid dispersions is directly related to the thermal motion and organization within the membrane. Such analyses would allow conclusions to be drawn as to the importance of membrane fatty acid composition and membrane physical state upon growth ability at high pressure and low temperature.

The results mentioned above indicated a role for MUFAs in growth at high pressures and low temperatures but the question remained as to the relative importance of PUFA production. To assess the role of EPA on the growth capabilities of SS9, a reverse-genetics methodology was employed to construct a mutant unable to synthesize EPA. Surprisingly, no significant deviations in growth were observed between an EPA$^-$ strain and wild-type SS9 under all pressure and temperature conditions examined. These results indicate that under the laboratory conditions used in this study, EPA was not vital to the growth of SS9 under high pressure and/or low temperature conditions, situations under which EPA levels are typically elevated. Increases in 16:1 and 18:1 were observed in the EPA$^-$ strain suggesting MUFA compensation for lack of EPA synthesis. In addition, no differences were noted in survival at extremes of pressure (100 MPa) or temperature (-20°C) in the EPA$^-$ strain. Given the presumed importance of MUFAs for growth of the EPA$^-$ strain at high pressure and low temperature, it would be of interest to examine the sensitivity of this strain to varying concentrations of cerulenin. It would be predicted that the minimum inhibitory concentration of cerulenin would be far less in this strain compared to wild-type SS9. Indeed, attempt to create a *pfa/fabF* double mutant in SS9 have not succeeded presumably due to the paramount importance of MUFAs in the EPA$^-$

mutant. If not specifically required for piezophilic or psychrophilic growth, a possible role of bacterially derived PUFAs may be as nutritional sources for higher organisms with which PUFA-producing microorganisms have established symbiotic associations. This suggests a specific role for *in situ* secondary production of PUFAs by piezophilic bacteria in deep-sea environments. This is consistent with the metazoan source of many piezophiles and warrants additional investigations.

Omega-3 PUFA synthesis was investigated in SS9 by the cloning and sequencing of the genes, termed *pfa* (*p*olyunsaturated *f*atty *a*cid), responsible for eicosapentaenoic acid (EPA) synthesis. As a function of varying hydrostatic pressure and reduced temperature EPA levels were found to increase at high pressure and low temperature however *pfa* transcript analysis by ribonuclease protection assays did not reveal transcriptional regulation. Interestingly, a chemical mutant which greatly overproduces EPA, strain EA2, was found to dramatically upregulate *pfa* gene transcription suggesting a *trans*-acting regulatory factor exists which influences EPA production in SS9. Furthermore, analysis of the *pfa* gene products provided insight into the potential mechanism of bacterial PUFA production suggesting a mechanism akin to polyketide antibiotic synthesis. Identification of the fifth gene required for EPA synthesis in SS9, *pfaE* (a phosphopantetheinyl transferase), would allow recombinant studies to be performed in alternative heterologous strains such as *E. coli*. Additional future experiments could involve analysis of PUFA synthesis discrimination in EPA vs. DHA (docosahexaenoic acid; 22:6) synthesizing bacteria. Making use of the genes responsible for EPA synthesis in SS9 and those required for

DHA synthesis in *Moritella marina* (11) genetic experiments in SS9 could be performed to engineer SS9 strains which produce DHA. Furthermore, the labeling of cells with various 13C labeled organic acids such as acetate and malonate would provide additional insight into the mechanism of bacterial PUFA synthesis. Lastly, a host of experiments could be performed involving combinatorial synthesis of recombinant, non-native PUFAs by the specific addition or deletion of Pfa enzymatic domains.

In addition to PUFA mechanistic studies, it would be of tremendous interest to identify the regulatory factor presumed to be involved in regulation of *pfa* gene transcription. Experiments are currently under way using a genetic methodology involving the creation of a *pfaA::lacZ* fusion strain in SS9 and EA2. In addition, anti-PfaA antibody will be used to identify SS9 transposon mutants containing decreased PfaA abundance by Western analysis. Identification of the site of transposon insertion in these mutant strains may reveal the *pfa* regulatory factor.

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APPENDIX 1

cAMP Receptor Protein (CRP) Mediated Modulation

of Unsaturated Fatty Acid Production

An interesting phenomenon observed in *Photobacterium profundum* strain SS9 involves reduced monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) levels when grown in the presence of glucose at atmospheric pressure. This "glucose effect" appears to be selective to hydrostatic pressure parameters as growth at elevated pressure in the presence of glucose does not result in reduced unsaturated fatty acid (UFA) levels. The observation that glucose supplementation specifically results in reduced 18:1 (*cis*-vaccenic acid) and 20:5 (eicosapentaenoic acid; EPA) levels at atmospheric pressure suggests some aspect of catabolite repression influences UFA levels in SS9. The focus of this appendix is to introduce findings implicating involvement of the global transcription factor cyclic AMP receptor protein (CRP) in influencing UFA levels in SS9 under varying hydrostatic pressure regimes and under certain carbon and energy states.

CRP together with cyclic AMP (cAMP) mediates catabolic repression or activation of a host of genes involved in the metabolism of carbon catabolites in a variety of prokaryotes (6). Briefly, the presence of glucose in the culture medium results in diminished cytosolic cAMP concentrations resulting in inactive CRP (cAMP-CRP dissociation) leading to the catabolic repression of genes involved in the utilization of alternative carbon sources and activation of glucose metabolic enzymes. Conversely, the absence of glucose results in enhanced cAMP concentrations which, when complexed with CRP, results in the activation of transcriptional initiation of genes involved in alternative carbon and energy utilization while repressing the expression of glucose utilization enzymes. In addition to carbon catabolic enzymes,

numerous proteins have been shown to be subject to catabolite repression including those involved in such diverse functions as antibiotic biosynthesis, bioluminescence, sporulation, photosynthesis, and pigment biosynthesis (12).

Previous studies in SS9 have revealed carbon and energy status to be an important determinant influencing gene expression at high and low pressure. Expression of the outer membrane protein gene *ompH* is regulated by a variety of parameters including elevated pressure, phase of growth, and carbon and energy status (5). The addition of glucose to the culture medium results in repression of *ompH* expression which could be alleviated by the addition of cAMP suggesting CRP involvement influencing *ompH* expression. However, glucose repression of *ompH* transcription was only observed at low pressure despite prevailing evidence that catabolite repression is active in SS9 at elevated pressure.

Figure 1 shows the percent composition of UFA species 18:1 and 20:5 in SS9 and 16:1, 18:1 levels in *E. coli* in the presence or absence of glucose and in *crp*$^-$ mutant strains. The presence of glucose at low pressure results in over a four-fold reduction in 18:1 (*cis*-vaccenic acid) levels and nearly a three-fold reduction in 20:5 (eicosapentaenoic acid; EPA) levels. This reduction in UFA content could be reversed to a significant extent by the addition cAMP to the growth medium, suggestive of cAMP-CRP-mediated regulation of genes influencing UFA levels. However, 18:1 and 20:5 levels were identical at high pressure with or without glucose supplementation suggesting lack of CRP-mediated regulation of UFA levels at high pressure, a phenomenon analogous to that observed for *ompH* in response to pressure treatment.

Furthermore, analysis of an SS9 strain harboring an insertionally inactivated *crp* gene (CRP$^-$) exhibited greatly diminished UFA levels at low pressure quantitatively similar to glucose supplementation. Analysis of the *crp*$^-$ mutant at high pressure revealed UFA levels identical to wild-type SS9 further supporting the notion of CRP-independent UFA modulation at high pressure.

Figure 2 depicts a working hypothesis of cAMP-CRP mediated control of UFAs at low pressure and regulation at high pressure. At atmospheric pressure cAMP-CRP complex formation is required to promote transcription of gene(s) affecting UFA levels. This explains why the presence of glucose (lack of cAMP) and/or lack of CRP results in diminished UFA levels at low pressure. At high pressure however the situation is slightly more complex. The process of gene activation is likely to occur irrespective of CRP at high pressure. This could be consistent with the induction of alternative sigma factors or activator proteins that influence gene expression specifically at high pressure.

Interestingly, evidence suggests catabolite repression of UFAs may be a common phenomenon among bacteria. Analysis of an *E. coli crp*$^-$ mutant and the isogenic parental strain revealed greatly diminished 16:1 and 18:1 at 18 and 37°C in the *crp*$^-$ strain. Catabolite repression in *E. coli* has been extensively studied (6, 12) however no evidence of an effect on UFAs has been previously described. Furthermore, of the more than 60 genes known to be regulated by CRP none encode proteins with obvious connection to UFA synthesis or regulation.

Of particular interest is identification of the gene or genes that are regulated by cAMP-CRP which influence UFA levels at low pressure. In *Escherichia coli*, regulation of UFA synthesis in is a complex process and has been shown to occur both at the transcriptional level and at the level of biosynthetic enzyme activity. The fatty acid synthase mechanism in *E. coli* has been well characterized and represents the paragon of type II (dissociated) fatty acid synthase systems (8). Genetic studies have revealed two genes specifically required for UFA synthesis in *E. coli*, *fabA* and *fabB*. FabA introduces the double bond into the growing acyl chains at the 10-carbon stage via its dual role as a β-hydroxyacyl-ACP dehydratase and a trans-2, 3-decanoyl isomerase. FabB (KAS I) acts to divert the *cis*-3 intermediate to UFA production by its efficient elongation of the *cis*-3-decanoyl intermediate. Furthermore, the regulation of membrane fluidity in response to environmental parameters such as temperature by manipulating the relative levels of saturated and unsaturated fatty acids occurs as a result of enhanced activity of the condensing enzyme FabF (KAS II) at reduced temperature resulting in, specifically, elevated *cis*-vaccenic (18:1) synthesis (9, 10).

Recent evidence in *E. coli* has shown two transcription factors with opposing influences direct UFA synthesis by regulation of *fabA* and *fabB* expression in *E. coli*. FadR acts as a positive regulator of *fabA* and *fabB* expression while repressing *fad* (fatty acid degradation) gene expression involved in the β-oxidation of exogenous fatty acids (7, 11). The other transcription factor, FabR, is a potent repressor of UFA synthesis by its negative regulation of *fabB*, and to a lesser extent, *fabA* expression

(13). It is through the combined influences of *fabA* and *fabB* expression and FabF activity which greatly determine the UFA composition of an *E. coli* cell.

Differential regulation of the *fabA*, *fabB* or *fabF* genes could potentially influence UFAs to the extent observed in response to glucose. Analysis of both the *fabB* and *fabF* genes have been investigated in SS9 by mutational analyses (1, 3). The fatty acid phenotype of a *fabF* mutant produces 18:1 and 16:1 levels comparable to SS9 grown in the presence of glucose or in the *crp*⁻ strain, approximately 2 and 40% respectively. However, 20:5 levels are significantly higher in the *fabF*⁻ strain. Furthermore, analysis of *fabF* transcript abundance at low pressure in the presence of glucose is identical to that without glucose and at high pressure. Consequently, *fabF* is not a likely target for cAMP-CRP-mediated regulation. An SS9 strain containing an insertionally inactivated *fabB* gene displays greatly reduced 16:1 and 18:1 levels and greatly up-regulated 20:5 levels. Based on the fatty acid data, *fabB* is not predicted to be under catabolite repression control in SS9. These results also likely preclude FadR or FabR involvement.

Identification of the cAMP-CRP regulated genes will not be an easy undertaking. One possibility is to identify SS9 mutant strains, derived for example by transposon mutagenesis, which are pressure sensitive, determine the site of transposon insertion and from this examine the possible involvement in UFA synthesis. If a mutant could be obtained which does not produce UFAs at high pressure, the nature of the mutation could reside in a gene resulting in a low pressure fatty acid phenotype. It is predicted that creation of a "low pressure plus glucose" fatty acid profile at high pressure would

result in high pressure sensitivity based on previous analyses of SS9 mutant growth at high pressure (2-4).

Alternatively, making use of DNA microarray technologies, genes exhibiting differential regulation at low pressure ± glucose, or wild-type vs. *crp*⁻, would reveal genes subject to catabolite repression. Subsequent mutational and transcriptional analyses would confirm the microarray results and verify the gene(s) involved in UFA regulation.

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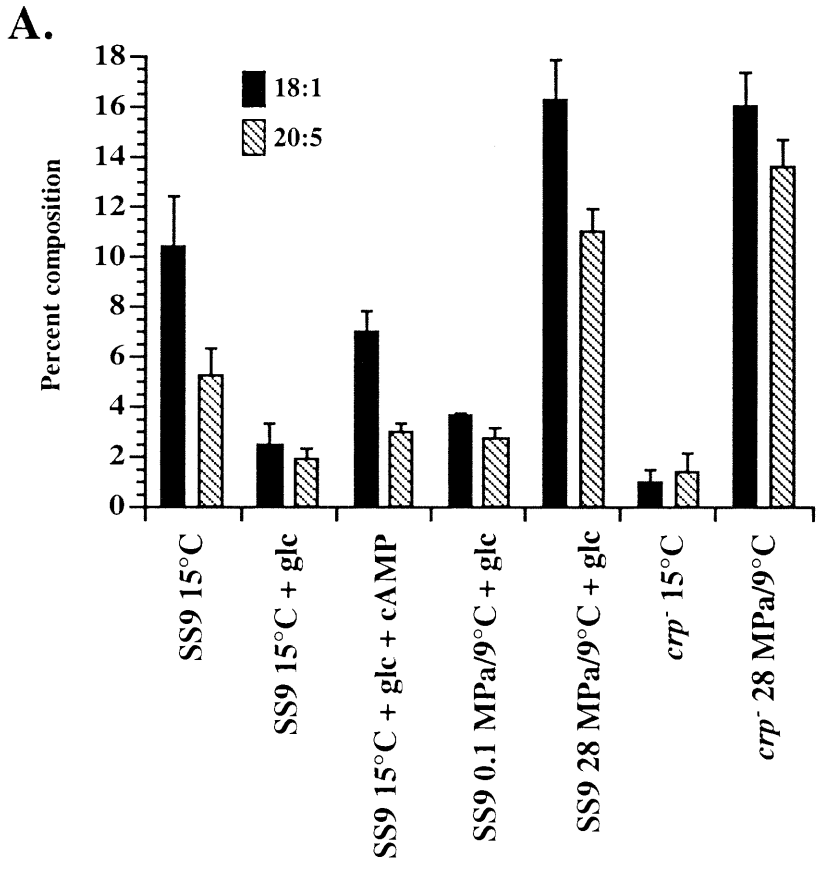
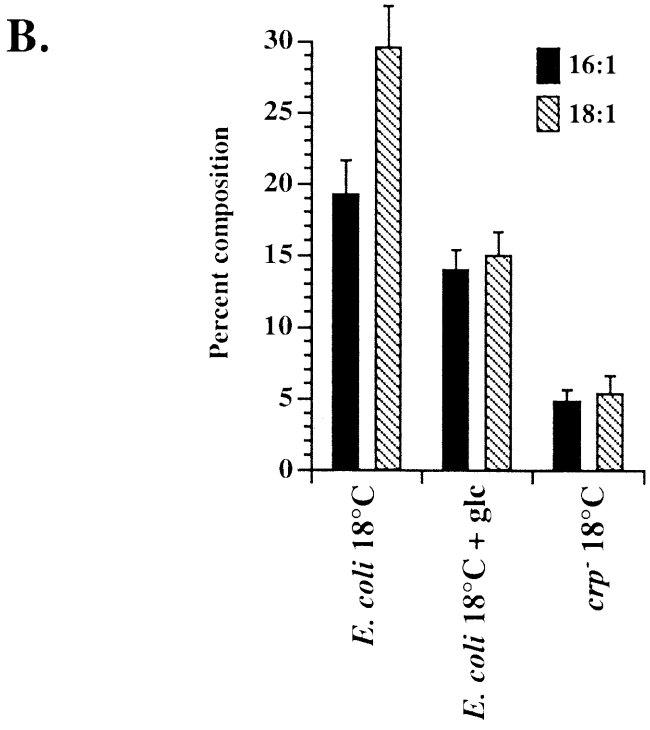
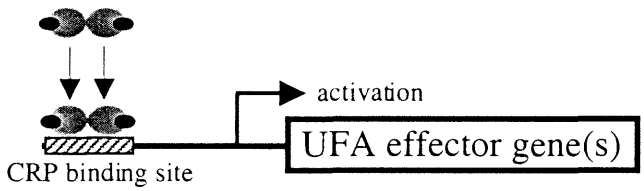

A.
Percent composition
18:1
20:5
0
2
4
6
8
10
12
14
16
18
SS9 15°C
SS9 15°C + glc
SS9 15°C + glc + cAMP
SS9 0.1 MPa/9°C + glc
SS9 28 MPa/9°C + glc
crp- 15°C
crp- 28 MPa/9°C

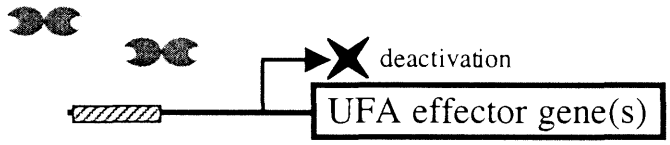

B.
Percent composition
16:1
18:1
0
5
10
15
20
25
30
E. coli 18°C
E. coli 18°C + glc
crp- 18°C

Low pressure minus glucose



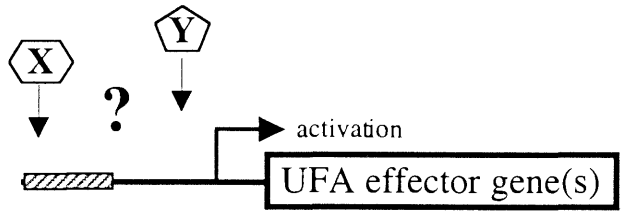
18:1 / 20:5

Low pressure plus glucose



decreased
18:1 / 20:5

High pressure



elevated
18:1 / 20:5

APPENDIX 2

Effect of *fabB* mutation on growth at high pressure in

Photobacterium profundum strain SS9

In the type II, dissociated fatty acid synthase system, β-ketoacyl-acyl carrier protein-synthase I (KAS I), product of the *fabB* gene, plays an essential role in monounsaturated fatty acid (MUFA) synthesis (3). In *E. coli*, the 10-carbon stage of fatty acid synthesis represents the branch point between production of unsaturated and saturated fatty acids. KAS I is responsible for elongation of the cis-3-decanoyl intermediate required for further synthesis of 16- and 18-carbon unsaturated fatty acids (UFAs). In *E. coli fabB* is an essential gene. *E. coli* mutants harboring temperature-sensitive *fabB* lesions are unable to grow at the non-permissive temperature without exogenous UFA supplementation to the growth medium.

It was predicted that disruption of *fabB* in SS9 would not be lethal due to the presence of two fatty acid synthases, the type II mechanism for MUFA synthesis and the PFA synthase system for polyunsaturated fatty acid synthesis. Furthermore, analysis of an oleic acid auxotrophic chemical mutant, strain EA2, exhibited a MUFA phenotype which may be consistent with *fabB* disruption. Strain EA2 is essentially devoid of MUFAs however greatly upregulates PUFA production and is capable of growth at low temperature and high pressure (2). The goals of this investigation were threefold: (1) investigate the role of KAS I (FabB) in growth of SS9 at high pressure; (2) determine if the nature of the EA2 mutation resided in the *fabB* gene; and (3) investigate the interplay between MUFA and PUFA synthesis in SS9.

The SS9 *fabB* gene was cloned by the generation of PCR primers designed by alignment of known *fabB* gene sequences present in GenBank including *E. coli*, *Haemophilus influenzae*, and *Pasteurella multocida*. Once an internal fragment of

fabB was obtained from SS9 genomic DNA, inverse PCR was used to attain additional upstream and downstream sequence. SS9 *fabB* was found to possess a high degree of identity and similarity to the *E. coli fabB* gene, 71 and 83% respectively over 392 amino acids. With the complete gene sequence at hand, the *fabB* gene was subsequently amplified from strain EA2 and sequenced. The sequence of the SS9 and EA2 *fabB* gene sequences were found to be identical indicating it is not the source of the UFA mutation in this strain. Further experiments are underway in the Bartlett Lab to identify the nature of the EA2 mutation.

Insertional inactivation of *fabB* was performed by the cloning of an internal fragment of the SS9 *fabB* gene into the suicide vector pMUT100 and conjugation into strain SS9R following previously described procedures (1, 2). Kanamycin resistant exconjugants were PCR screened using primers flanking the insertion site to confirm *fabB* disruption.

The engineering and stable propagation of an SS9 strain containing an insertionally inactivated *fabB* indicates it is not an essential gene in SS9, at least not under standard cultivation conditions (15°C, 0.1 MPa). Growth at 15°C, 0.1 MPa was nearly identical to wild-type SS9. Unfortunately, growth experiments at low temperature, ie. 4°C, have yet to be performed. Figure 1 shows the growth profiles of the *fabB* strain ± 0.025% Tween 80 (18:1) and parental strain SS9R at 28 MPa, 9°C. The *fabB* mutant represents perhaps the most pressure sensitive strain thus far isolated in SS9 exhibiting essentially no growth at high pressure. However, supplementation of the *fabB* mutant with 18:1 in the form of Tween 80 permits significant

complementation of the high pressure growth defect, an effect seen with other SS9 UFA mutants (1, 2).

Interestingly, maintenance of *fabB* high pressure cultures for extended periods results in prominent growth. It would appear that the acquisition of secondary *fabB* suppressor mutations allows for growth at high pressure. Subculturing of the *fabB* suppressor strains from extended high pressure cultivation results in nearly unaffected growth at high pressure, comparable to the parental strain (data not shown).

Figure 2 shows the fatty acid profiles of the *fabB* and SS9R strains at 0.1 MPa, 9°C and at high pressure in the *fabB* suppressor strain, designated *fabB**. Analysis of the fatty acid phenotype of the *fabB* mutant was similar to results previously attained with treatment of SS9 cultures with the antibiotic cerulenin (2). Cerulenin specifically and irreversibly inhibits fatty acid condensing enzymes KAS I and KAS II (FabF). The similarity in fatty acid profiles of a *fabB* strain and cerulenin-treated cells is not surprising considering *E. coli* KAS I is significantly more sensitive to the antibiotic than KAS II, effectively creating a *fabB*-like phenotype (4). *fabB* mutation resulted in significant reductions in 16:1 and 18:1 levels together with compensatory increases in 20:5. Surprisingly, the *fabB** strain at high pressure exhibited a fatty acid profile essentially devoid of MUFAs while producing nearly two-fold more 20:5 than the parental strain.

It is assumed that the inability to produce MUFAs at high pressure results in the high pressure sensitive phenotype in the *fabB* strain. However, why then is the *fabB** suppressor strain able to grow at high pressure in the absence of increased UFAs? It

would appear the nature of the suppressor mutation(s) do not influence UFA synthesis. Clearly, it will be of tremendous interest to map the location of the second-site suppressor mutation.

The fact that 20:5 percent composition is coordinately elevated in the presence of MUFA deficiency suggests interaction between the two fatty acid synthase systems in SS9. Furthermore, PUFA deficiency results in compensatory increases in MUFAs (2). Whether this coordination occurs at the level of precursor partitioning, direct activation of biosynthetic genes and/or gene products, or some auxiliary regulatory process is not known and awaits further characterization.

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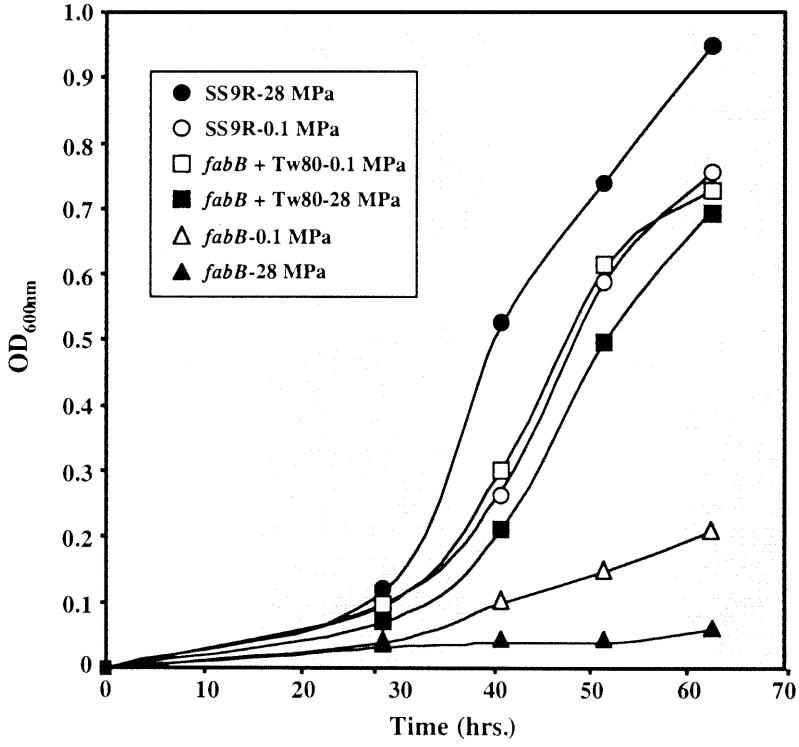
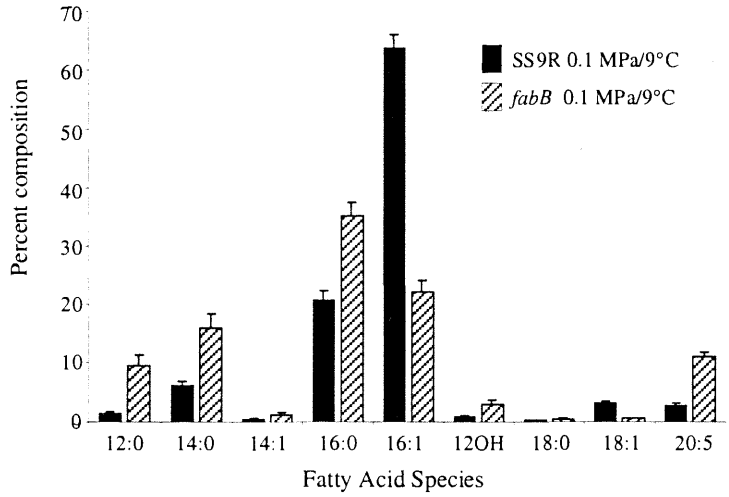
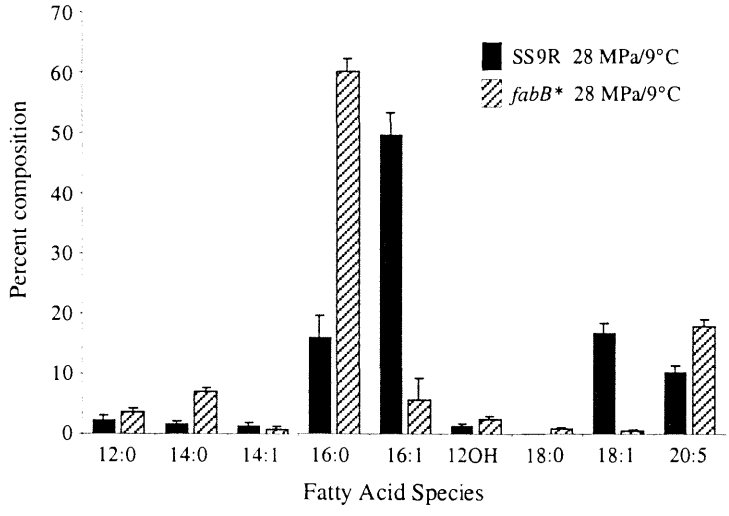

SS9R-28 MPa
SS9R-0.1 MPa
fabB + Tw80-0.1 MPa
fabB + Tw80-28 MPa
fabB-0.1 MPa
fabB-28 MPa
OD600nm
Time (hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
10
20
30
40
50
60
70

A. Low Pressure



B. High Pressure



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Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium *Photobacterium profundum* strain SS9

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Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) have been shown to be of major importance in the promotion of cardiovascular health, proper human development and the prevention of some cancers. A high proportion of bacterial isolates from low-temperature and high-pressure marine environments produce EPA or DHA. This paper presents the sequence of a 33 kbp locus from the deep-sea bacterium *Photobacterium profundum* strain SS9 which includes four of the five genes required for EPA biosynthesis. As with other bacterial *pfa* (polyunsaturated fatty acid) genes, the deduced amino acid sequences encoded by the SS9 genes reveal large multidomain proteins that are likely to catalyse EPA biosynthesis by a novel polyketide synthesis mechanism. RNase protection experiments separated the SS9 *pfa* genes into two transcriptional units, *pfaA–C* and *pfaD*. The *pfaA* transcriptional start site was identified. Cultivation at elevated hydrostatic pressure or reduced temperature did not increase *pfa* gene expression despite the resulting increase in percentage composition of EPA under these conditions. However, a regulatory mutant was characterized which showed both increased expression of *pfaA–D* and elevated EPA percentage composition. This result suggests that a regulatory factor exists which coordinates *pfaA–D* transcription. Additional consideration regarding the activities required for PUFA synthesis is provided together with comparative analyses of bacterial *pfa* genes and gene products.

Keywords: eicosapentaenoic acid, *pfa* genes, hydrostatic pressure, polyketide synthase, ribonuclease protection assay

INTRODUCTION

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5*n*-3: EPA) and docosahexaenoic acid (22:6*n*-3; DHA) are essential components of many animal membrane lipids, well documented for their beneficial effects in human health and role as precursors for many hormone and hormone-like regulatory molecules (Angerer & Schacky, 2000; Lauritzen *et al.*, 2001; Sauer *et al.*, 2001). PUFAs can be obtained directly from dietary sources or synthesized by chain elongation and aerobic desaturation of pre-existing fatty acids such as linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) (Beaudoin *et al.*, 2000; Parker-Barnes *et al.*, 2000). Marine algae and numerous fungal micro-organisms produce significant quantities of PUFAs via aerobic pathways involving elongation/desaturation (Bajpai & Bajpai, 1993).

PUFAs were once thought to be absent in bacterial membranes (Erwin & Bloch, 1964), but numerous bacterial species of marine origin have now been shown to produce very-long-chain PUFAs such as EPA and

Abbreviations: ACP, acyl carrier protein; AT, acyl CoA:ACP transacylase; CLF, chain length factor; DHA, docosahexaenoic acid; DH/I, dehydratase/isomerase; EPA, eicosapentaenoic acid; ER, enoyl reductase; FAS, fatty acid synthase; KR, β-ketoacyl-ACP reductase; KS, β-ketoacyl-ACP synthase; PKS, polyketide synthase; PPTase, phosphopantetheinyl transferase; PUFA, polyunsaturated fatty acid; RPA, ribonuclease protection assay.

The GenBank accession numbers for the sequences reported in this paper are AF409100 and AF467805.

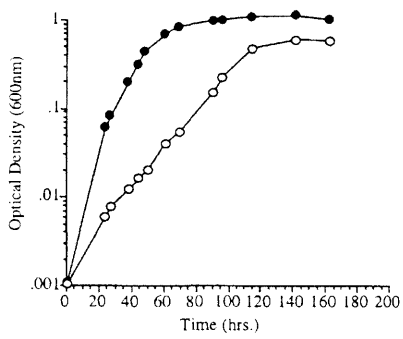

FIG. 3. Nutritional complementation of *P. profundum fabF* mutant EA40 at elevated pressure (28 MPa, 9°C). ●, plus 18:1 supplementation; ○, minus 18:1.

high-pressure growth phenotype in strain EA40. To address this possibility, strains EA40 and DB110 were grown at 4 and 15°C (0.1 MPa) under conditions identical to those used with pressure cultivation (in heat-sealable bulbs with glucose and HEPES added). These experiments showed no apparent cold sensitivity in strain EA40, suggesting that the high-pressure-sensitive phenotype of EA40 is the result of pressure effects and not the result of cultivation conditions (data not shown).

Nutritional complementation of strain EA40. If the basis of the high-pressure growth defect in EA40 stems from its reduced abundance of 18:1, it should be possible to nutritionally complement this defect by providing an exogenous supply of this fatty acid in the growth medium. This approach was previously used with partial success in overcoming the pressure-sensitive and cold-sensitive growth characteristics of a chemical mutagen-derived mutant of SS9 deficient in the production of both 16:1 and 18:1 fatty acids (1). The growth characteristics of mutant EA40 at 28 MPa (9°C) in the presence or absence of exogenous 18:1 in the form of 0.025% Tween 80 are shown in Fig. 3. In the presence of exogenous 18:1, mutant EA40 exhibited completely restored growth characteristics at elevated pressure. Fatty acid analysis of inner and outer membrane and total phospholipid fractions of strain EA40 grown in the presence of Tween 80 at 28 MPa (9°C) revealed incorporation of 18:1 into the membrane phospholipids, suggesting that the exogenous 18:1 supplied is actively utilized for membrane restructuring (data not shown). These results provide further evidence of the need for 18:1 fatty acid for growth at high pressure.

***E. coli* does not regulate fatty acid composition in an adaptive manner in response to pressure changes.** Although the effects of pressure on fatty acid composition in numerous piezophilic and piezotolerant bacteria have been well documented (1, 11, 12, 47), the effect of a pressure increase on the fatty acid composition of a non-pressure-adapted bacterial species has not yet been described. To determine whether the observed effect of elevated pressure on deep-sea bacterial fatty acids is reflective of an adaptive feature specific to microorganisms which have evolved in high-pressure, pressure-variable environments, or if the response simply reflects a similar physical effect of high pressure and low temperature on some aspect of cell structure or physiology (i.e., membrane fluidity), experiments were conducted using *E. coli* as a representative of a mesophilic bacterial species.

Table 3 shows the fatty acid profiles of *E. coli* strains MR86 (*fabF*::Km^r) and its parental strain SJ16 at 30 MPa (37°C) and 0.1 MPa (37°C) in pressurizable bulb cultures. As described in Materials and Methods, high-pressure cultivation necessitated growth under microaerobic conditions with glucose and HEPES added to the media. In contrast to the fatty acid changes observed in wild-type *E. coli* strains in response to temperature downshift, wherein increased production of UFAs 18:1 and 16:1 are observed at the expense of 16:0 (33), no changes in fatty acid profile were evident when either strain (SJ16 or MR86) was grown at high pressure (Table 2). The UFA/SFA ratios of strain SJ16 at the two pressures were essentially identical, 0.41 at 30 MPa and 0.38 at 0.1 MPa. Similar results were obtained for *fabF* strain MR86 at various pressures. At 30 MPa (37°C), strain MR86 experiences a slight reduction in UFA production and an increase in SFA content relative to 0.1 MPa (37°C) cultivation. Specifically, high-pressure incubation resulted in an overall increase in 16:0 in combination with a reduction in 16:1 content. Consequently, substantially reduced UFA/SFA ratios are observed at elevated pressure in this strain (0.16 at 30 MPa, compared to 0.38 at 0.1 MPa). Unlike pressure increase, temperature decrease (from 37°C to 15°C) elicited approximately a twofold increase in UFA/SFA ratios in both strains (approximately 0.6 to 1.2; our unpublished results).

Results comparable to those obtained with SJ16 and MR86 were obtained with other *E. coli* strains, specifically MG1655 (21) and 1100 (15), which also displayed lower UFA/SFA ratios at 30- than at 0.1-MPa cultivation. Moreover, fatty acid

TABLE 3. Fatty acid composition of *E. coli* strains as a function of varying pressure[a]

Fatty acid species	Mean wt% fatty acid species ± SD			
	Wild type		*fabF*	
	0.1 MPa	30 MPa	0.1 MPa	30 MPa
12:0	4.54 ± 0.3	4.89 ± 0.9	3.75 ± 0.6	3.96 ± 0.2
14:0	10.82 ± 1.7	9.81 ± 1.4	11.29 ± 0.0	11.07 ± 1.8
16:0	47.02 ± 4.5	49.22 ± 9.1	50.79 ± 6.2	62.54 ± 0.1
16:1	14.55 ± 5.3	15.24 ± 0.6	24.43 ± 9.6	12.60 ± 1.9
18:0	1.63 ± 1.0	1.74 ± 2.2	0.72 ± 0.4	1.51 ± 0.8
18:1	11.90 ± 2.1	9.70 ± 4.7	1.10 ± 0.1	0.46 ± 0.2
14-OH	9.54 ± 1.1	9.40 ± 1.6	7.92 ± 2.5	7.86 ± 0.2
UFA/SFA ratio[b]	0.41	0.38	0.38	0.16

[a] Data represents values derived from triplicate samples harvested in mid-exponential-phase growth. Cultures were grown at 37°C in LB medium containing 22 mM glucose buffered with 100 mM HEPES.

[b] UFAs, 16:1 and 18:1; SFAs, 12:0, 14:0, 16:0, and 18:0.

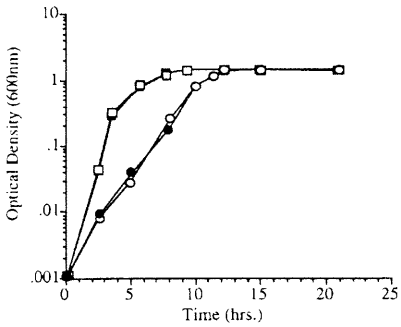

FIG. 4. Growth characteristics of *E. coli fabF* disruption mutant MR86 and parental strain SJ16 as a function of pressure. See Materials and Methods for cultivation conditions. Pressure experiments were conducted at a temperature of 37°C. ●, SJ16 at 30 MPa; ○, MR86 at 30 MPa; ■, SJ16 at 0.1 MPa; □, MR86 at 0.1 MPa.

analysis was conducted on all four of the *E. coli* strains studied at 30 and 0.1 MPa at a temperature of 30°C in addition to 37°C. No dramatic fatty acid content differences were observed in any of the *E. coli* strains at 30 MPa (30°C) compared to 0.1 MPa (30°C) cultivation (data not shown). The conclusion from these studies is that *E. coli* does not modulate fatty acid production in an adaptive manner in response to a pressure increase, and therefore the response of many piezophilic bacteria to increase UFA levels with pressure is likely to be an acclimatory feature specific to microorganisms inhabiting high-pressure environments.

Similar to arguments made with *P. profundum* strain EA40, it is possible that the lack of fatty acid regulation at elevated pressure in *E. coli* strains is the result of anaerobic rather than pressure effects. When grown at 15 and 37°C (0.1 MPa) in pressurizable bulbs, all *E. coli* strains (MR86, SJ16, MG1655, and 1100) exhibited approximately twofold increases in UFA/SFA ratios and no apparent decreased temperature sensitivities (data not shown). These results suggest that the lack of an adaptive fatty acid regulatory response of *E. coli* with exposure to elevated pressure is not a factor of the conditions used for pressure cultivation.

***E. coli fabF* mutants do not display increased pressure sensitivity.** Another contrasting feature between SS9 and *E. coli* concerns the role of KAS II in growth at elevated pressure. The growth characteristics of *E. coli* strain SJ16 and its *fabF* mutant derivative MR86 at various pressures are shown in Fig. 4. Previous reports have indicated that *E. coli fabF* mutants are not cold sensitive (20). Likewise, at both 0.1 and 30 MPa (37°C), the growth of strain MR86 was identical to that of parental strain SJ16. Finally, no differences in the growth characteristics of the two strains were observed even when pressure cultivation was performed at reduced temperatures (30 and 15°C, 30 MPa; data not shown). Thus, even the combined effects of elevated pressure and reduced temperature did not result in differential growth susceptibility in *fabF* strain MR86 relative to strain SJ16.

DISCUSSION

The state of the physical environment (i.e., pressure and temperature) influences the physical properties of biological membranes (22). Decreases in temperature or increases in hydrostatic pressure increase the molecular order of the fatty acyl chains and promotes tighter packing of the phospholipids. The biological response to such environmental conditions often entails the retailoring of membrane composition, most notably increases in fatty acid unsaturation (39, 49). This modification is believed to optimize membrane structure and function by offsetting the direct effects imposed by temperature and pressure.

Previous studies employing the deep-sea bacterium *P. profundum* strain SS9 have revealed direct correlations between UFA production and growth ability at low temperature or elevated pressure (1, 4). In the present study, we have targeted a key enzyme involved in UFA production, the KAS II product of the *fabF* gene. Results presented here indicate that SS9 KAS II is required for (i) the piezoregulation of *cis*-vaccenic acid (18:1) and (ii) piezophilic growth. In addition, our data indicate that pressure modulation of fatty acid levels is likely to be an adaptive feature unique to high-pressure-adapted microorganisms.

Since the *fabF* gene plays an essential role in thermal regulation of fatty acid composition in *E. coli*, we hypothesized that the SS9 *fabF* gene may play a similar role in the increased production of 18:1 observed in response to pressure increase. *P. profundum* strain EA40 containing an insertionally inactivated *fabF* gene was engineered using a reverse genetics methodology employing the suicide plasmid pMUT100 (7). Based on the fatty acid profile of strain EA40, a dual role for SS9 KAS II has been revealed. In addition to temperature regulation, the lack of 18:1 modulation in response to pressure increase in strain EA40 suggests that SS9 KAS II is responsible for the substantial increase in 18:1 levels observed in response to pressure increase in *fabF*$^+$ strains (Table 2).

Studies in *E. coli* have shown that thermal modulation of 18:1 production does not involve de novo enzyme synthesis (17). Such regulation resides at the level of enzyme activity, where KAS II exhibits increased catalytic efficiency at reduced temperature (16, 18). Northern analyses were performed using *P. profundum* strain DB110 cultivated under different pressure and temperature conditions in order to determine if the SS9 *fabF* gene exhibits differential expression. Identical transcripts (sizes and amounts) were detected under all pressure and temperature conditions examined, suggesting that the SS9 *fabF* gene is not transcriptionally regulated in response to various cultivation parameters (Fig. 1). In light of these results, we hypothesize that SS9 KAS II displays increased catalytic activity at elevated pressure just as *E. coli* KAS II does at reduced temperature. Given the high level of identity between KAS II of SS9 and that of non-pressure-adapted organisms (*E. coli* KAS II is 76/88% identical/similar to SS9 KAS II), it will be of interest to analyze the structure and function of SS9 KAS II in comparison to the *E. coli* enzyme. Of course, the fact that SS9 *fabF* is not transcriptionally regulated does not preclude the possibility that some posttranscriptional processing event occurs which influences KAS II abundance at elevated pressure, thereby influencing 18:1 synthesis.

In strain DB110, a pressure increase from 0.1 MPa to 28 MPa results in greater than a fourfold increase in 18:1 levels, rising up to 16% of total fatty acids (Table 2). However, under identical conditions, 18:1 comprised only 1.1% of the total fatty acids in mutant EA40. The inability to regulate 18:1 levels at elevated pressure resulted in pronounced high-pressure sensitivity in this strain (Fig. 2). The fact that supplementation of this mutant with exogenous 18:1 resulted in restoration of wild-type-like growth rates and yields at elevated pressure (Fig. 3) suggests that the 18:1 defect is responsible for its high-pressure-sensitive growth phenotype. These results represent the first reported growth alteration observed in any bacterial

strain in which *fabF* is the only lesion in lipid synthesis. It should be noted, however, that *E. coli* strains harboring a mutation in *fabF* as well as an additional temperature-sensitive mutation in *fabB* (KAS I) are incapable of producing any long-chain fatty acids at the nonpermissive temperature (19).

In contrast to high-pressure conditions, strain EA40 exhibited no apparent growth sensitivity at reduced temperature despite producing only trace levels of 18:1 at 4°C. Contrary to pressure increase, temperature decrease did not elicit substantially elevated 18:1 production in wild-type SS9 strains (Table 2). Hence, some aspect of high-pressure growth ability appears reliant upon 18:1 production which is not apparent at low temperature. The fact that EPA levels increase significantly in response to temperature downshift in mutant EA40 but not with pressure increase is puzzling. It would appear SS9 is capable of compensating for decreased 18:1 levels at 4°C by increased EPA production but not at increased pressure. It could be either the change in global membrane fluidity (or membrane phase state) or that within the local environment of a key membrane protein that accounts for the pressure sensitivity of 18:1-deficient strains. Membrane proteins which are known or implicated as important for piezophilic growth include CydD (required for the assembly of the cytochrome *bd* respiratory complex [26]), RseC (an inner membrane protein of unknown function [5, 9]), and the ToxR transcription factor (which regulates the differential expression of outer membrane protein encoding genes as a function of pressure [45]). It is possible that membrane perturbation resulting from altered 18:1 synthesis contributes to the decreased activity of some key membrane component and consequently pressure sensitivity.

Attempts to complement mutant EA40 by the introduction of the wild-type SS9 *fabF* gene into this strain were unsuccessful. Despite the creation of numerous SS9 *fabF* constructs, expression problems prevented complementation of either the 18:1 defect or the high-pressure-sensitive phenotype of mutant EA40 by *fabF* containing plasmids (our unpublished results). One possibility is that all of our plasmid constructs lacked a *fabF* promoter. It could be that the two *fabF* transcripts observed in SS9 by Northern blotting result from a single promoter upstream of *acpP* and that the *fabF*-specific message arises from a posttranscriptional processing event. However, even SS9 *fabF* containing plasmids which placed *fabF* transcription under the control of the Kan^r promoter on pKT231 (3) still failed to transcribe *fabF*, even when a potential Rho-independent terminator sequence was removed from *fabF* upstream DNA. These results suggest that some mechanism for tight control of *fabF* transcription may exist in SS9. Coordinating *fabF* expression with that of other *fab* cluster genes is critical to viability in *E. coli*. Excess *fabF* transcription leads to the cessation of fatty acid synthesis as a result of blockage of fatty acyl chain elongation (42). Because of these FabF toxicity effects, *E. coli fabF* mutants have yet to be genetically complemented.

Fatty acid compositional adjustment is a near-ubiquitous response to temperature change among bacteria (39). However, unlike temperature, pressure variation is a seldom encountered environmental parameter outside of the deep sea or deep subsurface. Previous studies which have documented pressure regulation of fatty acids in deep-sea bacteria have inferred that such changes reflect acclimation to pressure change (1, 11, 12, 47). However, another possibility is that because high pressure exerts a physical change on membrane structure similar to that of a drop in temperature (30), most microbes would perceive high pressure as low temperature and respond accordingly to restore membrane fluidity or phase. Our results with *E. coli* indicate that at least for this organism, the latter possibility is not the case even though this mesophile is quite piezotolerant, capable of growing at pressures up to 50 MPa (54).

Fatty acid profiling of *E. coli* was performed at various pressures to determine whether fatty acid composition in this mesophile is responsive to pressure change as it is to temperature change. Because various *E. coli* strains were discovered to exhibit similar fatty acid compositions at low and high pressures, the results indicated that the capacity for thermal regulation of UFAs in bacteria does not necessarily predispose microorganisms to respond in a similar fashion to pressure changes, despite the fact that both parameters can be manipulated to produce similar effects on membrane structure. These results are in accordance with observations made using cultures of the mesophilic protozoan *Tetrahymena pyriformis* NT-1 (28). As with *E. coli*, exposure of *T. pyriformis* to 26 MPa did not result in changes in fatty acid composition or fluidity of microsomal membranes. Of course, the possibility exists that not all surface-living bacteria would respond as *E. coli* does to elevated pressure. For example, bacteria which possess membrane-localized desaturases may increase UFA production at elevated pressure due to activation of such enzymes in response to membrane fluidity changes.

While investigating pressure effects on the fatty acids produced by *E. coli*, we also examined the effect of loss of KAS II on *E. coli* growth at elevated pressure (Fig. 4). Previous reports have described the growth characteristics of *E. coli* at elevated hydrostatic pressure (6, 53, 54). Upon pressurization, *E. coli* experiences retardation in growth and reproduction owing in part to inhibition in macromolecular synthesis and cell division (51, 55). The response of *E. coli* to elevated hydrostatic pressure results in a unique stress response which results in induction of numerous heat shock and cold shock proteins as well as many proteins which appear solely in response to high pressure (46). Despite numerous studies having investigated high-pressure effects on specific functions in *E. coli*, it is still unclear as to the key pressure point(s) which limits its growth ability at elevated pressure (32, 37). Due to the physical effects elevated pressure is known to exert on biological membranes (22), it was hypothesized that the state of the membrane could represent such a pressure point. Since an *E. coli fabF* mutant did not exhibit increased pressure sensitivity, something other than membrane phospholipid structure most likely limits *E. coli* growth at high pressure. Possible limiting factors include aspects of chromosome partitioning (6), macromolecular synthesis (51), cytochrome function (26), or proton translocation and ATP production (32).

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CHAPTER V

Structure and Regulation of the Omega-3 Polyunsaturated Fatty Acid Synthase Genes from the Deep-Sea Bacterium *Photobacterium profundum* strain SS9