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Evolutionary and Functional Genomics of Bacteria from the Cold Deep Sea

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Oceanography

by

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DEDICATION

In memory of Giorgio “Midget” Lauro

1946-2007

EPIGRAPH

"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales"

Marie Curie

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LIST OF ABBREVIATIONS

6–4PP	pyrimidine-pyrimidone 6–4 photoproduct
ACT	Artemis comparison tool
ATP	adenosine triphosphate
AABW	Antarctic bottom water
BLAST	basic local alignment search tool
CPD	cyclobutane pyrimidine dimmer
cDNA	complementary DNA
CDW	circumpolar deep water
c.f.u.	colony forming units
COG	cluster of orthologous groups
CS	cold sensitive
DHA	docosahexaenoic acid
DMT	drug/metabolite transporter
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EPA	eicosapentaenoic acid
EPS	extracellular polysaccharide
HSP	high-scoring segment pair
Km	kanamycin
LGT	lateral gene transfer
LPS	lipopolysaccharide
MCP	methyl-accepting chemotaxis protein
NADW	North Atlantic deep water
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acid
ppGpp	guanosine-5'-di-3'-diphosphate
PS	pressure sensitive
Rif	Rifampicin
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcription PCR
Sm	streptomycin
ssDNA	single stranded DNA
tRNA	transfer RNA
UV	ultra-violet

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ABSTRACT OF THE DISSERTATION

Evolutionary and Functional Genomics of Bacteria from the Cold Deep Sea

by

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Chapter 1 introduces the deep sea environment. Recent studies of sequence-independent diversity of deep-sea microbial communities are reviewed and re-analyzed. These are compared to sequence-dependent studies evidencing the need for new culturing and isolation methods for deep-sea bacteria. The chapter ends by reviewing the current state-of-the-art of deep-sea genetics and genomics and describes the general features of the genomes of piezophiles.

Chapter 2 describes the development of three new vectors for use in *Photobacterium profundum* and other Gram-negative bacteria. The first one (pEE3) is a suicide vector for insertional inactivation of genes. The second one is a general purpose cloning vector (pFL122) based on the broad-host-range replicon RSF1010. The third one is a broad-host-range vector carrying an arabinose-inducible promoter to use for gene expression studies.

Chapter 3 explores the diversity evolution of cultured piezophiles. Three novel strains of piezophilic bacteria were isolated, one of them being the first Gram-positive piezophile ever isolated. Most of the cultured piezophiles, belong only to a narrow range of phylogenetic lineages which cluster within clades of known psychrophiles. In the ensuing discussion the hypothesis that cold-adapted piezophiles might evolve from polar water psychrophiles and disseminated through deep-sea oceanic currents is presented. During the study specific ribosomal RNA features of the 16S of piezophiles were identified. These features are correlated with the pressure adaptation of the strains.

Chapter 4 describes a high-throughput transposon mutagenesis approach to identify genes important for growth at low temperature and high pressure. These genes belong to a wide variety of categories which are discussed in detail. A large fraction of the mutants impaired in growth at low temperature had gene disruptions in genes for the synthesis of extracellular polysaccharides, providing the first direct evidence of the role of the cell envelope in adaptation to low temperature.

Chapter 5 presents the results of the sequencing and the analysis of the genome of the shallow water strain *P. profundum* 3TCK. The genome is compared to that of the deep-water strain *P. profundum* SS9 identifying unique regions that might be conferring fitness in different habitats. Among these is a cluster of genes for motility important for swimming in high viscosity found only in the deep-water strain, a set of genes for UV resistance found only in the shallow water strain, and the specific rRNA features described in chapter 3 found only in the deep-water strain. The motility gene clusters are further characterized evidencing the role for particular flagellins and motor proteins for swimming at high pressure. The UV and ribosomal features are exchanged between the shallow- and the deep- water strains and the phenotypes of the resulting strains are characterized.

1.

Prokaryotic lifestyles in deep sea habitats.

Abstract.

Gradients of physicochemical factors influence the growth and survival of life in deep-sea environments. Insights into the characteristics of deep marine prokaryotes has greatly benefited from recent progress in whole genome and metagenome sequence analyses. Here we review the current state-of-the-art of deep-sea microbial genomics. Ongoing and future genome-enabled studies will allow for a better understanding of deep-sea evolution, physiology, biochemistry, community structure and nutrient cycling.

Introduction

The largest fraction of the ocean is at depths >200 meters and is believed to contain $6.5x10^{28}$ cells, accounting for 55% of all the prokaryotes found in aquatic habitats (101), but very little is known about the adaptations allowing for growth and survival of microbes in the deep sea. In general abyssal (3,000-6,000 m) and hadal (>6,000 m) environments are characterized by low temperature, high hydrostatic pressure and the absence of solar radiation (15). This physically uniform environment is believed to be populated by a high diversity but low abundance of organisms (37, 93) and is occasionally interrupted by outbursts of activity in the locations of hydrothermal vents (85), whale falls (92) and cold seeps (34). Here chemoautotrophic

metabolisms dominate, fuelled by the presence of sources of reduced inorganic compounds.

Outside of these deep-sea oases, microbial life was, until recently, believed to be largely heterotrophic and supported by sporadic influxes of nutrients from the overlying productive layers of the water column (104). However, recent studies about the unique nature of deep-sea organic carbon (8) and nitrogen (9, 25) combined with the fact that the dominant group of archaea (57) in the deep sea might be chemoautotrophic (36, 65, 96) could change this view.

In the dark cold abyss, the most unique physical parameter is hydrostatic pressure. It can be more than 3 orders of magnitude higher than on the ocean's surface, reaching a maximum at ~110 MPa at the bottom of Challenger Deep in the Mariana Trench. Vertical zonation has often been explained by differential adaptation of some phylogenetic groups to this physical parameter. In particular the term "piezophile" (from the greek verb *piezo*, to press (111)) has been created to describe those microorganisms with optimal growth at pressures >0.1 MPa.

The field of piezomicrobiology was born more than 100 years ago (for a brief history see (91) but has suffered from the requirement for specialized and expensive collection vehicles such as ROVs and free vehicles, in addition to high-pressure culturing equipment. For this reason the number of scientists and labs involved in high-pressure microbiology studies is still limited.

Piezophilic microorganisms have been isolated from many regions of the world, displaying pressure optima for growth that span the entire range of pressures existing in the ocean biosphere (80, 113). Most of these isolates belong to the genera

Colwellia, *Moritella*, *Photobacterium*, *Pyschromonas*, and *Shewanella* within the gamma proteobacteria (28, 59, 61, 77-82, 108, 109) with the notable exception of two sulphate reducing isolates of the genus *Desulfovibrio* (3, 13) and one Gram-positive member of genus *Carnobacterium* (68). It is believed that these isolates represent only a small fraction of the phylogenetic and physiological diversity present in hadal and abyssal environments. All of these "confirmed" inhabitants of the cold deep sea form distinct clades within phyla of microbes from polar regions suggesting common ancestry and that adaptations to low temperature could be a pre-requisite for the initial acclimation to the deep sea (68). However, these isolates probably only represent the copiothrophic opportunists (r-strategists) and new culturing approaches (86, 116, 117) will have to be developed in order to isolate other members of the community.

In recent years, advances in biodiversity, genomic technology, and the environmental biotechnology of secondary metabolites (91), has revived the interest in this field of microbiology. This short review summarizes the state of the art of deep-sea microbial genomics and metagenomics.

The molecular diversity of deep-sea microbial communities.

When delving into the ecology of any environment, the first question that comes to mind is invariably "who is there". Less than 1% of the marine microbes can be cultured (37, 87) and despite the fact that a considerable amount of effort has been put forth in recent years to "culture the unculturable" (65, 86) most of our knowledge of deep-sea microbial diversity comes from culture independent studies.

The use of 16S rRNA in microbial ecology is well established (83) and has been applied to ocean samples from surface waters for many years (26, 37, 40). Similar studies using deep-sea samples were not initiated until later (28, 60, 81). These investigations provided the first clues to the extreme complexity of low-temperature deep-sea microbial communities.

More recently DeLong and colleagues (29) end-sequenced ~5000 fosmid clones from metagenomic libraries generated at 7 different depths (10m, 70m, 130m, 200m, 500m, 770m, 4000m) from station ALOHA (56) in the North Pacific Subtropical Gyre. This study provided the first snapshot of the vertical zonation of genes and pathways in the oligotrophic open ocean.

When the sequences obtained were classified by their best TBLASTX high scoring sequence pair match (HSPs), the photic zone (10m, 70m, 130m) samples were enriched in *Prochlorococcus*-like sequences while the deepwater (500m, 770m, 4000m) samples were enriched in δ-proteobacteria-like, *Actinobacteria*-like and *Planctomycete*-like sequences.

Sequences of putative α-proteobacterial origin were highly abundant at all depths, while those of putative γ-proteobacterial origin only constituted a small fraction of the total at all depths. Readers interested in more detailed analyses of this work are referred to Worden et al. (2006).

A more comprehensive survey of the vertical zonation of phylogenetic groups of microbes in an oligotrophic marine setting was performed by Sogin and colleagues in the North Atlantic Ocean (93). To avoid PCR-generated biases and increase the number of samples analyzed only the V6 hypervariable region of the 16S rRNA was

sequenced using 454 technology (72). A previous study had shown that in most cases the sequence variation within the V6 region is sufficient to broadly characterize the phylogenetic lineage of an organism (66).

Here we re-analyzed this dataset (materials and methods are provided as supplementary material) to show the variation of community composition as a function of depth (figure 1.1) and temperature. Some of the same trends observed for the ALOHA station sample were found. In particular α-proteobacterial tags were highly abundant in samples from all depths and temperatures and the deepest low temperature sample (112R) showed an increase in the relative abundance of tags classified as δ-proteobacteria, *Actinobacteria* and *Planctomycete.*

However, in contrast to the ALOHA station results, sequence tags of γ-proteobacteria were highly abundant in all samples, with percentages increasing at higher depths in the cold-water samples (figure 1.1b). This increase with depth in the percentage of δ- and γ-proteobacteria at the expense of α-proteobacteria was also observed in a depth comparison of samples from the Mediterranean and the Greenland seas (115).

The discrepancy between the Delong et al. (2006) and Sogin et al. (2006) results may be explained in 3 ways: 1) a fundamental differences between the 2 sets of samples, 2) technical differences in the sampling and processing of the samples or 3) a bias in the V6-tag database.

16S-based quantitative assessments of microbial communities are inherently skewed by differences in copy number of ribosomal operons among different phyla (98). Members of the phylum α-proteobacteria, for example, usually contain only one

copy, as opposed to some γ-proteobacteria that can contain as many as 15, leading to an over-estimation of γ-proteobacteria when compared to other genetic markers.

Another complicating factor in the culture-independent studies of deep-sea samples is the problem of discriminating against surface derived bacteria. Many microbes will become attached to sinking particles, making their way to sediments and waters of the deep sea where they can survive in a metabolically inactive state for indefinite periods of time. For example, spores of surface-derived *Clostridium* sp. have been recovered from Japan Trench sediments at a depth of 6.3-7.3 km (67). Upon germination, the vegetative cells showed no pressure adaptation, but the spores were extremely pressure resistant. Non-sporeforming bacteria can also exhibit remarkable pressure tolerance when starved (19). The role of these "foreign" species in nutrient and energy cycling might be only passive, because of their contribution to the pool of organic matter that is removed from photic layer. Therefore, the understanding of biochemical cycling in the ocean requires means of distinguishing between autochthonous and allochthonous members of the community.

One solution to the problem is to pre-filter the collected water samples in order to remove surface-derived detritus (105), at the risk of losing those active members of deep-sea communities that colonize and degrade marine snow. Interestingly, one of the main differences in sample processing between the station ALOHA sample and the North Atlantic sample was that the first one was pre-filtered while the second one was not (Herndl and Sogin, personal communication). This difference between the two samples could have added or subtracted a large fraction of the population of γ-

proteobacteria in the water column which live attached to particles as opposed to the predominantly free-living lifestyle of α-proteobacteria (27).

High pressure, DNA damage and cell division.

A number of reviews have been published on the physiological adaptations of microorganisms to the deep sea (14, 16, 17, 58, 71, 91). These studies have traditionally focused on the effects of high hydrostatic pressure, a physical parameter whose increase will inhibit any process resulting in a positive volume change (44).

Deep-sea bacteria have been shown to possess piezo-specific adaptations in terms of membrane phospholipid fatty acid unsaturation (4, 6, 30) and cell division (20, 53, 55, 112). Moreover, hydrostatic pressure has been shown *in-vitro* to affect many protein-protein (90) and protein-DNA (24, 95) interactions, and, *in-vivo* to hinder essential cellular processes such as replication and translation (52, 100, 114).

One interesting observation is that when a microorganism is grown above or below its optimal hydrostatic pressure, it tends to become filamentous (55, 112). In *E. coli* a partial explanation for this phenomenon has been provided: high pressure enhances the activity of the Mrr cryptic endonuclease (1) which produces double stranded DNA damage, triggering an SOS response (2). One aspect of this signaling pathway is the increase of the SulA regulatory protein which in turn inhibits FtsZ-mediated septal ring formation (2). This pressure-triggered SOS response can be exacerbated by mutation of the gene encoding the lon protease, an enzyme which postranslationally limits SulA abundance. The only caveat in this otherwise elegant story is the fact that filamentation still occurs in *sulA* mutants (2, 53, 62), suggesting

that an alternative filamentation pathway must also exist. The second pathway could arise from high hydrostatic pressure inhibition of the polymerization of tubulin-like protein FtsZ (53).

The DNA damage signal for pressure-induced double-stranded DNA breaks is not known at this time but could involve RecD, which together with RecB an RecC composes exonuclease V, an enzyme with multiple activities centered on the control of genetic recombination (10). *recD* mutants in the moderate piezophile *Photobacterium profundum* SS9 are pressure sensitive (20, 23) and the introduction of the *recD* gene from *P. profundum* into *E. coli* inhibits cell filamentation at high pressure (20).

Interestingly, the piezo-sensitive *recD* mutant of *P. profundum* SS9 was isolated in a screen performed at atmospheric pressure for mutants altered in the production of the outer membrane protein OmpH (23) and only subsequently was found to be pressure sensitive. *ompH* gene expression is induced at low pressure under carbon and energy starvation (18), a condition that is known to trigger an SOS response (54). Consistent with this possible SOS connection, microarray studies of *P. profundum* SS9 indicate the upregulation of many genes associated with DNA repair (including *uvrA* and *uvrD*) when the cells are grown at atmospheric pressure but not when grown at 28 or 45 MPa (22). And, as previously noted by Aertsen et al. (2004) outer membrane proteins in *E. coli* are also known to be controlled by the SOS response (39). Understanding the pathway by which this non-traditional SOS response is pressure-triggered should be a priority for future studies.

High pressure and protein synthesis.

An essential cellular process inhibited by hydrostatic pressure is protein synthesis. This has been explained through the dissociation of the ribosomal subunits that has been detected both *in vitro* (43, 45, 89) and *in vivo* (7, 76). Transcriptome and proteome studies have shown that high hydrostatic pressure induces the synthesis of a number of ribosomal and heat-shock proteins in *Lactobacillus sanfranciscensis* (49, 84) and *E. coli* (52, 100). The triggering of a heat-shock response is particularly interesting as the pressures used in these studies (<100 MPa) are too low to cause pressure-denaturation of proteins (90). Instead, it is compatible with the hypothesis of Hormann et al. (49) that suggest it is due to a partial loss-of-function in the ribosomes that produce truncated and misfolded proteins. As was the case for the SOS response, in the deep-sea bacterium *P. profundum* SS9 the opposite effect is observed with heat-shock genes being overexpressed at atmospheric pressure (99).

16S ribosomal RNA comparisons between strains from different depths has identified structures that appear to be specific to the piezophiles (68), suggesting functional constraints for ribosomal function in the deep sea. Additional work will be required to clarify the extent of ribosomal adaptation to high pressure.

Genomic adaptations to the deep sea.

Recently we have initiated with colleagues the analysis of the first five genomes of psychropiezophilic (cold- and pressure-loving) bacterial isolates: *Photobacterium profundum* SS9 (99), *Shewanella* sp. KT99 (68), *Moritella* sp. PE36 (28), *Psychromonas* sp. CNPT3 (28), *Carnobacterium* sp. AT7 (68). Each of these

strains has a number of phylogentically closely related species/strains that are adapted to surface water conditions (Table 1).

To aid in comparing related species differing in adaptation to deep-sea environments we have adopted the term “bathytype” (from the greek word *bathos*, depth) to identify depth-specific ecotypes, defined as “a population of a species genetically adapted to a certain depth in the water column”. The very existence of closely related microbes differing in their bathytypes implies that relatively little genetic change is required to evolve and adapt to the deep sea. In fact, genomic comparison between strain SS9 (82) and strain 3TCK (22), the two bathytypes of *Photobacterium profundum* whose genome are completely sequenced, has revealed a high degree of synteny.

What are the genomic features that restrict the depth of each bathytype?

All the deep bathytytpes possess a high ratio of rRNA operon copies/genome size (figure 1.2) and larger-than-average intergenic regions (figure 1.3). The number of ribosomal operons in a genome has been correlated with the ecological strategy of bacteria (64): the highest numbers of operons are present in bacteria which respond most rapidly to environmental changes. Similarly, small intergenic regions have been correlated with an equilibrium strategy (K-strategy) (70) as observed in the cosmopolitan marine clade SAR-11 (41). Taken together these observations suggest that most of the deep bathytypes in culture have an opportunistic (r-strategy) lifestyle with a high degree of gene regulation. Future studies will help clarifying if this strategy is selected by deep-sea conditions or simply reflects a bias in piezophile isolation and cultivation. Interestingly, the analysis of two 16S-containing δ-

proteobacterial cosmid clones from a metagenomic library constructed from a 500m deep Antarctic water sample showed that the average intergenic size varied from 163 bp to 858 bp (74), suggesting that both strategies might coexist at intermediate depths.

Other hallmarks of all the current deep bathytype genomes are the presence of a large number of genes involved in membrane unsaturation, such as Δ-9 desaturases and polyketide synthase gene clusters (5, 6) for the synthesis of polyunsaturated fatty acids (Table 1), and the absence of photolyase (*phr*) genes. The *phr* gene product (110) uses blue-light energy in order to repair cyclobutane pyrimidine dimers in UV irradiated DNA. It is therefore expected to be absent from the genomes of any organisms that are never exposed to light. The previously mentioned metagenomic analysis of the vertical distribution of genes in the central Pacific Ocean (29) showed that *phr*-like genes are substantially under-represented below the photic zone.

This bias in *phr* distribution is not universal. Indeed, photolyase-like genes are present in the genomes of several bacterial isolates obtained from the deep sea. These are *Idiomarina loihiensis* (50), *Oceanobacillus iheyensis* (94) and *Alteromonas macleodii* (draft sequence). However, it should be noted that while these organisms were derived from deep-sea environments, there is no data available on their pressure adaptation. They could be allochthonous to the deep-sea environment or recently evolved piezophiles in which surface derived genes have not yet been purged by natural selection.

Additional genetic modifications are expected for coping with long periods of starvation (88) and for searching for nutrients in patchy oligotrophic environments (104). In fact, studies conducted with the moderate piezophile *Psychromonas* sp.

CNPT3 have shown interesting behavioral responses. Upon shifting to low-nutrient conditions, the cells decreased their biovolume and membrane unsaturated fatty acid content and increased their ability to attach to a glass substratum, particularly during incubations at high pressure. This latter attribute could reflect an adaptation for localization to nutrient-rich particles (88). How long can piezophiles survive with little or no food? In one long term experiment, a batch culture of *Psychromonas* HS11 (31) was maintained at high pressure for over 20 years (Chastain and Yayanos, personal communication). Upon decompression and dilution into new media, the culture displayed exponential growth within 2 weeks.

Motility is another important adaptation for marine bacteria, in particular to avoid grazing and for the continuous quest for nutrients (46). In hadal and abyssal environments, the hunt for dissolved (21) and particulate (11, 63) organic matter might explain the large number of methyl-accepting chemotaxis (MCP) proteins present in the genomes of all the deep bathytytpes. MCPs are signal transducing proteins that respond to gradients of chemicals in the environment, relaying a signal for directional swimming to the flagellar motor. These sensory systems must be able to detect miniscule changes in the surrounding chemistry in order to enable the cells to maximize their productivity and growth in environments of small amounts of spatially and temporally distributed food supplies (103).

Flagellar assembly and motor function is also worth studying in greater detail as a model for a high pressure-adapted system. Motility is arguably the most pressure-sensitive cellular process in surface-water prokaryotes (16, 73) and gene clusters for motility and chemotaxis are among the most divergent between bathytypes (22). In

fact, microarray-based genome comparison between three strains of *P. profundum* has shown that the deep bathytypes (SS9 and DSJ4 (82)) have an additional gene cluster that is lacking in the shallow bathytype (3TCK) which resembles genes for the production of lateral flagella (22). The function of this additional cluster is unknown, but preliminary results have provided evidence that the deep bathytype SS9 swims well under high pressure, but very poorly at atmospheric pressure, while the opposite phenotype has been observed in the shallow bathytype 3TCK (Eloe et al., unpublished results).

Future directions.

Genomic analyses of piezophilic microorganisms is only starting to crack the codes for deep-sea adaptations. Continued sequencing of key deep-sea bathytypes within all three domains of life will be essential along with reinvigorated attempts to culture a more representative fraction of the deep-sea microbial community. With advances in computer modeling it will be possible to detect more subtle global adaptations such as specific protein motifs that allow enzymes to work under high pressure and low temperature and to identify novel sensory, regulatory and metabolic pathways that allow growth under conditions of low and shifting nutrient conditions.

Another frontier will be the use of large-scale community sequencing to more fully understand deep-sea microbes at the community and ecosystem level (12). Metagenomics is still in its infancy because of the amount of sequencing and cyber-infrastructure required to extensively sample complex environments (98). However it

has been successfully applied to relatively simple systems such as acid mine drainage (97) and anaerobic methane oxidizing microbial communities (48).

With new technologies (72) and approaches (42) it is hoped that it will be possible in the near future to characterize the bulk of the microbial community even in the case of environments of high diversity and complexity such as the deep sea. Certainly the work of DeLong et al. (29), Ferrer et al. (35) and Grzymski et al. (47) are ground-breaking steps in this direction. In the meantime, insights into the function, the metabolic potential and the role of individual phylotypes in microbial populations could be gathered by using environmental microarrays (118). These come in various flavors: 1) Functional gene arrays (FGA) have probes designed to detect specific functional genes. They could be used to search for specific metabolic pathways that are expected to be over-represented in the deep sea (106, 107) and to analyze changes in the expression of those pathways in response to spatio-temporal changes in the physicochemical parameters (38). 2) SSU rRNA-based phylogenetic oligonucleotide arrays (POAs) could be used to analyze phylogenetic diversity and microbial community shifts in response to the same environmental fluctuations (33, 69, 102). 3) Arrays based on the whole complement of open reading frames of a reference genome could be used to rapidly identify genomic variations among a large number of closely related isolates. Understanding the role of strain-specific genes in deep bathytypes (22) and the significance of horizontal gene transfer in producing genome variation (32, 75) would provide valuable insights into the evolution of deep-sea genomes.

However these technologies must proceed in parallel with the development of new genetic tools and culturing approaches in order to test hypotheses stemming from the analyses of molecular data.

For example, it will be interesting to physiologically characterize deep-sea members of the group I Crenarchaea, whose numbers become dominant with depth (57). Based on the molecular and culturing data of a shallow-water representative from this group it appears that these microbes are chemoautothrophs which gain energy through the oxidation of ammonia (36, 65, 96). If true for the group in general, current models for carbon and nitrogen cycling in the deep ocean will need to be revised (9, 51).

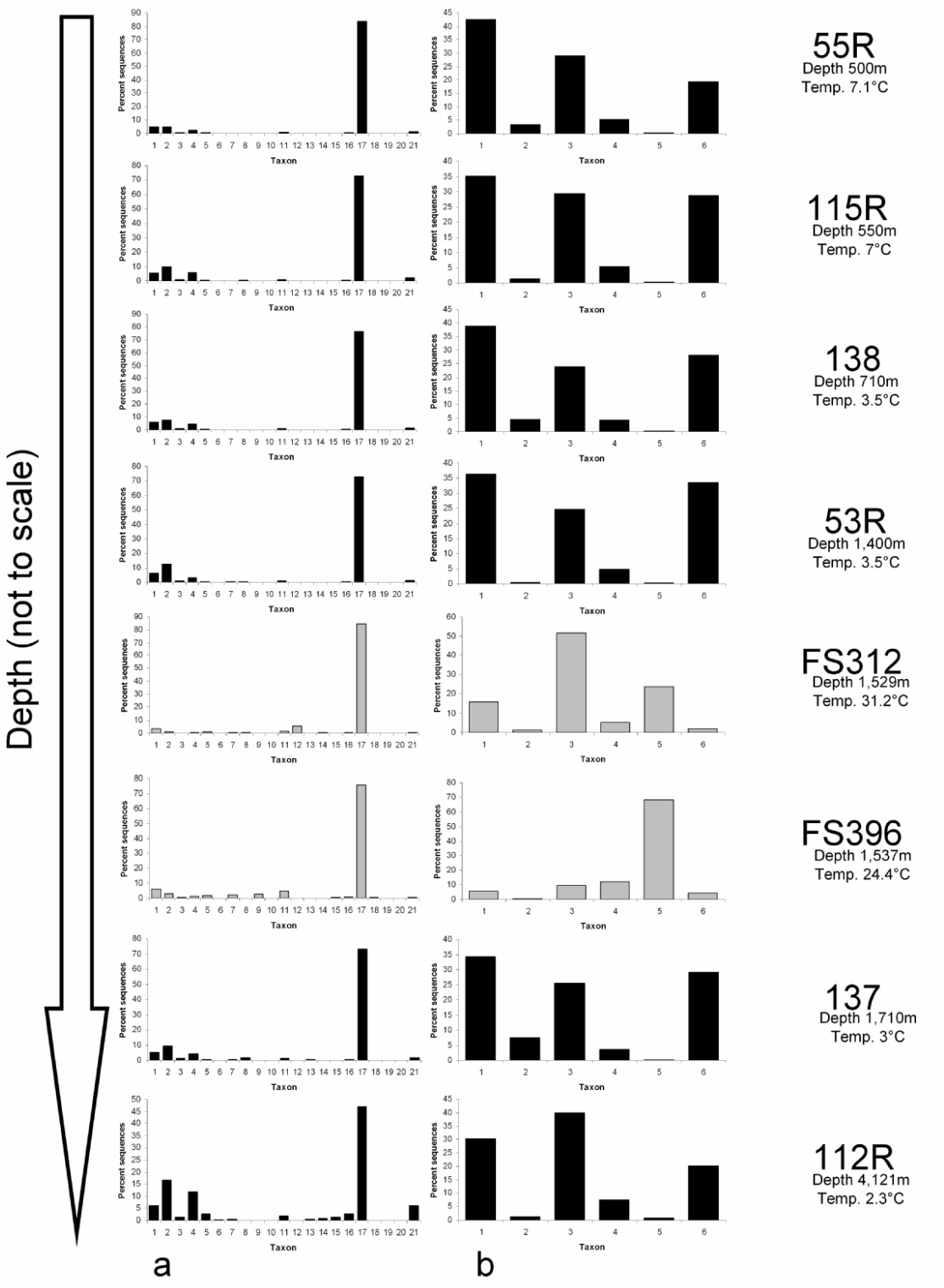
The text of chapter 1, in full, is a reprint of the material as it appears in Extremophiles DOI 10.1007/s00792-006-0059-5 with co-author Douglas H. Bartlett. The dissertation author was the primary investigator and author on this paper.

Table 1.1: Deep bathytype microbial species and corresponding comparison species from shallow waters. PUFA=polyunsaturated fatty acid. EPA=eicosapentaenoic acid (20:5n-3); DHA=docosahexaenoic acid (22:6n3). The analysis of fatty acid methyl esters was performed by gas chromotography-mass spectrometry.

* Traces of DHA

Strain	P_{opt}	T_{opt}	PUFA	Source	Phylogeny	Comparison strain
Photobacterium profundum SS9 (99)	28	16	EPA	Sulu Sea	Proteobacteria Gammaproteobacteria Vibrionales	*Photobacterium profundum* 3TCK *Photobacterium* sp. SKA34
Shewanella sp. KT99	~98	~2	EPA*	Kermadec Trench	Proteobacteria Gammaproteobacteria Alteromonadales	*Shewanella frigidimarina* NCIMB400 *Shewanella violacea* DSS12
Psychromonas sp. CNPT3	52	12	EPA*	Central North Pacific	Proteobacteria Gammaproteobacteria Alteromonadales	*Psychromonas ingrahamii*
Moritella sp. PE36	41	10	DHA	Patton Escarpment	Proteobacteria Gammaproteobacteria Alteromonadales	*Moritella marina*
Carnobacterium sp. AT7	20	20	ND	Aleutian Trench	Firmicutes Bacilli Lactobacillales	*Enterococcus faecalis*

Figure 1.1: Classification of the V6-region rRNA tags produced by Sogin et al. (2006) (a) Left column: percentage composition the total bacterial community. Taxa: 1) No significant HSPs 2) Only environmental HSPs 3) Acidobacteria 4) Actinobacteria 5) Bacteroidetes 6) Chlamydiae 7) Chloroflexi 8) Cyanobacteria 9) Deferribacteres 10) Deinococcus-Thermus 11) Firmicutes 12) Fusobacteria 13) Gemmatimonadetes 14) Lentisphaerae 15) Nitrospira 16) Planctomycetes 17) Proteobacteria 18) Spirochaetes 19) Thermodesulfobacteria 20) Thermotogae 21) Verrucomicrobia (b) Right column : percentage composition among the proteobacteria. Taxa: 1) α-proteobacteria 2) β-proteobacteria 3) γ-proteobacteria 4) δ-proteobacteria 5) ε-proteobacteria 6) unclassified proteobacteria. Solid black bars are samples from the North Atlantic Deep Water: 53R and 55R from 58.300ºN-29.133ºW; 112R and 115R from 50.400ºN-25.000ºW; 137 and 138 from 60.900ºN-38.516ºW. Grey bars (FS312 and FS396) are samples from hydrothermal vent fluids of the Axial Seamount in the Juan de Fuca Ridge, 45.916ºN-129.983ºW.


Depth (not to scale)
Percent sequences
Taxon
a
b
55R
Depth 500m
Temp. 7.1°C
115R
Depth 550m
Temp. 7°C
138
Depth 710m
Temp. 3.5°C
53R
Depth 1,400m
Temp. 3.5°C
FS312
Depth 1,529m
Temp. 31.2°C
FS396
Depth 1,537m
Temp. 24.4°C
137
Depth 1,710m
Temp. 3°C
112R
Depth 4,121m
Temp. 2.3°C

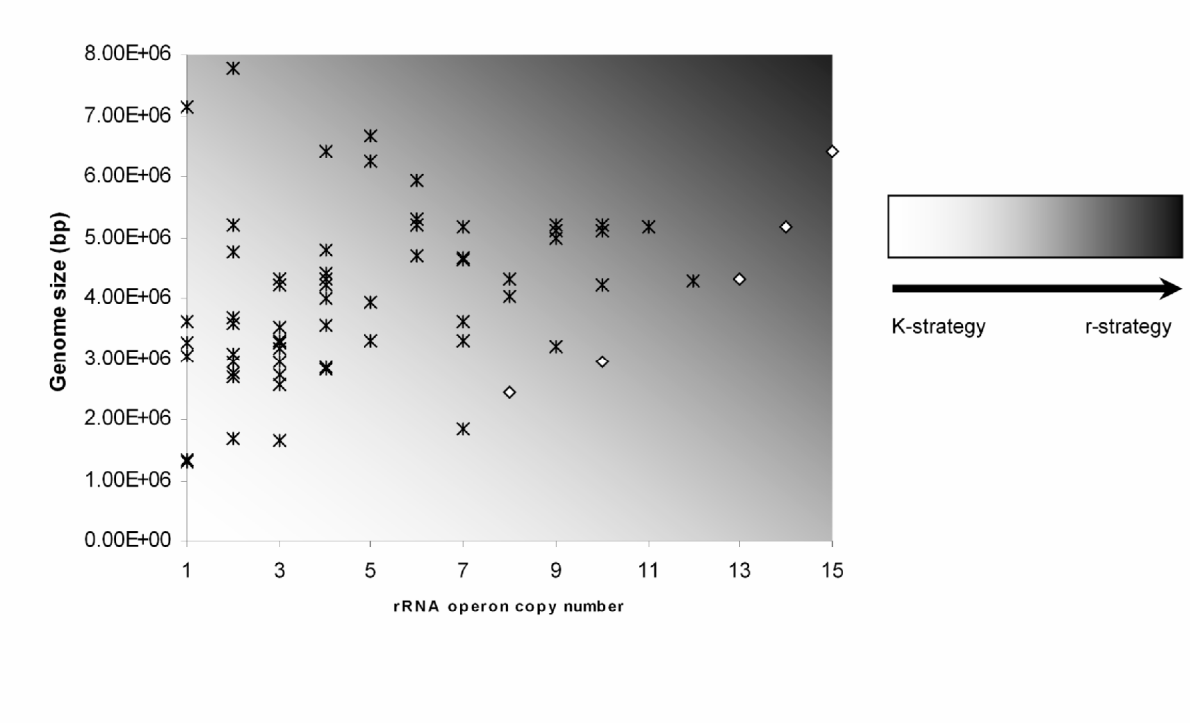


Figure 1.2: Relationship between genome size and rRNA copy number for 66 bacterial genomes. The open diamonds are the deep bathytypes.

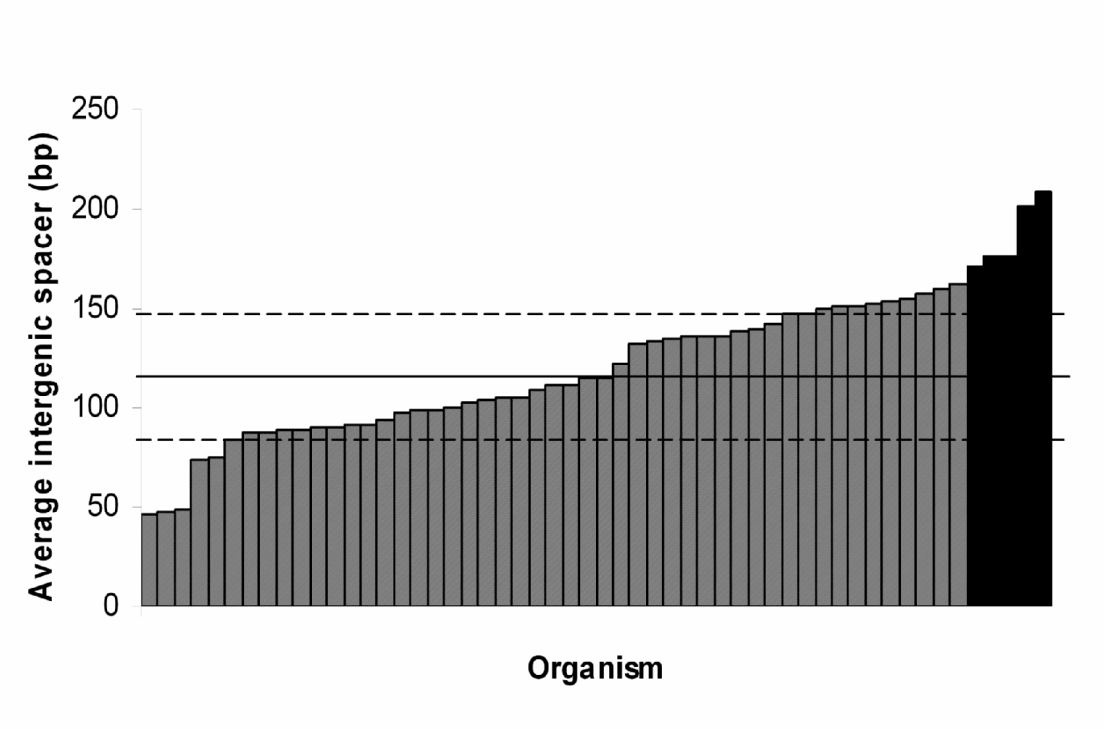


Figure 1.3: Average intergenic spacer for 54 bacterial genomes. The dark bars are the deep bathytypes. Solid line represents the mean, dashed lines the standard deviation.

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Supplemental material: Materials and methods

Classification of the North Atlantic V6-tag data

The phylogenetic diversity of the V6-sequence tags of Sogin et al. (5) was computed with a custom PERL script and is summarized in Figure 1.1.

Each trimmed set of sequences, as described in the original paper, was searched using BLAST (1) against the V6RefDB database (5). Only hits with an e-value of less than 0.001 were considered to be significant.

The accession number in the FASTA header of the best hit was then used to retrieve the full length sequence and that sequence was searched on the RDP-II (Ribosomal Database Project) database (2, 4) parsing the phylogenetic lineage.

Whenever the best hit was to sequences that could not be classified, the script would go back and scan the first 5 BLAST hits to retrieve the highest scoring sequence that could be classified by this method. If no classifiable sequence was found in the top 5 or the significance dropped below the threshold (e-value<0.001), the sequence was discarded from the analysis. By this method between 77% and 96% of each sample was classified.

Determination of intergenic regions and ribosomal operon copy numbers.

The average intergenic region spacer was calculated by dividing the number of non-coding nucleotides in each genome by the total number of predicted ORFs, ribosomal RNA genes and tRNA genes.

The data about number of ribosomal operons was retrieved either from completed genome projects or from the Ribosomal RNA Copy Number Database (3).

To limit the bias of phylogeny on the outcome of this comparative analysis, the genomes selected were primarily relatives of the deep-sea strains under study, with a resulting over-representation of gamma-proteobacteria in the dataset. Similarly, because the environment is expected to play a central role in the selection of ribosomal operon copy number and intergenic region size, most of the genomes selected were of marine origin, coming from the "Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project".

Draft sequences were used for the rRNA analysis only if they consisted of less than 10 scaffolds. In this case, additional ribosomal RNA operons were identified by searching the ends of the scaffolds as most RNA operons are expected to fall in sequence gaps because of their repetitive nature.

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2.

Conjugal Vectors for Cloning, Expression and Insertional Mutagenesis in Gram-Negative Bacteria.

Abstract

The development of a new set of conjugal vectors for cloning, expression and insertional site-directed mutagenesis is described. These vectors are based on the broad-host-range replicon of RSF1010. They were tested in the deep-sea γ-proteobacterium *Photobacterium profundum* but have potential application for use in many Gram-negative bacteria.

The ever increasing pace of genome sequence acquisition, annotation and transcriptional profiling is providing large data sets of possible open reading frame function. However detailed gene characterization still requires *in vivo* genetic manipulation. As a result there is a need for greater efficiency when applying these procedures to microbial systems. While high throughput genetic strategies have been described for most members of the family *Enterobacteriaceae* (1, 2), *B.subtilis* (3) and the yeast *S.cerevisiae* (4), all of which can be easily transformed, there is a need to develop similar approaches for those hosts that cannot be transformed. Here we report the development of a set of conjugal plasmids for insertional mutagenesis,

complementation and inducible expression in a wide variety of gram-negative bacteria and test their efficiency using the psychrotolerant γ-proteobacterium *Photobacterium profundum* strain SS9, for which the whole genome sequence has recently been completed (5).

Site-specific mutagenesis in *Photobacterium profundum* has previously been performed using conjugal narrow host range suicide plasmids for single-crossover insertional mutagenesis (6) or for double-crossover allelic exchange (7). While useful, these methods suffer from the need to first PCR clone fragments of interest into a vector such as pCR2.1 (Invitrogen Corp., Carlsbad, CA) and then subclone into the plasmid to be delivered into *P. profundum*. To overcome this limitation a TA-cloning (8) conjugal suicide vector (pEE3) was successfully engineered from parental plasmids pBBRI-MCS2 (9, 10) and the narrow host-range, mobilizable, ColE1 plasmid pMUT100 (11) which does not replicate in *P. profundum* SS9 (6). Initially, dual *Xcm*I sites were introduced within the *lacZ* gene of pBBRI-MCS2 allowing for the generation 3' T-overhangs upon *Xcm*I restriction. This construct was subsequently subcloned into the *Eco*RI site of the suicide vector pMUT100 yielding pEE3 (Figure 2.1A). With this plasmid PCR fragments internal to genes of interest can be amplified with *Taq* polymerase and directly ligated into *Xcm*I digested vector for single-crossover insertional mutagenesis. *Escherichia coli* transformants containing plasmids with inserts are readily identified on Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) containing media as white colonies. The desired recombinant plasmid can then be transferred via conjugation from *E.coli* into the appropriate gram-negative bacterium by tri-parental mating employing a second *E. coli* strain containing a helper

plasmid such as pRK2073 (12). Exconjugants obtained by kanamycin or tetracycline selection are likely to contain an insertion in the gene of interest. As with other gene-knockout systems, it is advisable to check that the correct insertion has been obtained by PCR amplification and sequencing of the junction points.

To date the pEE3 cloning system has been used to generate five knockouts in *P.profundum* with an average efficiency of 2.4×10^{-9} gene disruptions per conjugal recipient. In addition, this vector is also useful for restriction endonuclease-mediated cloning into the multiple cloning sites within *lacZ.*

A second plasmid, designated pFL122, has been designed for complementation studies (Figure 2.1B). It is an improved version of previous RSF1010-derived (IncQ) vectors in that it contains a *lacZ* marker with a multiple cloning site for convenient cloning and screening along with streptomycin resistance. The choice of selectable marker allows pFL122 to be used to complement most types of transposon mutants (for example see 13) as well as insertional knockouts obtained with pEE3. Conjugation efficiencies of pFL122 into *P. profundum* (Table 2.1) are comparable to those obtained with the widely used IncQ plasmid pKT231 (14) and vastly improved over the broad host range vector pBBR1-MCS2 (9,10). pFL122 has been successfully employed to complement the growth defect of four transposon mutants of *P.profundum* (unpublished results).

Finally, we have prepared a new expression vector (Figure 2.1C). While a wide variety of expression systems are available in *E.coli*, there are only a few reports of successful use of inducible expression systems in other hosts (15). A vector containing the P_{BAD}/*araC* system was prepared by subcloning the relevant portion of pJN105 (15)

into an RSF1010 (IncQ) derivative (pFL107) resulting in pFL190. pFL190 has been successfully used to provide arabinose-dependent β-galactosidase and GFP expression, respectively, in *P. profundum*. Table 2.2 provides β-galactosidase activity as a function of arabinose concentration in the P_{BAD}::*lacZ* derivative of pFL190.

Since IncQ plasmids have been shown to replicate in a wide variety of gram-negative bacteria including, *Agrobacterium*, *Alcaligenes*, *Pseudomonas, Rhizobium* and *Vibrio* (14,16) the above vectors should be applicable to studies in a variety of bacterial hosts. The presence of multiple unique restriction sites outside of the cloning region makes it easy to broaden their applicability by adding other antibiotic resistance markers. The physical map of all these vectors is reported in Figure 2.1 while nucleotide sequences are available under accession numbers AY785148, AY785149, AY785150.

The text of Chapter 2, in full, is a reprint of the material as it appears in Biotechniques 38:708-712 with co-authors Emiley A. Eloe, Nicoletta Liverani, Giulio Bertoloni and Douglas H. Bartlett. The dissertation author was the primary investigator and author on this paper.

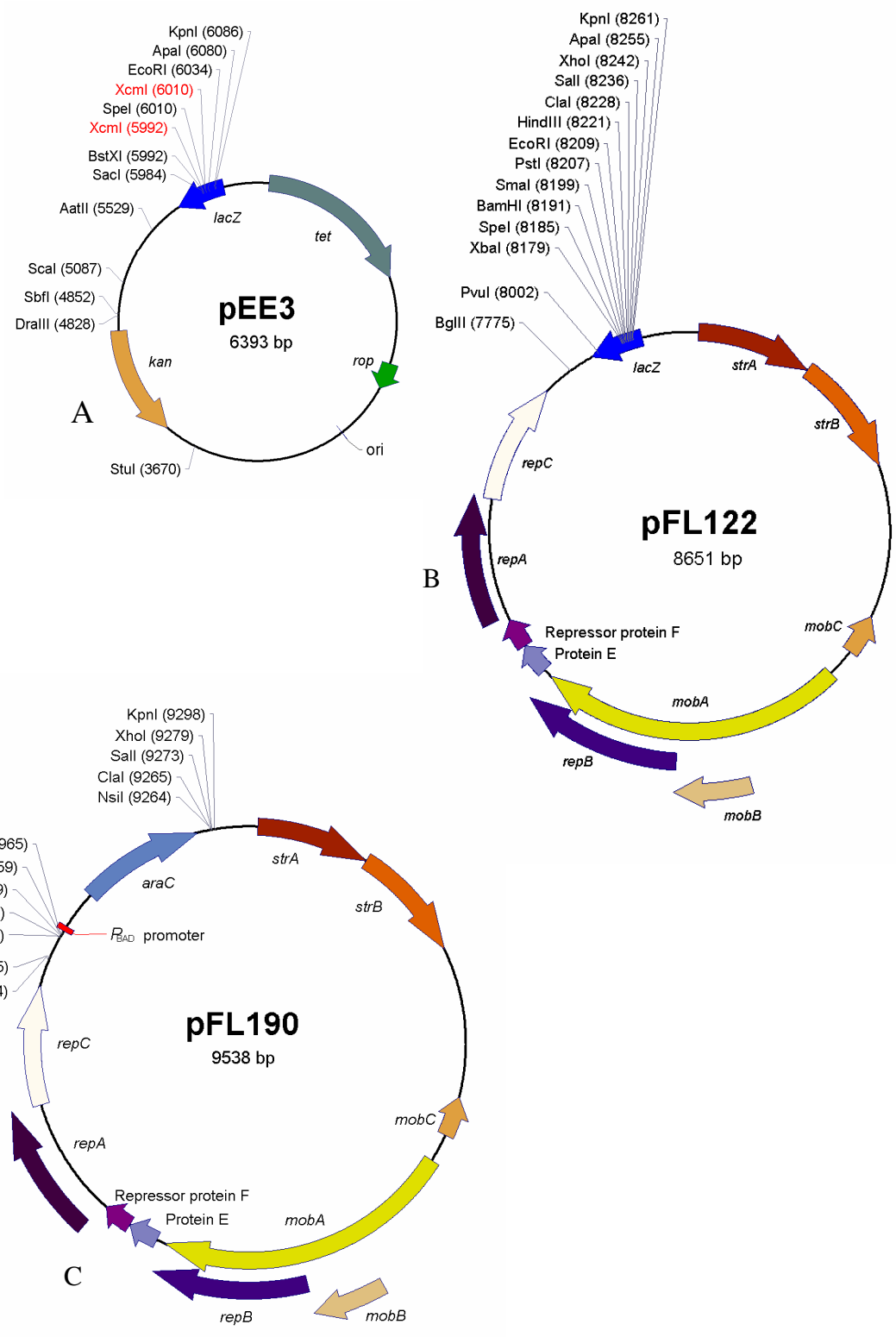
Table 2.1: Comparison of the conjugation efficiencies of pFL122 with two other broad host-range vectors in *P. profundum*. Values are in number of exconjugants per conjugal recipient cells with the relative standard error.

Sample	Conjugation efficiency (exconjugants/recipients)
pFL122	$1.779 \times 10^{-5} \pm 24.7\%$
pFL190	$1.623 \times 10^{-6} \pm 43.5\%$
pKT231	$4.689 \times 10^{-5} \pm 52.4\%$
pBBR1-MCS2	$< 1.2 \times 10^{-9}$

Table 2.2: β-galactosidase activity as a function of arabinose concentration in pFL191. This plasmid is a derivative of pFL190 where the P_{BAD} promoter drives *lacZ* expression. Activity is expressed in Miller units (17) with the relative standard error. *E. coli* DH5α was grown aerobically in LB (Luria-Bertani) broth with 100 µg/ml of streptomycin, at 37°C. *P. profundum* DB110 was grown aerobically in 2216 marine broth (Difco) with 150 µg/ml of streptomycin, at 15°C.

L-Arabinose concentration	**pFL191 in *E.coli* DH5α**	**pFL191 in *P.profundum* DB110**
0%	**<2**	**<2**
0.01%	**1153±12.2%**	**64±38.3%**
0.5%	**1296±9.6%**	**236±4%**

Figure 2.1: Physical map of the vectors presented in this study. (A) TA-cloning conjugal suicide vector, (B) Broad-host-range cloning vector, (C) Broad-host-range expression vector. Only the relevant restriction sites are shown. Antibiotic markers: *strA*, streptomycin resistance protein A; *strB*, streptomycin resistance protein B; *tet*, tetracycline resistance protein; *kan*, kanamycin resistance protein.


KpnI (6086)
ApaI (6080)
EcoRI (6034)
XcmI (6010)
SpeI (6010)
XcmI (5992)
BstXI (5992)
SacI (5984)
AatII (5529)
lacZ
tet
ScaI (5087)
SbfI (4852)
DraIII (4828)
pEE3
6393 bp
kan
rop
ori
StuI (3670)
A
KpnI (8261)
ApaI (8255)
XhoI (8242)
SalI (8236)
ClaI (8228)
HindIII (8221)
EcoRI (8209)
PstI (8207)
SmaI (8199)
BamHI (8191)
SpeI (8185)
XbaI (8179)
PvuI (8002)
BglII (7775)
lacZ
strA
strB
repC
pFL122
8651 bp
repA
mobC
Repressor protein F
Protein E
mobA
repB
mobB
B
KpnI (9298)
XhoI (9279)
SalI (9273)
ClaI (9265)
NsiI (9264)
NheI (7965)
EcoRI (7959)
SmaI (7949)
SpeI (7935)
XbaI (7929)
BglII (7775)
SapI (7764)
araC
strA
strB
P BAD promoter
pFL190
9538 bp
repC
mobC
repA
Repressor protein F
Protein E
mobA
repB
mobB
C

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3.

The Unique 16S rRNA Genes of Piezophiles Reflect both Phylogeny and Adaptation

Abstract

In the ocean's most extreme depths, pressures of 70-110 megapascals prevent the growth of all but the most hyperpiezophilic (pressure-loving) organisms. The physiological adaptations required for growth under these conditions are considered to be substantial. Efforts to determine specific adaptations permitting growth at extreme pressures have thus far focused on relatively few γ-proteobacteria, in part due to the technical difficulties of obtaining piezophilic bacteria in pure culture. Here, we present the molecular phylogenies of several new piezophiles of widely differing geographic origin. Included are results from an analysis of the first deep trench bacterial isolates recovered from the Southern hemisphere (9.9 km depth) and of the first Gram-positive piezophilic strains. This new data allowed both phylogenetic and structural 16S rRNA comparisons among deep-ocean trench piezophiles and closely related strains not adapted to high pressure. Our results suggest that (1) the Circumpolar Deep Water acts as repository for hyperpiezophiles and drives their dissemination to deep trenches in the Pacific Ocean and (2) the occurrence of elongated helices in the 16S rRNA genes increases with the extent of adaptation to growth at elevated pressure. These helix changes are believed to improve ribosome function under deep-sea conditions.

Introduction

Low temperature and high hydrostatic pressure structure deep-sea communities outside of hydrothermal vents. Tight selection by these and other environmental parameters is considered the cause of the conspicuous absence of many deep-sea taxonomic groups from the deepest ocean environments (8, 44).

Both temperature and pressure exert their effects at many levels of bacterial physiology, from the structure of macromolecules to the rate of metabolic reactions. Adaptations to low temperature include alterations of membrane phospholipids, such as increased fatty acid unsaturation (43), enzymes characterized by high catalytic efficiency and reduced activation enthalpy (15, 20, 37, 45) and high levels of cold shock proteins, RNA helicases (9) and post-transcriptional modification of tRNA molecules (16), all of which may reduce the formation of unfavorable nucleic acid secondary structures at low temperature. In contrast with enthalpy-based temperature effects, the underlying cause of pressure effects arise from the promotion of reduced system volumes, in accordance with Le Chatelier's principle (5). Despite these thermodynamic differences, low temperature and high pressure share a surprising number of influences on biological processes. For example, membrane fluidity, permeability and phase are similarly altered by both parameters.

As with psychrophiles, piezophiles contain lipids with highly unsaturated fatty acids (6, 7). Indeed the presence of unsaturated fatty acids is critical to growth ability at high pressure (3, 4, 19). Both low temperature and high pressure also alter protein quaternary structure (46) and nucleic acid secondary structure (50), and at the cellular

level both parameters exert a pronounced influence on DNA replication and protein synthesis (27, 59).

While considerable insight has been garnered concerning the biological adaptation to extremes of temperature (13, 20) the lack of large numbers of well characterized deep-sea organisms has limited analyses of the molecular details of pressure adaptation. This is in part a result of the technical difficulties of culturing piezophiles ("high pressure loving" microbes) including the need for pressurization systems, low temperature incubators and precautions to limit light exposure (57).

The recent genome analysis of the piezophilic model organism *Photobacterium profundum* strain SS9 (54) has suggested that adaptation to the deep sea involves a combination of modifications of gene structure and regulation. However no piezo-specific genes are yet known. Therefore, discerning general molecular trends associated with piezophily requires comparisons with other piezophilic extremophiles.

Here we present the isolation and characterisation of the first hyperpiezophiles (strains with an optimal growth pressure >60 MPa) obtained from the southern hemisphere, and from another trench environment the first isolation of piezophilic gram positive bacteria. The 16S rRNA-based phylogenetic assessments of these novel isolates and other geographically disseminated piezophiles reveal that 1) low temperature piezophiles appear to have descended from psychrophiles present in the polar regions and 2) stem elongation of helices 10 and 11 within the 16S rRNA molecule strongly correlates with the extent of piezophily.

Materials and Methods

Collection of trench material for bacteria enrichment. On October 17, 2001, scavenging amphipods of the species *Hirondellea dubia* were collected with insulated, baited, free-vehicle traps deployed to a depth of 9,856m in the Kermadec Trench (32° 01.07' S 177° 20.99'W). The temperature at this depth was recorded to be 1.8°C. The trap was estimated to be on the sea floor for 14 ± 0.5 hours with an ascent time of 4 h 36 min to reach the sea surface, at which time the trap contents were at atmospheric pressure with a temperature of less than 5°C. The trap was recovered within 15 min of surfacing and contents were immediately transferred to ice-chilled buckets. In a cold room (maintained at 4°C), several amphipods were placed into Kapak bags (Ampac Packaging, Cincinnati, OH) containing 5 ml of sterile, ice-cold filtered seawater. Bags were then sealed and subsequently pressurized to 99 MPa in pressure vessels which were maintained at 0°C for the duration of the cruise. Samples were at atmospheric pressure for less than 1 hr. Once back at Scripps Institution of Oceanography, samples were maintained at 2°C (in pressure vessels) except for periods of less than 30 min to allow inoculation of enrichment cultures which were in turn incubated at high pressure.

Strains AT7 and AT12 are in a collection of deep-sea piezophilic bacteria at Scripps Institution of Oceanography. These two strains originated from a water sample taken by John Burke from the Aleutian Trench at 52° 53.1' N 163° 0.0' W. The sample was from a depth of 2,500m where the temperature was 1.8°C. The sample was collected on June 22, 1981 and put in a narrow-mouth sterile plastic bottle, kept

on wet ice, and returned to Scripps Institution of Oceanography. Strains AT7 and AT12 are from enrichments incubated at 40 MPa and begun on August 14, 1981.

Isolation of piezophiles and growth characterization. The decomposing amphipods and surrounding medium were briefly depressurized and immediately inoculated into a beaker containing full-strength 2216 Marine Broth (Difco Laboratories, Detroit, MI). From this slurry, approximately 15 ml were loaded into sterile Samco transfer pipets (Samco Scientific Corp., San Fernando, CA), which were heat-sealed, and quickly pressurized to 99 MPa in pressure vessels (57, 60). The first enrichment, performed on December 19, 2001, was incubated in a rocking water bath at 2°C. A separate enrichment was performed on April 30, 2004, and cultures were grown in rocking water baths maintained at either 2°C or 8°C respectively. Enrichments were microscopically examined after three weeks for evidence of bacterial growth. Once these cultures were established, the following procedure was followed to obtain clonal isolates. Enrichments were serially diluted into fresh 2216 broth with 4% gelatin. This mixture is liquid at room temperature (~22°C), but hardens when cooled below 15°C, thus making it a suitable medium for growing and isolating colonies. The inoculated mixture was then loaded into fresh transfer pipettes, and quickly pressurized to 99 MPa. These new cultures were incubated at the same temperature (either 2°C or 8°C) as the initial enrichment and periodically examined until colonies were observed within the gelatin matrix. Colonies were extracted directly from the transfer pipette bulbs by piercing the walls with a sterile needle (21 gauge) and syringe. Isolates were extruded into ice-cold 2216 broth, reloaded into transfer pipettes, and incubated at the appropriate temperature and pressure for growth.

Established cultures were maintained by transferring them into fresh medium every 2-3 month. During one of these transfers *Colwellia* sp. KT27 was lost.

For growth rate determination as a function of pressure, cultures were inoculated into multiple heat-sealable bulbs and pressurized as described above. At prescribed time intervals one bulb was removed and the optical density (at 600 nm) of the culture was recorded.

16S characterisation of isolates. Genomic DNA was extracted from pure cultures using the DNeasy kit (QIAGEN, Valencia, CA). PCR amplification was performed using the general bacterial primers 27F and 1492R (17). Automated DNA sequencing was performed on an Amersham MEGABace 500 (Amersham Pharmacia biotech) using general eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 518R (5'-GTATTACCGCGGCTGCTG-3'), 530F (5'-GTGCCAGCAGCCGCGG-3'), 907R (5'-CCGTCAATTCATTTGAGT-3'), 926F (5'-ACTCAAAGGAATTGACGG-3'), 1492R (5'-GGTTACCTTGTTACGACTT-3'). The resulting chromatograms were assembled into contigs using Sequencher (Gene Codes Corp., Ann Arbor, MI). All the 16S rRNA gene sequences determined in this work were deposited in GenBank under accession numbers DQ027051-DQ027062.

Phylogenetic and structural analyses. Phylogenetic dendrograms were reconstructed with MEGA 2.1 (31) for neighbour-joining and minimum-evolution and Treefinder (29) for maximum-likelihood with an HKY (Hasegawa, Kishino, Yano) model of evolution (25). Since the topology of the trees was conserved independent of the method used, only the maximum-likelihood trees are shown. Bootstrap support is from 1,000 bootstrap replicates. Structure analysis of the 16S ribosomal RNA variable

regions was performed by dynamic programming energy minimization implemented in the VIENNA RNA package (26). A folding temperature of 15°C was used and GU pairing was allowed.

Semi-quantitative PCR and RT-PCR. Bacterial cells were grown in 2216 Marine Broth amended with glucose (20 mM) and HEPES (100 mM; pH 7.5). For RT-PCR, *P. profundum* SS9 was grown to an optical density at 600 nm of 0.1-0.2, harvested and total RNA was extracted using RNABee (Tel-Test, Friendswood, TX) and further purified with RNeasy columns (QIAGEN, Valencia, CA). Genomic DNA was removed using in column digestion with DNAse (QIAGEN, Valencia, CA).

RT-PCR was performed using the OneStep RT-PCR kit (QIAGEN, Valencia, CA) with partially degenerate primers PIEZOLOOPF (5'-GAAACGAYDGCTAATACCGCAT-3') and PIEZOLOOPR (5'-TTACCYYACCAACWAGCTAATC-3') that amplify the region containing helices 10 and 11 in most γ-proteobacteria.

Semi quantitative PCR was performed with *Taq* polymerase (Invitrogen, Carlsbad, CA) on genomic DNA, using the same primers but omitting the reverse transcriptase step in the PCR cycle.

For the quantification, PCR amplification products were removed at cycle 20, 25 and 30 and analyzed in 10% TBE acrylamide gels stained with ethidium bromide. The PCR program for amplification was 94°C for 5 min, 94°C for 25 s, 56°C for 25 s, 72°C for 35 s followed by a final extension for 5 minutes. The gel images were analyzed for peak intensity using the ChemiImager 5500 system (Alpha Innotech

Corp., San Leandro, CA) and the ratio between the intensity of the higher bands and the lowermost band was calculated.

Results and Discussion

Isolation of additional piezophiles. Four new piezophiles were isolated, two from the Aleutian Trench and two from the Kermadec Trench. Strains AT7 and AT12, isolated from the Aleutian Trench, were identified as members of the genus *Carnobacterium*. These strains are closely related to the recently isolated *Carnobacterium pleistocenium* (42) and to Antarctic isolates of the family Carnobacteriaceae (22). Members of this family have been previously detected in rRNA surveys of deep ocean sediments (38) but this is the first report of a piezophilic isolate of this species as well as the first Gram-positive piezophile ever identified. The pressure range for growth of AT7 and AT12 was 0.1-60 MPa with an optimum at 15 MPa (58). Interestingly the sister taxon *Marinilactibacillus* only contains two marine isolates, one of which is psychrophilic (28) and the other psychropiezotolerant (51).

Strains KT27 and KT99 were acquired from a depth of 9,856m (ambient temperature 1.8°C) in the Kermadec Trench located in the Southwest Pacific Ocean. Both isolates are piezophilic, and grow well at the *in-situ* pressure of 98 MPa . They represent the first psychropiezophiles from the southern hemisphere. Phylogenetic analysis revealed that the closest cultured relatives of KT27 and KT99 are the isolates *Colwellia* sp. MT41 (56) and *Shewanella* sp. PT99 (18, 56), respectively (Figure 3.1). Both MT41 and PT99 originate from extreme depths of deep-ocean trenches within the Northern hemisphere of the Pacific Ocean.

Origin and dispersal of psychropiezophiles. In order to obtain a broader perspective on the evolutionary history of existing psychropiezophiles, phylogenetic tree reconstruction was performed for a total of twenty psychropiezophiles and their closest Gram positive or Gram negative relatives (Fig. 3.1; also see Table S3.1 in the supplementary material). Interestingly, the 16S sequences of all the piezophiles share high similarity with non-piezophilic isolates from Antarctica. Moreover, some genera containing piezophiles (*Colwellia*, *Psychromonas* and *Moritella*) appear to be largely restricted to cold waters of various depths and are under-represented in rRNA surveys (1) and absent from environmental shotgun sequences (53) obtained from temperate waters.

These facts argue against piezophily arising from shallow-water mesophilic sources, an evolutionary path that would also seem unlikely on the grounds that it would require concurrent adaptations to both high pressure and to low temperature. Rather the most parsimonious explanation of the data is that psychrophiles can give rise to psychropiezophiles. Pressure and temperature exert overlapping effects on many microbial processes (18). In most microorganisms increasing pressure results in a shift to higher cardinal growth temperatures (11, 30, 35, 56), suggesting a partial compensation between the two physical factors. Thus, a logical hypothesis is that initial acclimation to high pressure is facilitated by pre-existing adaptations to low temperature. Genome comparisons within the well-studied γ subdivision of the phylum Proteobacteria might be very instructive in this context, since it contains two orders that contain psychropiezophiles: the Vibrionales and the Alteromonadales (18, 40, 41). The genome sequence of the piezophile *Photobacterium profundum* has

already been noted for its similar gene content to that of the cold-adapted Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (36).

Cold Antarctic waters and their associated shallow-water psychrophiles are a likely source of the piezophiles populating the deepest depths of the Pacific Ocean. Antarctic Bottom Water (AABW), mixing with North Atlantic Deep Water (NADW) forms the lower Circumpolar Deep Water (CDW), some of which escapes to the north, first encountering the Kermadec and Tonga Trenches (49, 52). Here strains acquiring mutations enhancing their fitness at depth could reproduce, and their descendants could then hitchhike on the "global conveyor belt" (49, 52) of deepwater circulation to other trenches. This dispersal mechanism could also explain the apparent paradox of the similarity between Arctic and Antarctic psychrophilic bacterial communities (48). Since it is unlikely for a psychrophile to survive crossing the equator in the warm surface waters, the two communities should be geographically isolated and should therefore be evolving independently. Deep-sea currents provide a plausible mechanism for mixing of Arctic and Antarctic psychrophilic communities. Alternatively, this community similarity could be a result of the current level of resolution in the available molecular data as suggested by Whitaker et al. (55). In-depth comparisons between the genomes of psychrophiles from the two hemispheres, along with related piezophiles, should be undertaken to better evaluate this hypothesis.

Convergent evolution of rRNA structure among piezophilic γ-proteobacteria. In the genera *Photobacterium*, *Colwellia* and *Shewanella* the majority of the 16S rRNA alignment disparities among sister strains from different depths comes from a few short insertions (Figure 3.2).

Folding models show that these insertions result in elongations to the stems of helices 10 (*Escherichia coli* positions 184-193) and 11 (*Escherichia coli* positions 198-219), while the loop sequence is conserved (Figure 3.3). Although these two helices are hypervariable regions of the 16S rRNA (12), the analysis of the alignment of 800 γ-proteobacterial sequences from the CRW website (12) (http://www.rna.icmb.utexas.edu) reveals that the longer stems are almost an exclusive feature of piezophiles, and hyperpiezophiles in particular. The only exceptions are *Pasteurella testudinis* and *Salinivibrio costicola*, which also possessed similarly structured longer loops.

This unique feature of the piezophilic ribosome is consistent with the well-documented sensitivity of mesophile ribosomes to elevated hydrostatic pressure (24, 32). *E.coli* ribosome piezo-sensitivity has been linked to ribosome dissociation, with the 30S subunit being the most sensitive (33, 39). In fact, hybrid ribosomes containing the 30S subunit from the piezotolerant *Pseudomonas bathycetes* coupled to the 50S subunit of *E. coli* showed remarkable pressure tolerance *in vitro* (33).

Functionally, the stems encompassing *E. coli* residues 122-239 have been implicated in interactions with protein S20 of the ribosome (14), which is essential for the assembly of a functional ribosome. Mutants defective in S20 synthesis are impaired in their capability of associating 30S and 50S subunits and in translation initiation (23).

Additional insight into the function of these helices comes from the genome analysis of *Photobacterium profundum* strain SS9 (54). This eurypiezophilic bacterium has the record number of ribosomal operons (15) with high intra-genomic

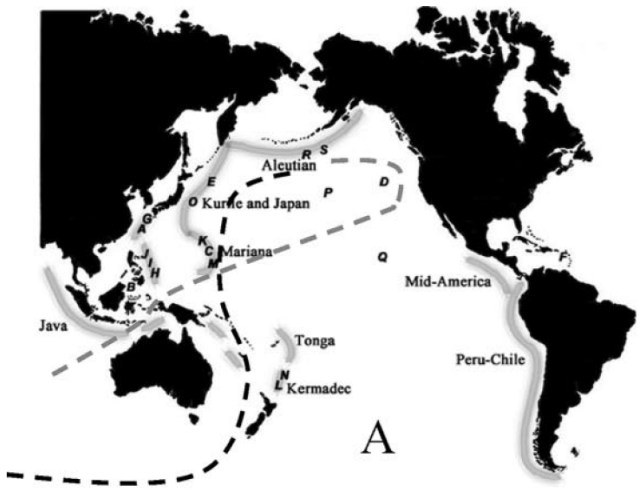
variability within the operons (4% nucleotide divergence). Most of the sequence variation within the 16S is due to indels in helices 10, 11 and 49 (*E. coli* nucleotide position 1409-1491), allowing for a total of 5 ribotypes (Figure 3.4). Helix 49 has also been implicated in the interaction with protein S20 and in interfacing the 30S and 50S subunits (21). Semi-quantitative RT-PCR performed on total RNA extracted from *P. profundum* SS9 cells showed that all ribotypes are constitutively expressed regardless of the pressure conditions (data not shown).

P. profundum contains both piezophilic and nonpiezophilic strains (10, 47). Among the different *P. profundum* strains, semi-quantitative PCR revealed that the proportion of ribotypes with longer stems is directly correlated to the optimal growth pressure (r^2=0.97; Figure 3.5), with the piezophilic strains of *P. profundum* (SS9, DSJ4) having a higher proportion of the longer stems. A similar trend is observed within *Shewanella* strains (r^2=0.79; Figure 3.5), another cosmopolitan oceanic genus with members adapted to various depths. It is possible that the longer loops could have been acquired through lateral gene transfer of ribosomal operons, a process thought to contribute to the high intra-genomic variability in genomes with multi-copy 16S rRNA genes (2). In this scenario, shallow water strains such as those belonging to the genera *Photobacterium* and *Shewanella* could acquire deep-sea piezophile ribosomal 16S rRNA genes and homologous recombination and gene conversion would then generate the various intragenomic ribotypes (34). Alternatively, the conditions in the deep sea might alter the selection pressure on loops 10, 11 and 49 generating the longer ribotypes. Interestingly the sequence of the elongated loops appears to be genus-specific in strains isolated thousand of miles apart (Figure 3.2). Notably, not all

piezophilic γ-proteobacterial 16S rRNA gene sequences available in Genbank exhibit the long loops. However, if only some of the rRNA operons within a given piezophile have the long loops then they might not be detected during 16S cloning and sequencing. Among the *Shewanella* strains examined, only F1A and KT99 have exclusively the longer ribotype (PCR analysis, Figure 3.5). Alternatively, other ribosome modifications might compensate for the lack of these long loops at high pressure in other bacterial genera. Additional studies are currently under way to assess the physiological importance of the piezo-specific loops.

The text of Chapter 3, in full, is a reprint of the material as it appears in Applied and Environmental Microbiology 73:838-845 with co-authors Roger A. Chastain, Lesley E. Blankenship, A. Aristides Yayanos and Douglas H. Bartlett. The dissertation author was the primary investigator and author on this paper.

Figure 3.1. Phylogenetic relationship between cultured deep-sea isolates and relatives from shallow waters. Maximum-likelihood trees were computed as described in materials and methods. Piezophilic and piezotolerant isolates are in bold with a letter referring to the approximate site of isolation on the world map. Psychrophilic and psychrotolerant strains are underlined. Reference and accession numbers for each sequence are given in Table S1. A) Map of the world with approximate site of collection for each strain. The dashed line is the approximate path of the global conveyor belt in the Pacific Ocean: in black the deep current and in grey the surface current. B) Phylogenetic tree depicting the relationship of the γ-proteobacteria strains. C) Phylogenetic tree depicting the relationship of the Carnobacteriaceae strains.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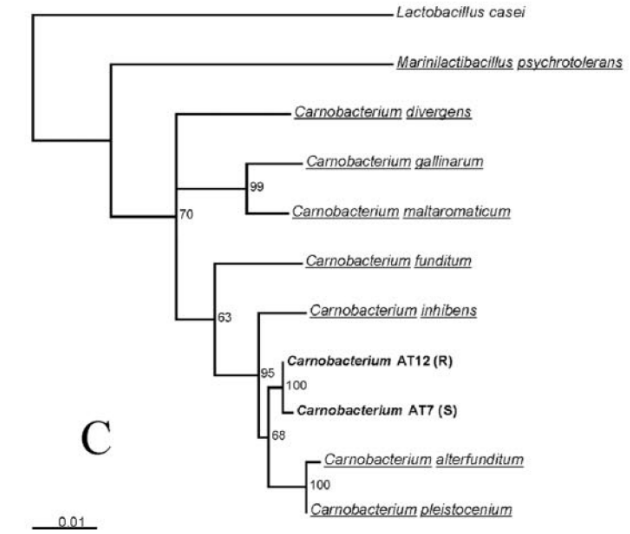
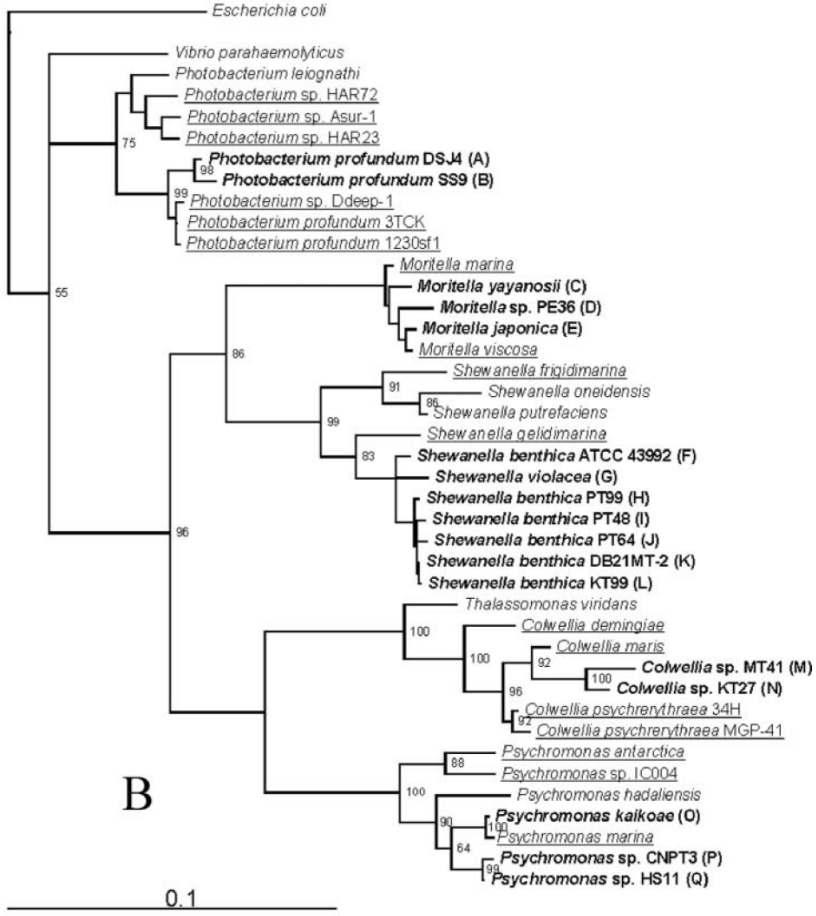
Kurile and Japan
Mariana
Mid-America
Java
Tonga
Peru-Chile
Kermadec
A


Lactobacillus casei
Marinilactibacillus psychrotolerans
Carnobacterium divergens
Carnobacterium gallinarum
Carnobacterium maltaromaticum
Carnobacterium funditum
Carnobacterium inhibens
Carnobacterium AT12 (R)
Carnobacterium AT7 (S)
Carnobacterium alterfunditum
Carnobacterium pleistocenium
0.01
C


Escherichia coli
Vibrio parahaemolyticus
Photobacterium leiognathi
Photobacterium sp. HAR72
Photobacterium sp. Asur-1
Photobacterium sp. HAR23
Photobacterium profundum DSJ4 (A)
Photobacterium profundum SS9 (B)
Photobacterium sp. Ddeep-1
Photobacterium profundum 3TCK
Photobacterium profundum 1230sf1
Moritella marina
Moritella yayanosii (C)
Moritella sp. PE36 (D)
Moritella japonica (E)
Moritella viscosa
Shewanella frigidimarina
Shewanella oneidensis
Shewanella putrefaciens
Shewanella gelidimarina
Shewanella benthica ATCC 43992 (F)
Shewanella violacea (G)
Shewanella benthica PT99 (H)
Shewanella benthica PT48 (I)
Shewanella benthica PT64 (J)
Shewanella benthica DB21MT-2 (K)
Shewanella benthica KT99 (L)
Thalassomonas viridans
Colwellia demingiae
Colwellia maris
Colwellia sp. MT41 (M)
Colwellia sp. KT27 (N)
Colwellia psychrerythraea 34H
Colwellia psychrerythraea MGP-41
Psychromonas antarctica
Psychromonas sp. IC004
Psychromonas hadaliensis
Psychromonas kaikoae (O)
Psychromonas marina
Psychromonas sp. CNPT3 (P)
Psychromonas sp. HS11 (Q)
0.1
B

```
Colwellia sp. MT41                ATAACGTGCTTTTTG-TA-GTGAAAAGTACCAAAGGGGGGGAT-C----------TTCGGA-C
Colwellia sp. KT27                ATAACGTGCTTTTTG-TA-GTGAAAAGTACCAAAGGGGGGGAT-C----------TTCGGA-C
Colwellia maris                   ATAACGTC--------TACG-------GACCAAAGGGGGGGAT-C----------TTCGGA-C
Colwellia psychrerythraea 34H     ATAACGTC--------TACG-------GACCAAAGGGGGGGAT-C----------TTCGGA-C
Colwellia psychrerythraea MGP-41  ATAACGTC--------TACG-------GACCAAAGGGGGGGATTC---------TTCGGAAC
Colwellia demingiae               ATAACGTC--------TACG-------GACCAAAGGGGGGGAT-C----------TTCGGA-C
Thalassomonas viridans            ATAATGTC--------TACG-------GACCAAAGCGGGGGAC-C----------TTCGGG-C
Moritella japonica                ATACGACC--------TACG-------GGTGAAAGGGGGCCTCTT---------CTTGAAAG
Moritella marina                  ATACGACC--------TACG-------GGTGAAAGGGGGCCTCTT---------CTTGAAAG
Moritella sp. PE36                ATACGACC--------TACG-------GGTGAAAGGGGGCCTCTT---------CTTGAAAG
Moritella viscosa                 ATACGACC--------TACG-------GGTGAAAGGGGGCCTCTT---------CTTGAAAG
Moritella yayanosii               ATACGACC--------TACG-------GGTGAAAGGGGGCCTCTT---------CTTGAAAG
Photobacterium sp. HAR72          ATAATCTC--------TTCG-------GAGCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium leiognathi         ATAATCTC--------TTCG-------GAGCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium profundum 3TCK     ATAACGTC--------TTCG-------GACCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium profundum 1230     ATAACGTC--------TTCG-------GACCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium profundum DSJ4     ATAACGTCCTTGTTCATTAC-GAGCGGGACCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium profundum SS9      ATAACGTCCTTGTTCATTAC-GAGCGGGACCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium sp. Asur-1         ATAATCTC--------TTCG-------GAGCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium sp. ddeep-1        ATAACGTC--------TTCG-------GACCAAAGAGGGGGAC-C----------TTCGGG-C
Photobacterium sp. HAR23          ATAATCTC--------TTCG-------GAGCAAAGAGGGGGAC-C----------TTCGGG-C
Vibrio cholerae                   ATAACCTC--------GCAA-------GAGCAAAGCAGGGGAC-C----------TTCGGG-C
Vibrio parahaemolyticus           ATGATGCC--------TACG-------GGCCAAAGAGGGGGAC-C----------TTCGGG-C
Escherichia coli                  ATAACGTC--------GCAA-------GACCAAAGAGGGGGAC-C----------TTCGGG-C
Psychromonas antarctica           ATAATGTC--------TACG-------GACCAAAGGTGGCCTCTA---------TTTATAAG
Psychromonas hadaliensis          ATAATATC--------TTCG-------GATCAAAGTGGGGGAC-C----------TTCGGG-C
Psychromonas kaikoae              ATAATGTC--------TACG-------GACCAAAGCAGGGGACCT---------TAGGGC-C
Psychromonas marina               ATAATGTC--------TTCG-------GACCAAAGCAGGGGACCT---------TCGGGC-C
Psychromonas sp. CNPT3            ATAATGTC--------TACG-------GACCAAAGCAGGGGACCT---------TCGGGC-C
Psychromonas sp. HS11             ATAATGTC--------TACG-------GACCAAAGCAGGGGACCT---------TCGGGC-C
Psychromonas sp. IC004            ATAATGTC--------TACG-------GACCAAAGGTGGCCTCTA---------TTTATATG
Shewanella benthica ATCC43992     ATACGCCC--------TACG-------GGGGAAAGGAGGGGAC-C----------TTCGGG-C
Shewanella benthica PT48          ATACG--C--------TACG-------GGGGAAAGGAGGGGA--C----------TTCGCG-C
Shewanella benthica DB21MT-2      ATACGCCC--------TACG-------GGGGAAAGGAGGGGACGCG-TTTTTCGAAGTGCG-C
Shewanella benthica KT99          ATACGCCC--------TACG-------GGGGAAAGGAGGGGACGCGCTTTTTCGAAGTGCG-C
Shewanella benthica PT64          ATACGCCC--------TACG-------GGGGAAAGGAGGGGACGCGCTTTTCCAATGTGCG-C
Shewanella benthica PT99          ATACGCCC--------TACG-------GGGGAAAGGAGGGGACCCG-TTTTTCGAACTGCG-C
Shewanella benthica F1A           ATACG--C--------TACG-------GGGGAAAGGNGGGGACGCAC-THTTCGAAGTT-G-C
Shewanella frigidimarina          ATACGCCC--------TACG-------GGGGAAAGGAGGGGAC-C----------TTCGGG-C
Shewanella gelidimarina           ATACGCCC--------TACG-------GGGGAAAGGAGGGGAC-C----------TTCGGG-C
Shewanella oneidensis             ATACGCCC--------TACG-------GGGGAAAGAGGGGGAC-T----------TTCGGG-C
Shewanella putrefaciens           ATACGCCC--------TACG-------GGGGAAAGGAGGGGAC-C----------TTCGGG-C
Shewanella violacea               ATACGCCC--------TACG-------GGGGAAAGGAGGGGAC-C----------TTCGGG-C
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Figure 3.2. Alignment of the relevant portion of the 16S rRNA gene highlighting the elongated loops in the deep-sea strains.

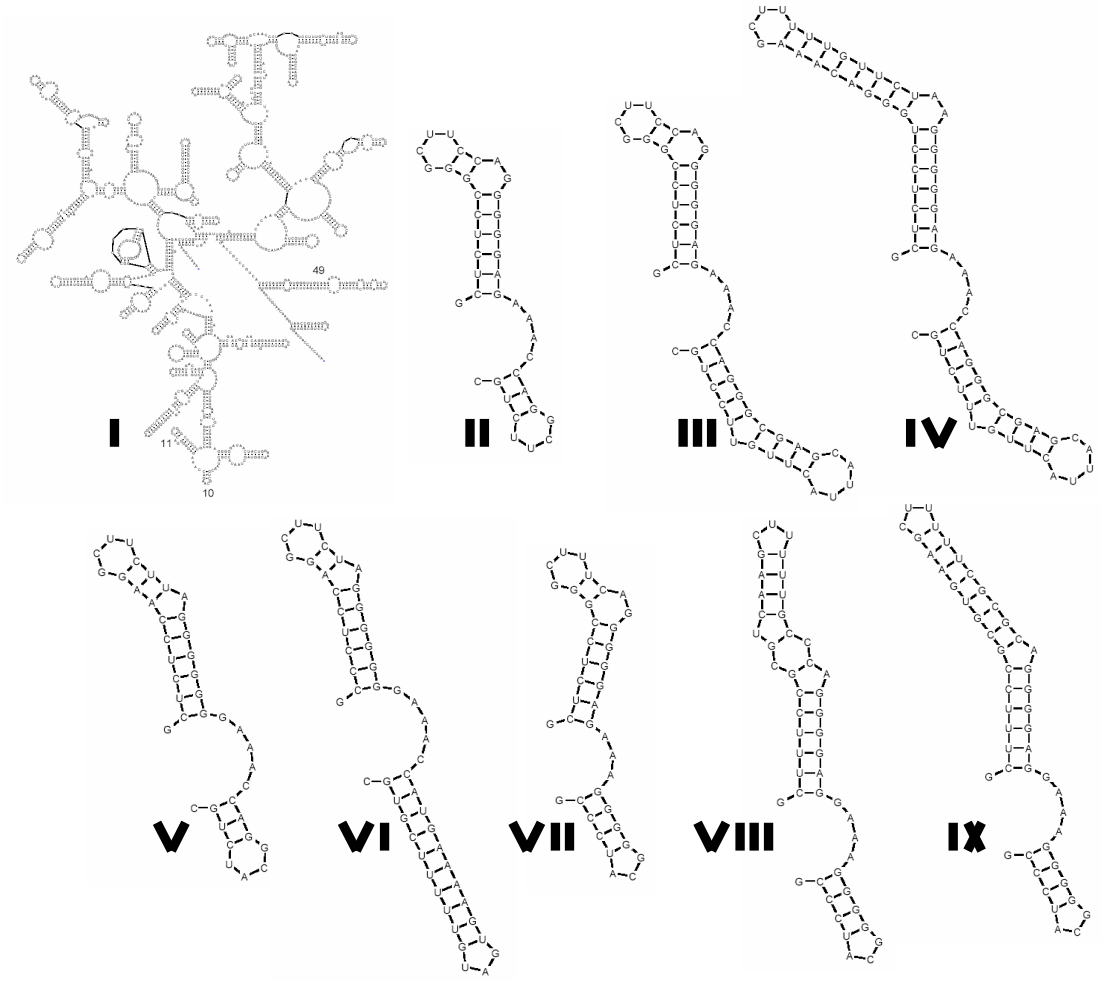
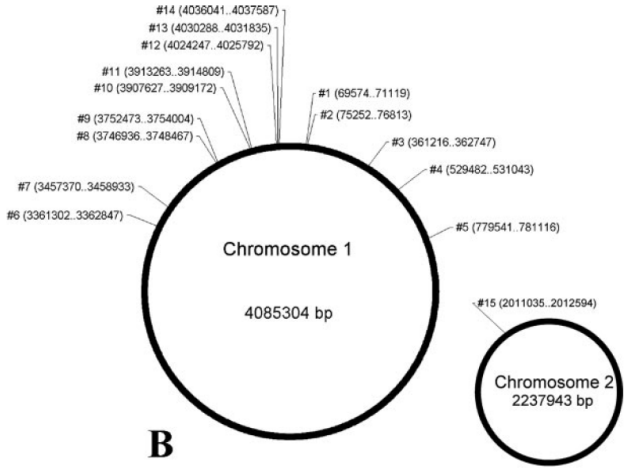


Figure 3.3. Structural comparison of the relevant portion of the 16S rRNA (helices 10 and 11) of piezophilic and non-piezophilic strains. Alignment of the same regions are shown in Fig 2. I) 16S structure of *Escherichia coli* with indication of helices 10, 11 and 49, II-IV) *Photobacterium profundum* SS9 helices 10-11 from different ribotypes, V) *Colwellia psychrerythraea* 34H, VI) *Colwellia sp.* MT41, VII) *Shewanella oneidensis* MR1, VIII) *Shewanella benthica* PT99, and IX) *Shewanella benthica* KT99.

16S	Helix #10	Helix #11	Helix #49
#1	Long	Short	Short
#2	Long	Short	Long
#3	Short	Short	Short
#4	Long	Short	Long
#5	Long	Long	Long
#6	Long	Short	Short
#7	Long	Short	Long
#8	Short	Short	Short
#9	Short	Short	Short
#10	Long	Short	Short
#11	Long	Short	Short
#12	Long	Short	Short
#13	Long	Short	Short
#14	Long	Short	Short
#15	Long	Long	Short

A



B

```
179 ATAACGTCGCAA-------------GACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTTGCCATC =//= 1439 GTAGCTTAACC------TTC----------GGGAGGGCGCT     E_coli_K12
179 ATAACGTCGCAA-------------GACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTTGCCATC =//= 1439 GTAGCTTAACC------TTC----------GGGAGGGCGCT     E_coli_O157
169 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1444 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#1
169 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1444 ATAGCTTAACCCCGATTTTTTAGGAAAAAGGGGAGGGCGTT     SS9_16S_#2
169 ATAACGTCTTCG-------------GACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1430 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#3
169 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1444 ATAGCTTAACCCCGATCTTTTAGGAGAAAGGGGAGGGCGTT     SS9_16S_#4
169 ATAACGTCTTTGTTCATTACGAGCGGGACCAAAGAGGGGGAATCTTGTTTTTCGAAACAGGGTCCTCTCGCGCTA =//= 1458 ATAGCTTAACCCCGATCTTTTAGGAGAAAGGGGAGGGCGTT     SS9_16S_#5
170 ATAATGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1445 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#6
172 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1447 ATAGCTTAACCCCGATCTTTTAGGAGAAAGGGGAGGGCGTT     SS9_16S_#7
170 ATAACGTCTTCG-------------GACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1431 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#8
170 ATAACGTCTTCG-------------GACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1431 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#9
170 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1445 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#10
170 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1446 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#11
170 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1445 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#12
172 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTTTCGCGCTA =//= 1447 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#13
170 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGG-------ACCTTCGGG-------CCTCTCGCGCTA =//= 1446 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#14
171 ATAACGTCCTTGTTCATTACGAGCGGGACCAAAGAGGGGGAACCTTGTTTTTCGAAATGGGGTCCTCTCGCGCTA =//= 1460 ATAGCTTAACC------TTC----------GGGAGGGCGTT     SS9_16S_#15
```

Figure 3.4. Ribotype (A), location (B) and alignment (C) of the relevant portions for the fifteen 16S rDNA of *P. profundum* strain SS9. The data is derived from (54) using the genome assembly of February 2004.

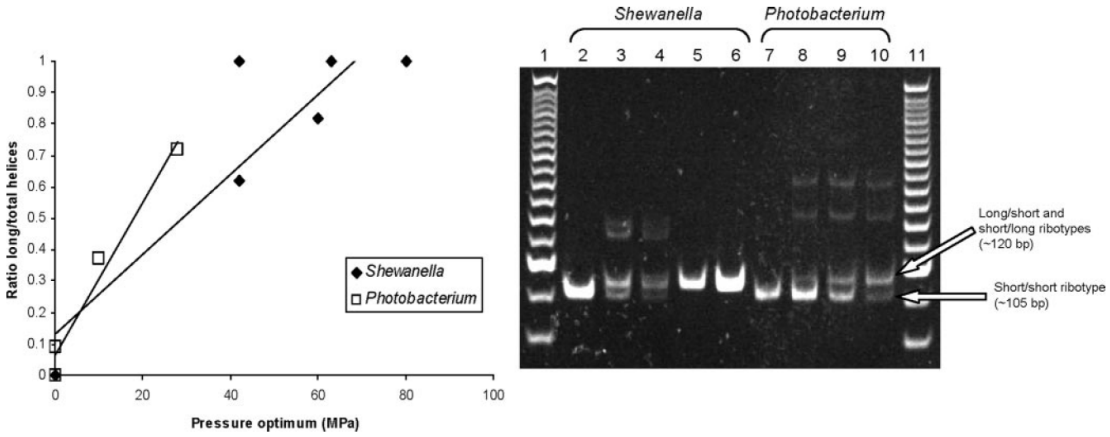


Figure 3.5. A) Relationship between optimal growth pressure and loop elongation in the genera *Shewanella* and *Photobacterium*. B) Detection of ribotype composition in various strains by semi-quantitative PCR. The assay was performed (25 cycles) as described in materials and methods (1-25bp ladder; 2-PE36; 3-PT48; 4-PT64; 5-F1A; 6-KT99; 7-3TCK; 8-1230; 9-DSJ4; 10-SS9; 11-25bp ladder). Because of chimera formation, in *Photobacterium* strains only the short/short, the short/long and the long/short ribotypes could be detected with the general primers. The long/long ribotype had to be amplified with specific primers.

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Supplementary material

TABLE S3.1. References and accession numbers for the sequences used in this study. The char. column refers to the strain characteristics: (P) Psychrophile (PP) Psychropiezophile (M) Mesophile.

Strain	Isolated from	Isolation depth	Char.	Primary citation	Genbank accession
Carnobacterium alterfunditum ACAM311	Ace Lake, Antarctica	Surface	P	(11, 33)	L08623
Carnobacterium divergens DSM20623	Vacuum-packaged beef	Surface	P	(7, 13)	M58816
Carnobacterium funditum DSM5970	Ace Lake, Antarctica	Surface	P	(11)	S86170
Carnobacterium gallinarum DSM4847	Ice slush on chicken carcasses	Surface	P	(7)	AJ387905
Carnobacterium inhibens K1	Atlantic salmon, Sweden	Unknown	P	(16)	Z73313
Carnobacterium maltaromaticum DSM20342	Milk with malty flavour	Surface	P	(21)	M58825
Carnobacterium pleistocenium FTR1	Permafrost tunnel, Alaska	Surface	P	(29)	AF450136
Carnobacterium sp. AT12	Aleutian Trench	2,500 m	PP	This study	DQ027062
Carnobacterium sp. AT7	Aleutian Trench	2,500 m	PP	This study	DQ027061
Colwellia demingiae ACAM459	Sea ice, Antarctica	Surface	P	(3)	U85845
Colwellia maris ABE-1	Hokkaido coast	Surface	P	(39)	AB002630
Colwellia psychrerythraea 34H	Greenland continental shelf	305 m	P	(14)	AF396670
Colwellia psychrerythrea MGP-41	Eastern Antarctica	709-964 m	P	(4)	AF530145
Colwellia sp. KT27	Kermadec trench	9,856 m	PP	This study	DQ027052
Colwellia sp. MT41	Mariana trench	10,476 m	PP	(37, 38), this study	DQ027051
Lactobacillus casei NCDO161	Unknown	Surface	M	(8)	X61135
Marinilactibacillus psychrotolerans M13-2	Miura peninsula, Japan	Surface	P	(15)	AB083406
Moritella japonica DSK1	Japan Trench	6,356 m	PP	(24)	D21224
Moritella marina ATCC15381	North Pacific Ocean	1,200 m	P	(35)	AB038033
Moritella sp. PE36	North Pacific Ocean	3,584 m	PP	(9, 37), this study	DQ027053
Moritella viscosa	Atlantic Ocean, Norway	Unknown	P	(1)	Y17574
Moritella yayanosii DB21MT-5	Mariana trench	10,898 m	PP	(17)	AB008797
Photobacterium leiognathi ATCC25521	Fish light organ	Unknown	M	(28)	D25309
Photobacterium profundum 1230sf1	Peru Trench	5,086 m	P	(2), this study	DQ027055
Photobacterium profundum 3TCK	San Diego coast	Surface	P	(6), this study	DQ027054
Photobacterium profundum DSJ4	Ryukyu Trench	5,110 m	PP	(27)	D21226
Photobacterium profundum SS9	Sulu Sea	2,551 m	PP	(36)	AB003191
Photobacterium sp. Asur1	Pacific Ocean	Surface	P	(30)	AB055784
Photobacterium sp. Ddeep1	Pacific Ocean	1,000-8,000 m	P	(30)	AB055793
Photobacterium sp. HAR23	Pacific Ocean, Japan	3,009 m	P	(34)	AB038031
Photobacterium sp. HAR72	Pacific Ocean, Japan	9,706 m	P	(34)	AB038032
Psychromonas antarctica star-1	McMurdo Ice Shelf	Surface	P	(22)	Y14697
Psychromonas hadaliensis K41G	Japan Trench	Unknown	?	(23)	AB094413
Psychromonas kaikoae JT7304	Japan Trench	7,434 m	PP	(25)	AB052160
Psychromonas marina 4-22	Okhotsk Sea, Japan	Surface	P	(18)	AB023378
Psychromonas sp. CNPT3	Central North Pacific Ocean	5,800 m	PP	(9), this study	DQ027056
Psychromonas sp. HS11	Hawaii Coast	5,800 m	PP	(10), this study	DQ027057
Psychromonas sp. IC004	Sea ice, Antarctica	Surface	P	(3)	U85849
Shewanella benthica ATCC43992	South Atlantic Ocean	4,575 m	PP	(26)	X82131
Shewanella benthica DB21MT-2	Mariana trench	10,898 m	PP	(17)	AB008796
Shewanella benthica F1A	North Atlantic	4,900 m	PP	(9)	U91592
Shewanella frigidimarina ACAM591	Prydz Bay, Antarctica	Surface	P	(5)	U85903
Shewanella gelidimarina MGP-71	Eastern Antarctica	709-964 m	P	(4, 5)	AF530149
Shewanella oneidensis MR-1	Oneida Lake, N.Y.	Surface	M	(12)	AE015906
Shewanella putrefaciens LMG26268	Sippewisset marsh, Woodshole	Surface	M	(31)	X81623
Shewanella sp. KT99	Kermadec trench	9,856 m	PP	This study	DQ027058
Shewanella sp. PT48	Philippine Trench	6,163 m	PP	(9), this study	DQ027059
Shewanella sp. PT64	Philippine Trench	7,100 m	PP	(37), this study	DQ027060
Shewanella sp. PT99	Philippine Trench	8,600 m	PP	(9, 19)	AB003189
Shewanella violacea DSS12	Ryukyu Trench	5,110 m	PP	(26)	D21225
Thalassomonas viridans XOM25	Valencia, Spain	Surface	M	(20)	AJ294748
Vibrio parahaemolyticus ATCC17802	Contaminated seafood	Surface	M	(32)	X74720

Supplementary References

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4.

Large-scale transposon mutagenesis of *Photobacterium profundum* SS9 reveals new genetic loci important for growth at low temperature and high pressure.

Abstract

Microorganisms adapted to piezopsychrophilic growth dominate the majority of the biosphere that is at constant low temperature and high pressure, but the genetic bases for these adaptations are largely unknown.

Here I report the use of transposon mutagenesis to isolate 27 mutant strains impaired in growth at low temperature and 10 mutant strains with altered growth as a function of hydrostatic pressure. The largest fraction of loci associated with temperature sensitivity was involved in the biosynthesis of the cell envelope, in particular of the extracellular polysaccharide (EPS). The largest fraction of loci associated with pressure sensitivity was involved in chromosomal structure and function. The adaptation to both temperature and pressure was affected by mutations in a number of sensory and regulatory loci, suggesting the importance of signal transduction mechanisms in the adaptation to these physical parameters.

The results represent the first global genetic analyses of genes conditionally required for low temperature or high pressure growth in a deep-sea microorganism.

Introduction

Most deep-sea environments select for microorganisms capable of growth at low temperature (psychrophiles), high hydrostatic pressure (piezophiles) and sporadic inputs of organic nutrients (9, 10).

Photobacterium profundum strain SS9 (71) is a psychrotolerant and moderately piezophilic bacterium. It was first isolated from amphipod homogenate enrichment in the Sulu Sea (28). This microorganism, suitable for a variety of systems biology investigations, 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 to nearly 90 MPa (optimal pressure 28 MPa). The analysis the SS9 genome and transcriptome suggests that the two most important aspects of its deep-sea adaptations are the nature of its pressure- and temperature-responsive genes and the high degree of metabolic diversity and redundancy (89).

Like other *Vibrionaceae*, the genes of *Photobacterium profundum* are partitioned on two circular chromosomes (19, 89). The two chromosomes are thought to be functionally distinct with most "established" and essential genes located on chromosome 1, and most strain-specific and horizontally acquired genes on chromosome 2 (39, 89). In addition, *P. profundum* SS9 has a dispensable 80kb plasmid carrying mostly genes of unknown function (19, 89).

Campanaro *et al.* (19) analyzed the intraspecific variation between three strains of *P. profundum* with different degrees of piezoadaptation, speculating how some of the variable regions might be important for growth at high pressure.

Genetic approaches to elucidate mechanisms of deep-sea adaptation in *P. profundum* SS9 have also been conducted. During the course of investigating factors influencing the pressure regulation of outer membrane protein abundance, the membrane-localized transcription factor ToxR was identified as a pressure sensor (94), and the *rseC* and *recD* genes have been found to be important for low temperature/high pressure growth and high pressure growth, respectively (15, 23). More recently site-directed insertional mutagenesis was employed to identify genes involved in fatty acid unsaturation important for high pressure growth (4-6). None of the above studies have included a global analysis of genes conditionally required for growth at high pressure or low temperature.

To complement and expand upon these studies a collection of mini-Tn5 and mini-Tn10 transposon mutants was screened for high pressure-sensitivity and cold temperature-sensitivity. In this work I describe the isolation and characterization of these mutants.

Materials and Methods

Bacterial strains, plasmids, growth conditions and mutant screening. *P. profundum* strains were routinely cultured under aerobic conditions in 3/4 strength 2216 medium (28 g/l; Difco Laboratories) at 15°C. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium.

Antibiotics were used in the following final concentrations: rifampicin (Rif) 100 µg/ml; kanamycin (Km), 100 µg/ml *(E. coli*) or 200 µg/ml (*P. profundum*); streptomycin (Sm), 50 µg/ml *(E. coli*) or 150 µg/ml (*P. profundum*). X-Gal (5-bromo-

4-chloro-3-indolyl- [beta] -D-galactopyranoside) was added to solid medium at 40 µg/ml in N,N-dimethylformamide.

Bacterial strains and plasmids used in this study are listed in Table 4.1. Plasmids were introduced in *Photobacterium profundum* strain SS9 by bi-parental conjugations with *E.coli* S17-1 or tri-parental conjugations using an *E.coli* strain containing the helper plasmids pRK2073 or pRK2013 as previously described (24).

Transposon mutagenesis was performed by mating *E.coli* S17-1 containing the mini-Tn10 donor plasmid pLOF (40) or *E.coli* BW20767 containing the mini-Tn5 donor plasmid pRL27 (54) with *P.profundum* SS9R strain (a rifampicin resistant derivative of SS9) and screening for exconjugants growing with double selection (kanamycin+rifampicin) on 2216 Marine agar plates (Difco laboratories, BD, Franklin Lakes, NJ, USA).

The screening for auxotrophic mutants was done by replica-plating onto MOPS Glucose Minimal Marine Medium (11). Colonies unable to grow on this medium were characterized as auxotrophs.

The screening for mutants impaired in low temperature growth was performed by replica-plating transposon mutants onto 2216 agar plates and incubating at 15°C and 4°C. The clones that grew poorly compared to the wild-type after 120hrs at 4°C were saved. They were subsequently re-screened in liquid medium and their growth-plot was determined in triplicate. A cold sensitivity ratio was computed as follows: considering a=[hours necessary to reach mid-log in the 15°C culture] and b=[hours necessary to reach mid-log in the 4°C culture] then CS_{ratio}=[a/(b-a-50)]x100. This ratio was approximately 100 for a wild-type *P. profundum* SS9 culture grown in 2216

marine broth (with 20 mM of glucose, 10 mM of HEPES pH 7.5). Clones displaying a CS_{ratio}<70 were considered cold-sensitive. However, in those cases where a mutant reproducibly displayed the cold-sensitive phenotype on plates but not in liquid, it was still recorded as cold-sensitive.

High pressure growth of *P.profundum* SS9 transposon mutants was performed at 17°C in 2216 medium supplemented with 20 mM of glucose, 10 mM of HEPES pH 7.5, 200 µg/ml of kanamycin and 50 mg/L of phenol red as a color indicator for fermentative growth. Transposon mutants were individually inoculated in wells of a 96-elevated wells PCR plate (Aygen Scientific, Union City, CA, USA) and filled to the top with the above described high-pressure growth medium. The plate was sealed with a 96-well PCR Axymat (Axygen Scientific, Union City, CA, USA) being careful not to trap any air bubbles. The microtiter plates were then pressurized using water and a hydraulic pump in a custom-designed stainless steel 2 liter pressure vessel (Autoclave Engineers, Erie, PA, USA). The screening for pressure sensitivity was done by replica plating transposon mutants at high (45 MPa) and low (0.1 MPa) pressure (at 15° C) and looking for wells that retained the red color, indicating no fermentative growth had occurred, after 48 hours. Those mutants were saved and re-screened by determining their growth plot in triplicate.

Pressure-dependent growth curves were performed by 1000 fold dilutions of 48 hrs old cultures of the mutants that were aliquoted in heat sealable plastic bulbs and grown at 0.1 and 45 MPa. At each time point, one bulb was removed from the stock, transferred to a glass tube and the turbidity of the culture was measured at 600nm on a Spectronic20 spectrophotometer (Milton Roy, New York, USA).

The pressure-sensitivity ratio of the strains was assessed as previously described (23). Briefly, when the low pressure culture entered early logarithmic phase (OD_{600}=0.1-0.3), two values were recorded: a=[OD_{600} of the 45 MPa culture] and b=[OD_{600} of the 0.1 MPa culture]. The PS_{ratio}=a/b was then computed. Under these conditions the PS_{ratio} of the wild type was 1-1.1. Clones displaying a PS_{ratio}<0.5 were considered pressure-sensitive. Clones displaying PS_{ratio}>1.5 were considered pressure enhanced.

DNA extraction, purification, manipulation and sequencing. Genomic DNA was extracted from 3 ml of 48 hrs old *P. profundum* SS9 cultures by using the Wizard Genomic kit (Promega, Madison, WI, USA). The DNA was then further purified by extracting it once with a phenol/chloroform mixture and once with chloroform alone as described elsewhere (82).

Plasmid DNA was extracted using the Qiagen (Valencia, CA, USA) Miniprep (high copy number plasmids) or Midiprep (low copy-number plasmids) kits following manufacturer's instructions.

All enzymatic reactions were prepared following standard protocols (82). Enzymes were purchased from Invitrogen (Carlsbad, CA, USA) or from New England Biolabs (Beverly, MA, USA)

DNA sequences were determined by thermal cycle fluorescent dideoxy sequencing with a MegaBACE 1000 (Amersham Biosciences, Piscataway, NJ, USA) automated sequencer as instructed by the manufacturer.

Arbitrary PCR amplification and transposon cloning. Rapid identification of the flanking sequences to the transposon insertions in *P. profundum* was accomplished by a rapid arbitrary PCR method similar to the one used by Watnick and Kolter (92). This method involves two rounds of PCR. Briefly, during the first round genomic DNA from the mutant is PCR amplified with a primer unique to either end of the mini-Tn10 (10extdx2 [5'-AGAGCATTACGCTGACTTG-3'], 10extsx [5'-CACCCCTTGTATTACTGTTTATGT-3']) or mini-Tn5 (pRL27Extdx [5'-CCAGAAAGTGAGGGAGCCA-3'], pRL27Extsx [5'-GACAACAAGCCAGGGATG-3']) in combination with a degenerate primer (SS9arb1 [5'-GACCACGAGACGCCACACTNNNNNNNNNNCATGC]

SS9arb2-[5'- GACCACGAGACGCCACACTNNNNNNNNNNACTAG-3'] and SS9arb8 [5'-GACCACGAGACGCCACACTNNNNNNNNNNGATAT-3']) that is designed to hybridize to an arbitrary sequence on the chromosome and carrying a 5' GCclamp. Cycle conditions for the first round of amplification were: 94˚C for 30 sec, 30˚C for 30 sec, 72˚C for 60 sec repeated 5 times followed by 94˚C for 30 sec, 45˚C for 30 sec, 72˚C for 60 sec repeated 30 times. 2 μL of PCR product were subjected to a second round of amplification (95˚C x 30 sec, 55˚C x 30 sec, 72˚C x 45 sec for 30 cycles) using a nested primer unique to the mini-Tn10 end (10int [5'-GTATGAGTCAGCAACACCTTCTTC-3']) or the mini-Tn5 end (pRL27Intdx1 [5'-GAGTCGACCTGCAGGCATGC-3'] and pRL27Intsx [5'-CGCACTGAGAAGCCCTTAGAGC-3']) and a primer with sequence identity for the 5' GC clamp of the arbitrary primer (arb3 [5'- GACCACGAGACGCCACACT-3']). After running the product of the second amplification on a gel, the PCR showing

single bands were purified using the UltraClean PCR Clean-up Kit (MoBio Laboratories, Solana Beach, CA, USA) and sequenced using a third transposon specific nested primer (10end).

The mini-Tn10 insertion points difficult to characterize by arbitrary PCR were obtained by transposon cloning. Genomic DNA from the mutant was digested overnight with *Eco*RI that does not cut inside the transposon. The fragments obtained were ligated into the *Eco*RI site of pUC18, transformed in *E.coli* TOP10 cells and selected with kanamycin (100 µg/ml) and ampicillin (100 µg/ml). The transformants growing on this selection contained the pUC18 plasmid with the cloned kanamycin resistance gene from Tn10 together with flanking sequences. The flanking sequences were then determined by sequencing with primers specific to the multiple cloning site of pUC18.

Similarly, the mini-Tn5 mutants difficult to characterize by arbitrary PCR were analyzed by performing a genomic DNA extraction followed by complete digestion with *Bam*HI, religation and transformation into *E. coli* EC100D *pir*+ (Epicentre, Madison, WI, USA) and plating onto LB agar plates containing kanamycin (50µg/ml).

Sequence flanking the transposon insertion was searched with BLASTN (7) on the nucleotide sequence of the *P. profundum* SS9 genome allowing the retrieval of whole ORF sequences. Downstream genes were analyzed using the genome browser available at http://SS9.cribi.unipd.it. The translated ORF sequence was classified according to the COG database (86) and analyzed for cellular localization using SubLoc 1.0 (42) and the presence of signal peptides using SignalIP (13).

Complementation of selected mutants. A small number of the mutants obtained was further characterized by re-introducing the wild-type copy of the disrupted gene. The ORF sequence, including the predicted promoter and additional upstream sequence, was PCR amplified using Expand Long Template PCR kit (Roche, IN, USA) and ligated into the mobilizable broad host-range vector pFL122 (56). The recombinant clones selected after blue-white screening were sequenced for accuracy of the insert. This construct was conjugated into the appropriate mutant by tri-parental matings according to published procedures (23). The CS_{ratio} and PS_{ratio} of the merodiploid mutant were then re-determined as described above and compared to that of the isogenic parental mutant strain containing the plasmid vector alone.

Results and Discussion

Twenty thousand transposon mutant derivatives of *P. profundum* SS9R were obtained using either mini-Tn10 (6,000 mutants) or mini-Tn5 (14,000 mutants) transposable elements. During the preliminary screening, a Southern blot analysis of 12 auxotrophic mutants obtained by mini-Tn10 mutagenesis revealed the presence of hot spots for the mini-Tn10 transposition, because the 12 insertions were in just four genes: orotate phosphoribosyltransferase (PBPRA0199; 3 hits), acetylornithine aminotransferase (PBPRA0289; 4 hits), argininosuccinase and n-acetylglutamate synthase (PBPRA0269; 3 hits); shikimate kinase (PBPRA0280; 2 hits). Tn10 has been previously noted to exhibit strong sequence insertion bias (47). The same analysis performed with mutants obtained by mini-Tn5 did not show the same bias (data not

shown). Despite these limitations, the Tn10 mutants evaluated in this study provided a disproportionately large number of the cold-sensitive and pressure-sensitive mutants obtained (see below).

Mutants were examined for defects in growth at low temperature (4°C, atmospheric pressure) and high pressure (28 MPa, 15°C). In the latter case a Phenol Red fermentation color screening method in pressurizable microtiter plates was used (Fig. 4.1).

Putative cold-sensitive (CS) and pressure-sensitive (PS) mutants were rescreened and bona fide CS and PS mutants were further evaluated by generating a detailed growth curve: 1.5% (11/720) of the mutants tested were auxothrophs, 0.13% (27/20000) were cold sensitive and 0.04% (8/20000) were pressure sensitive.

In addition to the above mutants two pressure-enhanced mutants were also recovered. These mutants were initially isolated as weak fermenters at high pressure, but subsequent analyses revealed that they actually possessed better growth rates and yields at high pressure, indicating that high pressure partially compensated for their growth deficiencies. This further reinforces transcriptome data indicating that *P. profundum* SS9 is under greater stress at atmospheric pressure than at elevated pressure, perhaps reflecting its deep-sea origin (89).

Considering the cold-sensitive and pressure-affected mutants together, 77% of the transposon insertions were located on chromosome 1, 23% were located on chromosome 2 and none was located on the plasmid. The particular gene disruptions and their locations are shown in Table 4.2 and Figure 4.2. The presence of a larger percentage of loci required for growth at high pressure and/or low temperature on

chromosome 1 holds true even after normalization for the larger amount of ORFs encoded on this replicon. Transcriptome and codon adaptation index analyses indicate that the most actively transcribed genes are also located on chromosome 1 of *P. profundum* SS9 (89).

The effects of pressure and temperature on microorganisms are pleiotropic. For example, in the case of *Escherichia coli*, which is mesophilic with respect to both temperature and pressure, decreased temperature or increased pressure perturb membrane structure and function, DNA replication, transcription and translation (9). Although temperature and pressure are distinct thermodynamic parameters, in many cases, the effect of pressure is synergistic with that of low temperature (28). 16% of the mutants obtained here are altered in growth ability both at low temperature and at high pressure.

Isolation of cold-sensitive mutants.

Mutants in 27 loci displayed a cold-sensitive phenotype (Figure 4.3 and Table 4.2). Among these, 21 were exclusively cold-sensitive, 5 were also pressure-sensitive and 1 was also pressure-enhanced.

These mutants could be functionally divided into 6 categories based on the COG identity of the disrupted gene. However, genes belonging to the poorly characterized COG functional classes [R, S] were clustered based on additional information provided by other analyses.

1- ***Signal transduction mechanisms [T]*** (Strains FL1, FL4, FL11, FL13, FL21). Microorganism sense and coordinate metabolic functions during cold-stress

(74). These responses occur through temperature-induced changes in DNA supercoiling (30, 51, 66), via effects on ribosomes (88) through the modulation of ppGpp levels (46) and by gene regulation through the activity of alternative sigma factors (76, 96).

The expression of additional genes required for low temperature growth in SS9 might be modulated by specific regulators such as PBPRB2014 (strain FL1), a transcriptional regulator of the LuxR family, and PBPRB1757 (strain FL21), a hypothetical protein with a response regulator receiver domain (pfam00072) and a partial domain of a signal transduction histidine kinase (COG0642) suggesting its possible role as a hybrid two-component system. Sensory mechanisms of this sort have been observed in *B. subtilis* and some cyanobacteria (81). In these organisms, the expression of cold-inducible genes, in particular those responsible for membrane unsaturation, are controlled by specific two-component systems.

2- ***Cell envelope biogenesis, outer membrane [M]*** (Strains FL3, FL5, FL7, FL9, FL14, FL25, FL26, FL27). The involvement of capsular polysaccharide in adaptation to low temperature has been speculated for some time (25, 62, 64).

FL26 contains a disruption in the gene encoding PBPRA0218, a predicted O-antigen ligase for lipopolysaccharide (LPS) synthesis whose expression is reduced at low temperature (19, 89). Of the remaining mutants, 4 are part of a large gene cluster that is highly divergent within the members of the species *P. profundum* (19). This cluster is approximately 35 kb in size and contains many genes that are involved in various aspects of the LPS O-antigen biosynthesis, extracellular polysaccharide (EPS) biosynthesis and flagellar filament glycosylation (36). Some of these genes are also

differentially expressed by temperature and pressure (PBPRA2692, PBPRA2701 and PBPRA2710). Their functions are currently being further characterized (Ferguson *et al.*, in preparation). Strain FL7 (disruption of PBPRA2684, hypothetical protein, COG functional class R) is included within this category of mutants based solely on the fact that its insertion location is within the predicted polysaccharide biosynthesis cluster.

By a similar reasoning I included strain FL27 among the envelope mutants. The mutated gene in strain FL27, PBPRA0674 is homologous to the haemolysin coregulated protein (Hcp) of *Vibrio* species. While the function of Hcp is still uncharacterized (97), Enos-Berlage *et al.* have shown that a mutation in *hcp* results in alterations in the formation of *Vibrio parahaemolyticus* biofilms and thus could be associated with polysaccharide production (32).

Interestingly strains FL25, FL26 and FL27 displayed the cold-sensitive phenotype only when grown on agar plates, but not in liquid media (Fig. 4.3) suggesting different EPS/LPS requirements for growth at low temperature under the two conditions.

In *E. coli* a deletion of the *rfa* locus, responsible for the assembly of the core oligosaccharide of LPS is cold-sensitive and non-motile (72). The role of LPS in stabilizing the bacterial cell envelope is exerted both through its interaction with the extracellular polysaccharide (EPS) and through the fluidizing effects that its lipid components can have on the outer membrane (52).

Corsaro *et al.* (25), have suggested that the psychrophile *Pseudoalteromonas haloplanktis* TAC125 is unable to complete the biosynthesis of the lipooligosaccharide

(LOS) at sub-optimal temperature while the phosphorylation of both LOS and EPS decreases with temperature.

Within these structures, phosphate groups bind to divalent cations such as Ca^{2+} and Mg^{2+}, stabilizing the extracellular leaflet of the outer membrane (52, 78) and modulating its permeability. At the same time, LPS integrity is essential to the correct incorporation of many proteins in the outer membrane such as porins (72). It is possible that changes in LPS and EPS, by changing the surface properties of bacteria are used in processes affected by low temperature such as membrane fluidity, substrate transport or interaction with other bacterial cells (32).

3-***Carbohydrate transport and metabolism [G]*** (Strains FL10, FL12, FL16, FL17, FL22, FL23). Low temperature growth was affected by mutations in a number of genes of for the transport and the central metabolism of carbohydrates. These included the glycolytic enzyme pyruvate kinase I (PBPRA0428), a component of a group-translocation transporter specific for cellobiose (PBPRB2009) and the conserved hypothetical protein PBPRA2282 which is a homolog of HI0227 from *Hemophilus influenzae*, and may participate in the metabolism of N-acetylneuraminic acid and its derivatives (49).

Two mutants deserve special attention. PBPRA0747 clusters in this category because it is homologous to *suhB* whose gene product was shown to possesses inositol monophosphatase activity (70). However, its role in the cell appears to be that of modulating the processing activity of RNAse III, and because of this, in *E. coli*, a *suhB* mutation suppresses a wide variety of other mutations (43). In addition a *E. coli suhB* mutant alone is cold-sensitive (43).

Strain FL23 has an insertion in PBPRA3229 which was originally annotated as a phosphoheptose isomerase (89), but after a closer inspection turned out to be orthologous to *diaA*, a gene required during the initiation of chromosome replication (44). Both loci (PBPRA0747 and PBPRA3229) also affect growth at high pressure in *P. profundum* SS9.

4- ***Protein export [U].*** (Strains FL18, FL19, FL24) Strain FL19 is mutated in a gene encoding a hypothetical protein with a well defined signal peptide (13). While it is possible that the gene product of PBPRB1941 is directly involved in some aspect of temperature adaptation, another hypothesis is that this insertion has resulted in a blockage of the general secretory pathway (sec). This pathway is inherently cold-sensitive in *E. coli* (75).

Other secretion routes could also be important for low temperature growth. Strain FL18 contains an insertion in PBPRA0667, a homolog of *impC*, a gene of *Rhizobium leguminosarum* (16) that is part of a locus important for temperature dependant secretion and establishment of the symbiotic interaction with the host plant roots. Strain FL24 has a mutation in PBPRA0917, an orthologue of the flagellar chaperone *fliS* of *E. coli*. FliS functions as a substrate-specific chaperone facilitating the export of flagellin axial-filament subunits and preventing their polymerization in the cytosol (8). The cold-sensitive phenotype of strain FL24 is visible only on plates and might be a result of the deleterious accumulation of flagellin oligomers in the cytosol, especially at low temperature when the activity of proteases involved in recycling of non-functional peptides is lower.

5- ***Stress proteins [L, O]***. The fourth functional category (Strains FL2, FL6, FL15, FL20) includes a diverse set of genes involved in response to stress which belong to COG categories L (DNA replication, recombination and repair), O (Posttranslational modification, protein turnover, chaperones), and the poorly characterized functional classes R and S.

Strain FL2 had a mutation in PBPRB0212. This gene was annotated as a member of a family of ATP-dependant helicases known as DEAD box for their characteristic aminoacid motif (Asp-Glu-Ala-Asp). RNA helicases are involved in unwinding duplex RNA and, because of their regulation and role in ribosome biogenesis and translation initiation, have been linked to cold stress in both cyanobacteria (20) and *Archaea* (58).

The insertion in strain FL6 is in PBPRA1774, coding for a LA-related protease. Similar ATP-dependent proteases have been shown to degrade non-functional proteins in the cytoplasm of *E. coli* (80) and to be important for cold acclimation in the marine cyanobacterium *Synechococcus* (77).

When a protein becomes unfolded, it exposes hydrophobic surfaces, normally buried in the core, which can interact with other hydrophobic surfaces of neighboring molecules to produce large multi-molecular ensembles. Protein aggregates are considered `dead ends' and their accumulation may cause severe damage to the organism (80). This unfolding and destabilization can be induced by a cold shock (33) and it is possible that a null mutation in the PBPRA1774 protease leads to cytoplasmic accumulation of protein aggregates that affect growth.

Strain FL15 is interrupted in the gene for the hypothetical protein PBPRA3239 that is upstream and in the same trancriptional unit as the periplasmic serine proteases *degQ* and *degS.*

The mini-Tn10 insertion of mutant FL20 is in the coding region for a transposase. Transposable elements are highly abundant in the genome of *P. profundum* SS9 (89). The basis for the cold-sensitivity of this mutant is unclear at this time.

6- ***Unknown [R].*** (Strain FL8) The basis for cold sensitivity of strain FL8 is also unknown. The interrupted gene, PBPRA0396, codes for a hypothetical integral membrane protein and is part of an operon with two ribosomal proteins, L21 and L27.

Isolation of pressure-sensitive mutants.

Table 4.2 and Figure 4.4 list the transposon mutants displaying a pressure-altered phenotype together with their pressure-sensitivity ratio.

The number of loci recovered in this screen was less than 1/3 of that of the cold-sensitives. Three separate hypotheses could account for this difference: the screen for pressure-sensitive mutants was less sensitive, adaptation to high hydrostatic pressure requires fewer genes or adaptation to high hydrostatic pressure requires a higher proportion of essential genes.

Two lines of evidence point towards the latter two hypotheses: 1) previously identified pressure-sensitive site-directed mutants were identified using this screen, thus demonstrating its sensitivity and arguing against hypothesis 1, and 2) with the exception of PBPRA2596 (L-asparaginase) and PBPRA2658 (3-oxoacyl-[acyl-carrier-

protein] synthase I) all the genes conferring piezoadaptation have been shown to have pleiotropic effects in other bacteria because of their central role in the cell (12, 23, 41, 43, 60, 61, 67, 85), which is consistent with hypothesis 3.

PBPRA2658 (*fabB*) codes for the KASI enzyme (79) and has been linked to piezophily because of its role in the production of monounsaturated fatty acids which are essential for proper membrane physical state at high-pressure (3).

The basis for pressure-sensitivity in strain FL29 which contains an insertion in PBPRA2596 is less clear. The enzyme is essential under conditions in which asparagine is the only nitrogen source (27). It is possible the L-asparaginase activity is a key route for nitrogen assimilation at high pressure under the glucose and peptide fermentation conditions used. The culture medium employed contains only 15μM nitrate. The importance of an organic nitrogen source for metabolism is further reinforced by the upregulation at 45 MPa of PBPRA1174 (periplasmic L-asparaginase), PBPRA3391 (aspartate-ammonia lyase) and PBPRA2173 (histidine-ammonia lyase), all of which contribute to the catabolism of amino acids.

Another target of pressure resulting in a global alteration of metabolism is observed in strain FL10, carrying a mutation in pyruvate kinase I (PBPRA0428). Pyruvate kinase has been previously found to be a pressure-sensitive enzyme and to undergo adaptational changes in deep-sea animals (26, 59). Once again, this could be an experimental artifact of growth under glucose fermenting conditions. Alternatively, because of the role of pyruvate kinase in glycolytic regulation (67) it could be a regulatory mutant. Transcriptome studies have shown that 5 of the 9 steps of

glycolysis have pressure- temperature- regulated genes, one of them being PBPRA0428. This mutant is also impaired in growth at low temperature.

Almost half of the pressure-sensitive mutants were associated with some step of chromosome structure and partitioning during cell division. Cell division and chromosome replication and segregation are among the most pressure-sensitive processes in a bacterial cell (9, 98). Therefore proteins underlying these cellular functions are expected to be under a strong selective pressure at depth. Bidle and Bartlett (15) reported the impairment in growth at high pressure of a *recD* mutant of *P. profundum*. They were also able to show that high pressure impairment of cell division in *E. coli* could be rescued by the heterologous expression of the *recD* gene from *P. profundum* (15).

PBPRB0001 is a gene considered essential for the replication of chromosome II within the family *Vibrionaceae* (31). Egan and Waldor (31) were unable to obtain a null mutant of VCA0002, the orthologue of PBPRB0001, in *V. cholerae*. Because the insertion in strain FL31 is close to the 3' end of the ORF, I hypothesize that it results only in a partial loss of function. This further implies that the C-terminus of PBPRB0001 is required only under high-pressure conditions.

Two additional genes whose products are important for chromosome replication at high pressure were also uncovered. The first one, *seqA* (PBPRA1039), is a negative regulator of the cell cycle and a strain carrying a mutation in PBPRA1039 is pressure-enhanced. SeqA mutants in *E. coli* have irregular growth caused by asynchronous patterns of replication (60). The stress condition imposed by growth at low pressure might exacerbate this phenotype in the case of SS9, but it is

unclear if this is due to the role of SeqA as a cell-cycle regulator (60), its importance for chromosome partitioning (57) or for its effect on the structure of cell membranes (93).

While a *seqA* mutant resulted in a pressure-enhanced phenotype, pressure- and cold-sensitivity was observed for strain FL23 which contains an insertion in *diaA*. This gene has been recently identified as a novel DNA-binding protein involved in ensuring the initiation of chromosomal replication at the right time (44). Interestingly, studies conducted in *E. coli* have shown that both *seqA* and *diaA* can suppress temperature-sensitive phenotypes associated with mutations in *dnaA*, which encodes for the initiator of chromosome replication (44, 90).

The inactivation of *rseB* (PBPRA3093), a gene belonging to the *rpoE* cluster, results in a pressure- and cold-sensitive phenotype. Chi and Bartlett (23) have suggested that this is due to a polar effect on *rseC*. At the present time it is unclear if the observed phenotype is determined by RseC involvement in the regulation of the alternative sigma factor RpoE (65) or in its role in other cellular functions such as thiamine synthesis as observed in *Salmonella typhimurium* (12).

Mutations in PBPA0747 (*suhB*), PBPRB0212 (DEAD-box helicase) and PBPRA0189 (*spoT*) are predicted to affect the structure/function of the ribosome.

It is not surprising to find targets for pressure- and temperature- sensitivity in a complex structure like the ribosome. In fact mesophilic ribosomes are extremely sensitive to high hydrostatic pressure (35, 53). *Photobacterium profundum* SS9 has the record number of ribosomal operons with a high amount of intragenomic variation (55, 89). The intragenomic variation in the 23S rRNA of *P. profundum* SS9 is

concentrated in helices 25 and 45. In these two helices, insertion sequences have been previously observed in *Campylobacter* (50). There is no evidence that these sequences are retained in the processed ribosome and if the intervening sequences are removed, it is by the action of RNAse III. Interestingly RNAse III activity is modulated by SuhB (43), the product of PBPRA0747.

Helix 25 is also the major site of interaction with ribosomal protein L13, an essential protein for the assembly of the 50S subunit of the ribosome. In the correctly assembled ribosome, L13 is located within a few angstroms of loop 2475 (68). Two members of the DEAD-box family of RNA helicases have been implicated in this step of ribosome biogenesis. The first one, DbpA, was shown to interact with residues 2454-2606 of the 23S rRNA *in vitro* (87). The second, SrmB, was implicated directly in the assembly of L13 because an *srmB* deletion results in accumulation of incomplete large ribosomal subunits (40S) lacking L13 (22). Interestingly, such mutants are also cold-sensitive (22). Therefore, if PBPRB0212 was performing similar functions in the cell, this possibly explains the cold–sensitive phenotype of its mutant. But why isn't strain FL2 also pressure-sensitive?

One possible explanation relies on functional redundancy: the genome of *P. profundum* SS9 codes for at least 9 DEAD-box helicases (PBPRA0562, PBPRA1748, PBPRA3542, PBPRB0199, PBPRB0212, PBPRB0427, PBPRB1008, PBPRB1232, PBPRB1761), almost twice as many as *E. coli* where some of these proteins have been shown to have unique but partly overlapping functions in the cell (21). A similar expansion of the DEAD-box family of helicases is also observed in the psychrophilic γ-proteobacterial genomes of *Colwellia psychrerythraea 34H* (64) and

Pseudoalteromonas haloplanktis TAC125 (63) suggesting that it might be important for temperature adaptation.

In *P. profundum,* microarray analysis shows differential expression of some of these DEAD-box helicases under certain conditions of temperature/pressure: the orthologue (PBPRB0427) most closely related to the *E. coli* DbpA (NCBI accession P0A9P6; (17)) is underexpressed at 28 MPa, 45 MPa and 4ºC (19, 89), PBPRB1232 is underexpressed at 45 Mpa, PBPRB1761 is underexpressed at 4ºC. Notably this pattern of expression with the DEAD-box helicases being repressed at lower temperatures is opposite to what has been observed in the Antarctic methanogen *Methanococcoides burtonii* (58).

Functional overlap might also exist at the level of the ribosome where pressure changes could be sensed in a similar way to temperature (88) via the modulation of the stringent response. This phenomenon results in a dramatic down-regulation of ribosomal components following a variety of stresses and has been first observed in *E. coli* cells subjected to amino acid starvation (83). A cell not undergoing the stringent response is said to be in a relaxed state. The effector molecule of the stringent response is guanosine tetraphosphate or ppGpp, that is generated by the gene products of *spoT* and *relA*.

In *E. coli* a temperature downshift induces a relaxed state through a decrease in the levels of ppGpp (46). Similarly, one might predict that a pressure-induced drop in ppGpp levels would result in the production of increased amount of ribosomal proteins L7/L12, S6, the elongation factor EF-G and cold-shock proteins (46). Most of these markers can in fact be detected by proteomic analysis of *E. coli* subjected to a sudden

pressure upshift (95) and microarray analysis of high-pressure shocked *Lactobacillus sanfranciscensis* (73).

In *P. profundum*, a mutant in the *spoT* orthologue PBPRA0189 is both cold- and pressure- sensitive. This phenotype might be caused by the disruption of the delicate interplay between the stringent-, cold- and pressure- responses. SpoT is responsible for both the synthesis and the degradation of ppGpp and *spoT* mutants have higher basal levels of ppGpp even under steady state conditions (84).

An alternative hypothesis for the cold and pressure sensitivity of a *P. profundum spoT* mutant originates from the observation made in *Vibrio cholerae*, that a mutant producing lower-than-normal levels of ppGpp transcriptionally represses *toxR* (38). ToxR functions as a piezosensor in SS9. It is therefore feasible, that upregulation of *toxR* might occur in a *spoT* mutant background and overexpression of *toxR* in *P. profundum* does indeed result in a pressure-sensitive phenotype (Bartlett, unpublished results). Moreover, because in *E. coli* RelA generates ppGpp in response to amino acid starvation while SpoT is responsible for sensing other stresses (61) it would be interesting to analyze the pressure- and cold- sensitivity of a *P. profundum relA* mutant and a *spoT relA* double mutant.

A confounding factor when considering the basis for the phenotype in these mutants is that most of the available data on the genetic effects of pressure comes from the study of mesophilic bacteria. However, stress regulons have evolved to respond differently in piezophilic bacteria. For example, in *E. coli* grown at high hydrostatic pressure, both a heat- and a cold-shock response is induced (45, 95). Conversely, *P.*

profundum SS9 mounts a heat shock response when grown at atmospheric pressure (89) but not when grown at supraoptimal pressure (19).

Ishii *et al.* (45) have observed that a delta-*hns* strain of *E. coli* is at least 1,000 fold more sensitive to high hydrostatic pressure than the isogenic wild-type. On the contrary, mutant strain FL11 in the *hns* othologue PBPRA1082 grows better at high hydrostatic pressure than at atmospheric pressure. Instead H-NS deficient mutants are cold sensitive in both *E. coli* (29) and *P. profundum* suggesting that this gene plays different roles in the adaptation to temperature and pressure and has evolved piezo-specific traits in *P. profundum*.

The basis for the cold sensitivity of FL11 might be caused by the role of H-NS in modulating the cold-shock response (18, 45), or, alternatively, Enos-Berlage *et al.* (32) have reported that insertional inactivation of H-NS results in modifications of the capsular polysaccharide and the reason for the cold sensitivity of the *hns* mutant in *P. profundum* could reside in a regulatory alteration of the EPS/LPS matrix. Interestingly FL11 displays a visibly thicker biofilm when grown in liquid media.

Complementation of selected mutants

A subset of mutant strains (FL2, FL12, FL15, FL21, FL29) were selected for complementation analysis. In most cases (strains FL2, FL12, FL29) the re-introduction of the wild-type copy of the gene resulted in a wild-type phenotype (Figure 4.5) confirming the role of the disrupted ORF in growth at high hydrostatic pressure or low temperature.

In one case (FL21 - PBPRB1757), the cold-sensitive phenotype was intermediate, suggesting the possibilities of a partially dominant negative mutant or that a downstream gene might also perform a role in low temperature growth.

In another case (FL15 - PBPRA3239) the re-introduction of the wild type copy did not complement the mutant phenotype. The insertion in PBPRA3239 could exert a polar effect on transcription of the downstream genes PBPRA3240 or PBPRA3241, coding for orthologues of DegQ and DegS, serine proteases, involved in recycling of non-functional proteins in the periplasmic space (91). Curiously, *degS* has been shown to have a function in the regulation of *rpoE* activity by proteolytically degrading the periplasmic domain of RseA (34). A *degS* mutant in *E. coli* is unable to respond to extra-cytoplasmic stress (2). In *P. profundum,* this effect might be more dramatic at low pressure when both the *rpoE* operon and the cytoplasmic counterpart of DegS, RseP (PBPRA2961) are transcriptionally repressed, but the cells are under stress as evidenced by the steady-state up-regulation of heat-shock genes (89).

Alternatively, it is possible that PBPRA3239 functionally interacts with PBPRA3240 in the periplasm. In fact PBPRA3239 is predicted to be localized in the periplasmic space (42) and the synteny conservation of the two ORFs in all the members of the family *Vibrionaceae* suggests they are part of the same regulon. In this case the effect would be due to *trans*-dominance of the mutant allele.

Conclusions

Previous to this work, only Abe and Iida (1) had described a comprehensive analysis of nonessential genes influencing the growth of any microorganism at high

hydrostatic pressure. In their case the mesophile *Saccharomyces cereviseae* was examined. This study expands the number of *P. profundum* CS mutants from 2 (6, 23) to 28 and PS mutants from 4 (3, 4, 15, 23) to 10.

During this high-throughput screening some transposon insertions were in genes previously implicated in low temperature or high pressure growth (3, 23). However not all the piezoadaptive genes obtained previously were recovered. This can be explained by the limited coverage of our transposon screen. Based upon Poisson statistics I infer that the minimal number of mutants to be screened in order to have a 95% probability of hitting every ORF in the genome is ~16,500. Taking into consideration the existence of hotspots for the mini-Tn10 transposition the screening of 20,000 mutants is not likely to have been saturating. Indeed, of the 31 PS/CS loci discovered, only 7 were hit more than once.

This study also highlights that while some functional overlap exists between the adaptive response to temperature and pressure, each condition affects microbial cells in a unique way. Most cold-sensitive mutants are most pressure-sensitive and most pressure-sensitive mutants are not cold-sensitive. Almost all the CS mutants uncovered could be clustered in six COG functional classes (T, Signal transduction mechanisms ; M, Cell envelope biogenesis, outer membrane; G, Carbohydrate transport and metabolism; U, Intracellular trafficking, secretion, and vesicular transport; L, DNA replication, recombination and repair; O, Posttranslational modification, protein turnover, chaperones). The fewer PS mutants were more diverse, although at least two of them could be associated with chromosome partitioning and two with signal transduction mechanism (COG functional class T).

This is consistent with the hypothesis that pressure-regulated gene expression plays a central role in growth under deep-sea conditions (23, 48, 94).

This is also the first study to provide direct genetic evidence for a crucial role of EPS genes in adaptation to low temperature (62). It has been hypothesized that a function of EPS at low temperature might be that of cryoprotectants under freezing conditions (62). This doesn't appear to be the case for *P. profundum* as the CS phenotype of the EPS mutants appears well above the freezing point of water. These results highlight the need for further studies to understand the relation between EPS structure/function and cold-sensitivity.

Additionally, because of the wide variety of sensory and regulatory mutants affecting growth at high pressure and low temperature, it will be interesting to use microarray technology to identify the genes under transcriptional control of each of these regulators. There is increasing evidence that piezophilic bacteria are under stress at atmospheric pressure. Keeping this in mind, it might also be interesting to embark upon a mutant hunt for low-pressure-sensitive genes.

Table 4.1: Strains and plasmids used in this study

Strain or plasmid	Description	Reference
P.profundum		
SS9R	Rif^r SS9 derivative	(24)
E.coli		
ED8654	Used for pRK2073 maintenance	(69)
DH5α	*recA* strain used for cloning	(37)
XL1-Blue	*recA* strain used for cloning	Stratagene, La Jolla, CA, USA
TOP10	*recA* strain used for cloning	Invitrogen, Carlsbad, CA, USA
Plasmids		
pRK2073	Carries tra genes for conjugal transfer	(14)
pRK2013	Carries tra genes for conjugal transfer	(14)
pLOF	Mini-Tn10 transposon Km^r	(40)
pRL27	Mini-Tn5 transposon Km^r	(54)
pFL122	Broad host-range vector, Sm^r	(56)
pFL400	PBPRB0212 in pFL122	This study
pFL401	PBPRA0747 in pFL122	This study
pFL402	PBPRA3239 in pFL122	This study
pFL403	PBPRB1757 in pFL122	This study
pFL404	PBPRA2596 in pFL122	This study

Table 4.2: Mutants obtained in this study. TN=Type of transposon insertions recovered in that ORF. PH=Phenotype: cold sensitive (CS), pressure-sensitive (PS) or pressure-enhanced (PE). The ORF numbers were those assigned by Vezzi *et al.* (89).

FL ID	Gene	Annotation	COG	TN	PH	Notes
1	PBPRB2014	Transcriptional regulator; LuxR family	T	a-Tn5, b-Tn10	CS	
2	PBPRB0212	ATP-dependent DEAD-Box RNA helicase	LKJ	Tn5	CS	Complementation analysis
3	PBPRA2678	Hypothetical protein	M	a-Tn5, b-Tn5	CS	Missing/divergent in *P. profundum* strains 3TCK and DSJ4
4	PBPRA3093	*rpoE* regulatory protein RseB	T	Tn5	CS PS	Previously observed (23) Suppressors; Overexpressed at 28 Mpa
5	PBPRA2681	Glycosil transferase	M	a-Tn5, b-Tn5	CS	
6	PBPRA1774	ATP-dependent protease; LA-related	O	Tn10	CS	Overexpressed at 28 Mpa
7	PBPRA2684	Hypoth. protein involved in polysaccharide biosynth.	R	Tn5	CS	Missing/divergent in *P. profundum* strains 3TCK and DSJ4
8	PBPRA0396	Hypothetical integral membrane protein	R	a-Tn5, b-Tn5	CS	Pfam00892 DUF6 domain
9	PBPRA2686	Tyrosine-protein kinase	M	Tn5	CS	Missing/divergent in *P. profundum* strains 3TCK and DSJ4
10	PBPRA0428	Pyruvate kinase I	G	a-Tn10, b-Tn10	CS PS	Suppressors; Underexpressed at 28 Mpa
11	PBPRA1082	DNA-binding protein H-NS	R	Tn5	CS PE	Enhanced biofilm mutant
12	PBPRA0747	Suppressor protein SuhB	G	Tn10	CS PS	Complementation analysis
13	PBPRA0189	Guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase *spot*	TK	Tn5	CS PS	
14	PBPRA2407	Periplasmic linker protein	M	Tn5	CS	
15	PBPRA3239	Hypothetical protein	S	Tn5	CS	Pfam06295; DUF1043 domain; Upstream of *degQ/degS* orthologues; Complementation analysis
16	PBPRA2282	Conserved hypothetical protein	G	Tn5	CS	Pfam04074 DUF386 domain
17	PBPRB2009	PTS cellobiose-specific component IIc	G	Tn10	CS	Possibly missing/divergent in *P. profundum* strain DSJ4
18	PBPRA0667	Conserved hypothetical protein	S	Tn10	CS	Missing/divergent in *P. profundum* strain 3TCK; pfam 05943; DUF877
19	PBPRB1941	Hypothetical protein	-	Tn10	CS	Signal peptide
20	Unknown	Transposase	L	Tn10	CS	Multiple copies of the sequence: 8 copies on chr2; 5 copies on chr1
21	PBPRB1757	Hypothetical protein	T	Tn10	CS	Signal peptide; Missing/divergent in strain DSJ4; Complement. analysis
22	PBPRB0828	Beta-hexosaminidase	G	Tn10	CS	
23	PBPRA3229	Phosphoheptose isomerase	G	Tn10	CS PS	Probable *diaA* (44)
24	PBPRA0917	Polar flagellar protein FlaJ	NUO	Tn10	CS	Phenotype only on plates
25	PBPRA2700	Hypothetical protein	M	a-Tn5, b-Tn5	CS	Pfam04464; Phenotype only on plates
26	PBPRA0218	O-antigen ligase	M	Tn5	CS	Underexpressed at 4°C; Phenotype only on plates
27	PBPRA0674	Conserved hypothetical protein	S	Tn5	CS	Missing/divergent in *P. profundum* strain 3TCK; pfam 05638.3 (*hcp*); Phenotype only on plates
28	PBPRA1039	SeqA protein	L	Tn10	PE	
29	PBPRA2596	L-asparaginase	EJ	a-Tn5, b-Tn5	PS	Complementation analysis; Possibly overepressed at 45 MPa
30	PBPRA2658	3-oxoacyl-[acyl-carrier-protein] synthase I	IQ	Tn5	PS	Previously observed (3)
31	PBPRB0001	Chromosome II replication protein RctB	-	Tn5	PS	(31)

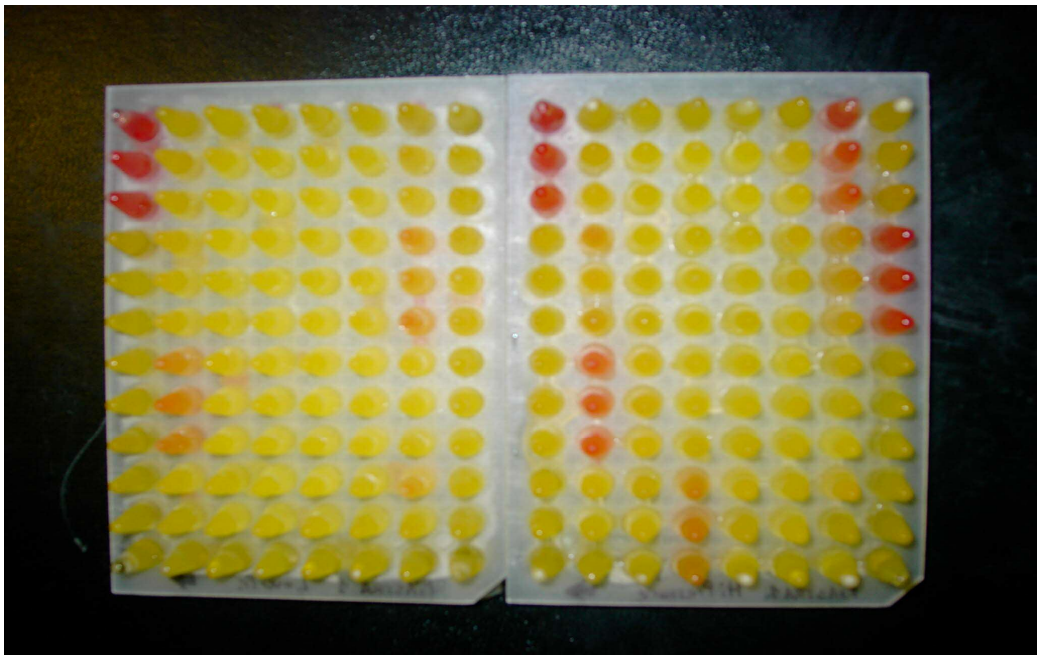

Figure 4.1: Microtiter plate growth assay used in the first screening of the transposon mutants. Growth was detected by the addition of phenol-red to the growth medium that changed color following the acid production by the bacteria growing fermentatively. Yellow=growth; red=no-growth.

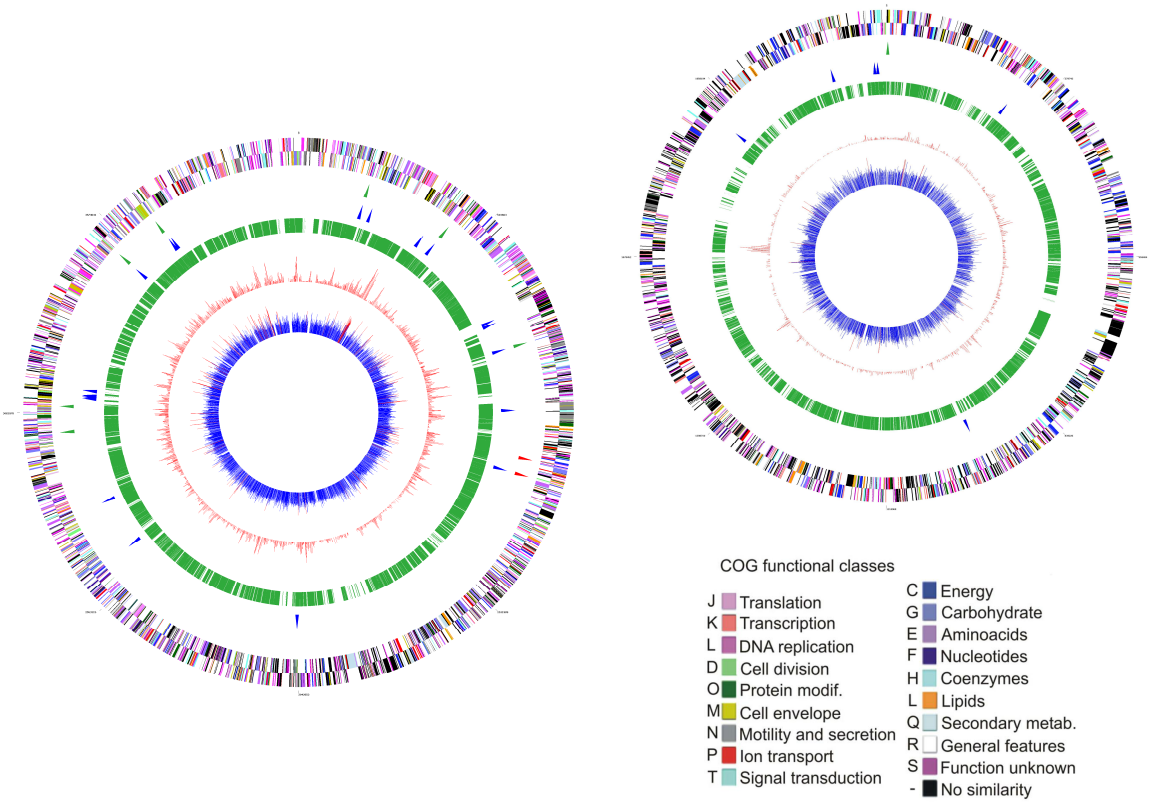

Figure 4.2: Genomic localization of the transposon insertions in the 2 chromosomes of *P. profundum* SS9 (not to scale). On the left chromosome 1 and on the right chromosome 2. From the outside inward: the first two circles represent the predicted protein-coding on the two strands, colored according to their COG functional class. The third the location of the pressure-sensitive (green) and pressure-enhanced (red) genes. The fourth the location of the cold-sensitive (blue) genes. The fifth the syntheny with the draft genome of *P. profundum* 3TCK (www.venterinstitute.org). The sixth the mean fluorescence intensity from the microarray experiments at 28 MPa and the sixth the codon adaptation index with scores>0.5 shown in red (89).

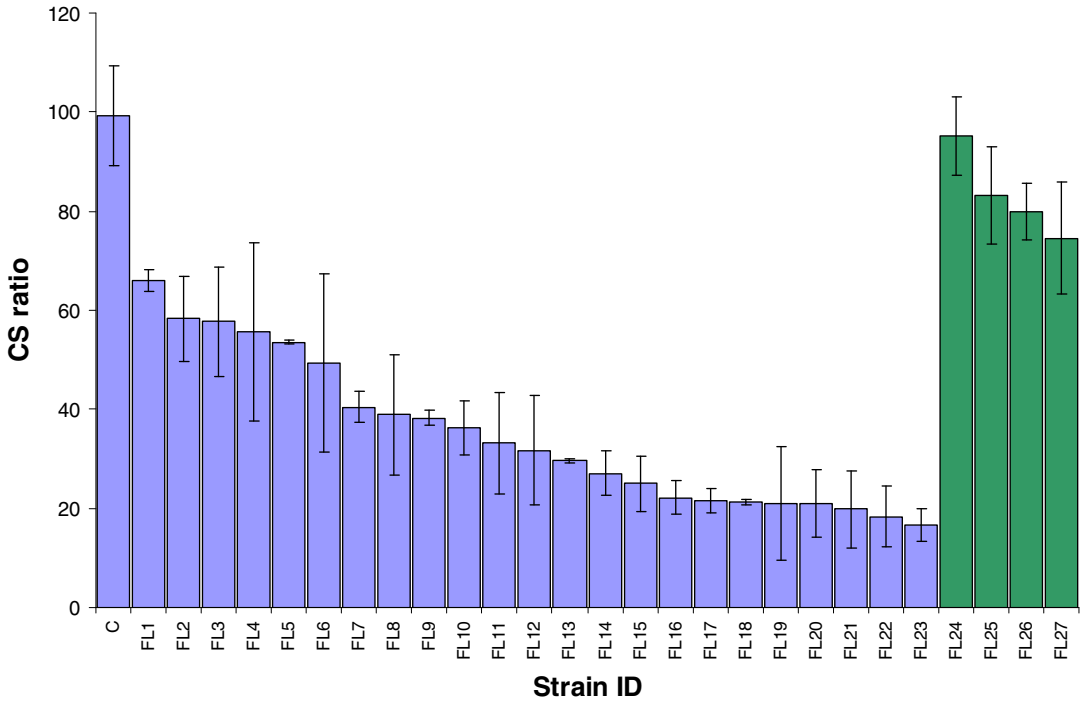

Figure 4.3: Cold sensitivity (CS) ratio for the mutants isolated in this study. The values were computed as described in materials and methods. The green bars are the ratios for the mutants that displayed a reproducible phenotype only on plates. C=control strain. Error bars represent one standard devation.

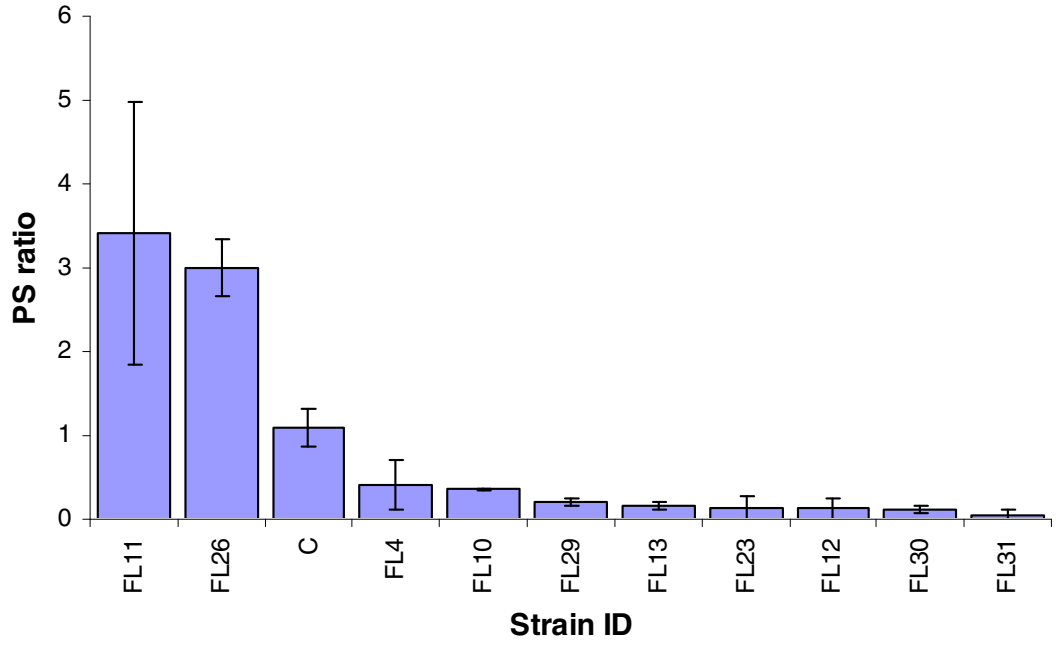

Figure 4.4: Pressure sensitivity (PS) ratio for the mutants isolated in this study. The values were computed as described in materials and methods. C=control strain. Error bars represent one standard deviation.

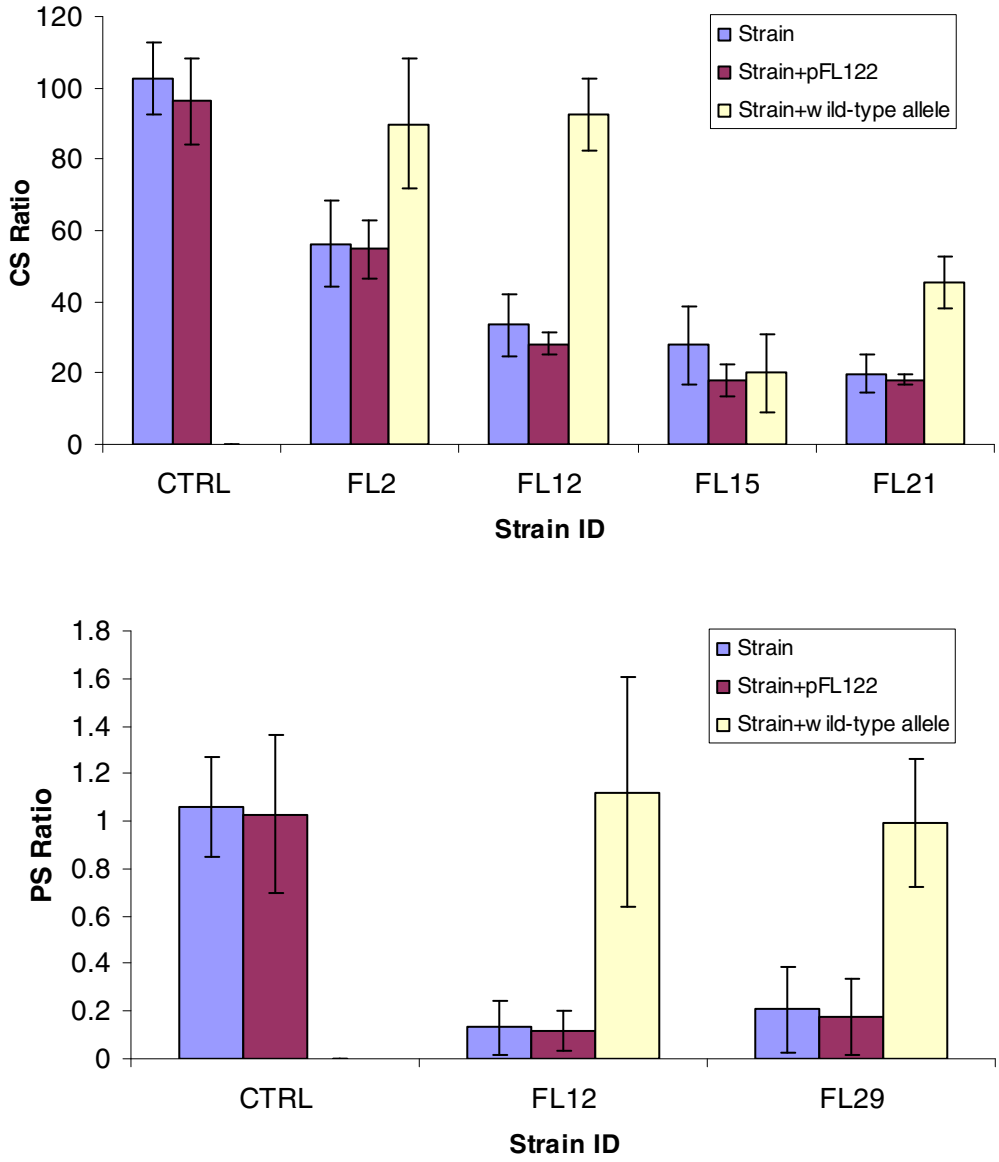

Figure 4.5: Cold- and pressure- sensitivity ratio of a selected subset of mutants after re-introduction of the wild-type copy of the allele on the plasmid vector pFL122. Top panel: complementation of cold-sensitive mutants. Bottom panel: complementation of pressure-sensitive mutants. The error bars represent one standard deviation.

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5.

Comparative genomics between closely related strains of *Photobacterium profundum* reveals adaptations to the stratified chemo-physical environments in the water column.

Abstract

Members of the bacterial species *Photobacterium profundum* have been isolated from different depths of the ocean environment and display remarkable differences in their growth responses to hydrostatic pressure. The genome sequence of the deep-sea piezopsychrophilic strain *Photobacterium profundum* SS9 (67) has provided some hints of the genetic features required for growth in the deep sea. Here I report the sequencing and analysis of the genome structure of *Photobacterium profundum* strain 3TCK, a non-piezophilic strain isolated from a shallow-water environment. I used a combination of comparative genomics and genetic approaches to identify peculiarities between the two genomes that relate to the chemo-physical differences of their environments. Variations in gene composition translate into differences in growth and swimming capabilities under conditions of high hydrostatic pressure. Furthermore specific ribosomal RNA signatures are unique to the deep-sea strain SS9 and the genes required for the photorepair of UV-induced damage to nucleic acids are only found in the shallow water strain 3TCK. Genetics studies have provided additional details regarding the role of particular flagellin and motility genes

to flagellum structure and function at high pressure, and gene exchange experiments between SS9 and 3TCK have indicated the importance of 16S rRNA ribotype to growth at elevated pressure and of DNA photolyase genes in survival in response to ultraviolet light exposure.

Introduction

Genome sequences are now available for organisms able to withstand extremes of temperature, pH, radiation and salinity (1, 26, 39, 42, 46, 54, 59, 60, 71). However, despite the fact that approximately 90% of the ocean's volume is at depths >1000m, deep-sea genomics is still only in its initial phase. The vast majority of this large portion of the biosphere is at a relatively constant low temperature, high hydrostatic pressure and is constrained by the small amounts of refractory organic nutrients that arrive in pulses from the overlying photic zone (73).

These conditions promote and maintain a diverse microbial community as detected by culture-independent approaches (58), but the small number of isolates in culture span only a narrow phylogenetic range, consisting mostly of γ-proteobacteria (2, 32).

Within the γ-proteobacteria, the genus *Photobacterium* is part of the family *Vibrionaceae.* Because of their wide distribution, the *Vibrionaceae* are thought to be contributing significantly to nutrient cycling and organic matter degradation (43, 64, 74) and many genome sequences for members of the family are now completed and available for comparison purposes (68). Members of this family have exploited many diverse ecological marine niches and are often found in symbiotic relationships

(either parasitic or pathogenic) with marine metazoans, although it is not uncommon to isolate them free living from ocean samples (50). The genus *Photobacterium* comprises a group of Gram-negative, facultatively anaerobic, plump and straight rod-shaped bacteria that require sodium ions for growth. Some members of the genus are bioluminescent, hence the name *Photobacterium*, and two species, *P. profundum* and *P. frigidiphilum* are known to incorporate eicosapentaenoic acid (EPA) in their membranes and to possess piezophilic growth (57).

P. profundum is a cosmopolitan member of the genus: to date at least four separate strains (SS9, DSJ4, 3TCK and 1230) have been isolated and characterized from multiple sites in the Pacific ocean (5, 10, 47). While strains SS9 and DSJ4 have been isolated from deep-sea environments and are adapted to high hydrostatic pressure, strains 1230 (unpublished results) and 3TCK (5) have been recovered from shallower waters and are inhibited by elevated hydrostatic pressure. These different ecotypes, which vary in their adaptation to depth in the water columns have been defined as bathytypes (31). The very existance of these closely related strains suggests that only subtle modifications are necessary and sufficient to evolve novel bathytypes (32).

In collaboration with the University of Padua (Italy) we have completed the genome sequencing and analysis of a deep bathytype of *Photobacterium profundum* strain SS9 (67). This information has been mined in a number of ways, including transcriptome analysis (5), proteome analysis (unpublished) and large-scale transposon mutagenesis (chapter 4) to better understand the genetic basis for deep-sea adaptations in bathyal bacteria.

Comparative genomics studies employing microarray hybridization indicate that 3TCK, the atmospheric pressure-adapted strain of *P. profundum*, shares approximately 90% of the SS9 genes (5). The lack of adaptation to increased pressure by 3TCK could stem from one or more categories of differences with the SS9 genome: (1) the lack of key genes, (2) the presence of key genes which inhibit growth at high pressure, (3) the lack of select or global gene sequence changes, or (4) the lack of appropriate gene regulation. Here I show that no single gene is likely to be responsible for determining the depth adaptation of the two bathytypes, and that a number of genetic features are specific to each strain. Some hypotheses developed as a result of comparing the two genome sequences have been genetically tested. These include depth-related differences in motility, rRNA structure and ultraviolet light damage repair.

Moreover I present evidence of the undergoing evolution of novel bathytypes of *P. profundum* through lateral gene transfer and suggest that the shallow bathytype strain 3TCK might have evolved from a deep-sea strain.

Materials and methods

Strains and growth conditions. The bacterial strains obtained in this study are listed in Table 5.1. The strains of *P. profundum* were cultured in 3/4 strength 2216 medium (28 g/l; Difco Laboratories) at 15°C and 0.1 MPa, unless otherwise specified. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium. High pressure growth experiments were performed by inoculating in heat-sealable plastic

bulbs containing media and no gas space. The heat-sealed bulbs were placed in pressure vessels and pressurized as previously described (77, 78).

When needed, antibiotics were used in the following final concentrations: rifampicin (Rif), 100 µg/ml; kanamycin (Km), 100 µg/ml (E. coli) or 200 µg/ml (P. profundum); streptomycin (Sm), 50 µg/ml (E. coli) or 150 µg/ml (P. profundum). X-Gal (5-bromo-4-chloro-3-indolyl- [beta] -D-galactopyranoside) was added to solid medium at 40 µg/ml in N,N-dimethylformamide. The introduction of plasmids in *P. profundum* was achieved by tri-parental conjugations using the helper *E. coli* strain pRK2073 as previously described (7).

Genome sequencing, closure and finishing. Genomic DNA was obtained from a culture of *P. profundum* strain 3TCK in mid-exponential growth. Approximately 1 liter of a liquid culture was harvested by centrifugation for 15 minutes at 5,000xg and the pellet was resuspended in 5 ml buffer A (50 mM Tris, 50 mM EDTA, pH 8.0). The suspension was incubated overnight at -20° C and thawed at room temperature with the addition of 500 µl of buffer B (250 mM Tris, pH 8.0, 10 mg/ml lysozime). After 45 min of incubation on ice, 1 ml of buffer C (0.5% SDS, 50 mM Tris, 400 mM EDTA, pH 7.5, 1 mg/ml Proteinase K) was added and the mixture was placed in a 50°C water bath for 60 minutes. Additional 750 µl of buffer C were added followed by additional 30 minutes of incubation at 50°C. The genomic DNA was extracted twice with 5 ml of phenol:chloroform:isoamyl alcohol (24:24:1), and precipitated with 0.8 volumes of isopropanol. The DNA pellet was recovered by spooling on a glass rod, and rehydrated overnight at 4°C in 4 ml of buffer D (50 mM Tris, 1 mM EDTA, 200 ug/ml RNAse A, pH 8.0). A further purification was

performed by extracting once with an equal volume of chloroform, then precipitating with 0.8 volumes of isopropanol. The DNA pellet was recovered by centrifugation, washed once with 70% ethanol and stored dry at -20°C.

The initial sequencing was done by a conventional whole-genome sequencing approach by preparing two genomic libraries with insert sizes of 4kb and 40kb as described in Goldberg et al. (15). This initial shotgun phase was performed by the J. Craig Venter Science Foundation Joint Technology Center on Applied Biosystems 3730XL DNA sequencers (Applied Biosystems, Foster City, CA) with funding from the GBMF Marine Microbial Genome Sequencing project. The initial assembly obtained using the Celera assembler (45) was imported in the Phred/Phrap/Consed (13, 14, 16) software package and the Autofinish (17) program was used to retrieve the fosmid clones necessary to re-sequence low coverage areas and gaps by primer walking. The gaps not covered by at least two independent fosmid clones were closed by a combination of combinatorial and long-range PCRs.

Genome analysis. The closed and finished sequence was submitted to the TIGR/JCVI Annotation Engine (www.tigr.org/AnnotationEngine) where it was analyzed with TIGRs annotation pipeline. Briefly, the pipeline performs an ORF finding with Glimmer (9) followed by searches of those ORFs with Blast-extend-repraze (BER), HMM, TMHMM, Signal IP and the assignments of gene names and functions with AutoAnnotate. All the information associated with each ORF was stored in a MySQL relational database and accessed for manual annotation through MANATEE (http://manatee.sourceforge.net/) installed locally at the Scripps Genome

Center. After each ORF was manually curated, the sequences and associated informations were retrieved and further analyzed with custom PERL scripts.

The assignment of each ORF to COG functional classes (63) was performed with AutoFACT (28). Codon usage was calculated as described by Karlin (25) using a custom PERL script.

Cloning and deletions. All restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). All the PCR amplifications were performed using the Expand Long Template PCR system (Roche Applied Science, Indianapolis, IN, USA).

The long-loop ribotype of *P. profundum* SS9 was excised with *Xho*I restriction of a fosmid clone (8BF3) of the large-insert sequencing library (67) of *P. profundum* SS9. This cut resulted in the appearance of multiple bands on an agarose gel. The 9.7 kbp band contained the ribosomal operon and was gel purified and ligated the *Xho*I site of pFL122 (33). The resulting plasmid was named pFL301.

The *lacZ*-overexpressing plasmid pFL185 was created as follows. The whole length *lacZ* gene was PCR amplified from *E. coli* MG1655 genomic DNA with the primers BGALRXba (5' – AGCTCTAGACAGGAAACAGCTATGACCATGATTA – 3'), which adds an *Xba*I restriction site, and BGALFBsa (5' – CCGCGTGGTCTCTAATTAAAGAATAAACCGAACATCCAAAAG – 3'), which adds *Eco*RI overhangs after restriction with *Bsa*I. The amplicon was cut with *Bsa*I+*Xba*I and ligated with a gel purified p519n*gfp* (36) cut with *Eco*RI+*Xba*I resulting in pFL185. This procedure effectively substitutes the *gfp* with the *lacZ* gene in p519n*gfp*, that is now under the control of the hyperactive promoter *pnptII*.

The generation of in-frame deletions of the motility genes was accomplished by marker exchange-eviction mutagenesis (51). Two to three kbp of the exact flanking regions of each ORF (PBPRA0041, PBPRA0048, PBPRA0808, PBPRA0912, PBPRA0913) from the nucleotide preceding the start codon to the nucleotide following the stop codon, were PCR amplified. The internal primers for each pair of amplicons contained a *Not*I site. Each pair of amplicons was digested with *Not*I, ligated and the product was re-amplified the Expand Long Template PCR system (Roche Applied Science, Indianapolis, IN, USA). This fragment, which contained the flanking regions to each of the ORFs and a *Not*I scar in place of each ORF was cloned in the suicide vector pRL271 (4). Each of the resulting plasmids was then cut with *Xho*I and ligated with a *Sal*I fragment from pMB2190 that contained the Km resistance gene generating pEAE1-pEAE5. These plasmids were mobilized in *P. profundum* strain SS9R by conjugation as described by Chi and Bartlett (7). Selection with kanamycin allowed the recovery of the clones where each construct was integrated within the gene of interest. Counterselection on plates containing no antibiotic and 10% sucrose, which is toxic to cells expressing the *sacB* gene, identified those clones that had undergone a second recombination event, approximately 50% of which contained the deletion. Each deletion in strains EAE1-EAE5 was confirmed by standard thermal cycle dideoxy sequencing with fluorescently labeled terminators (Applied Biosystems, Foster City, CA, USA).

The genes conferring UV resistance were cloned in pFL122 as follows. A fosmid clone (GCLNU_G4) from the *P. profundum* 3TCK sequencing library containing NT03PP_1444 (ORF01429; RNA polymerase sigma-70 factor, ECF

subfamily) NT03PP_1443 (ORF01428; Conserved Hypothetical Protein) NT03PP_1442 (ORF01427; deoxyribodipyrimidine photolyase) was cut with *Xho*I+*Kpn*I. The 7.2 kbp band contained the genes of interest and was gel purified and ligated in pFL122 (33) cut with *Xho*I+*Kpn*I yielding pFL303. The deletion Δ22 was done by cutting pFL303 with *Eco*RI and re-ligating, which effectively eliminates ORF01429, most of ORF01428 and the region with the two divergent promoters between the two. This deletion construct was named pFL304. The promoter region was PCR amplified from pFL303 using primers PROMPHO2F (5' – GTCGAATTCCTTTTCTTGCAGCGTCAGT - 3') and PROMPHO2R (5' – GTCGAATTCTAGTAAGCGAATAGCAGGAC -3').

Similarly the promoter region and the whole length ORF1429 was amplified with primers SIGMAPHO2F (5' – GTCGAATTCGTATTCAAGATGGGCACTCA – 3') and the same reverse primer as above PROMPHO2R. Both these amplicons were digested with *Eco*RI and cloned in the *Eco*RI site of pFL304 yielding pFL305 (promoter only) and pFL306 (promoter and ORF1429) respectively. The directionality of the inserts was checked by PCR and confirmed by standard thermal cycle dideoxy sequencing with fluorescently labeled terminators (Applied Biosystems, Foster City, CA, USA).

For the arabinose-inducible UV resistance experiments, the *phr* gene with its RBS was amplified with primers expPHO2F (5' – ATGGCCGTCTGCAAGATCCTGTA -3') and expPHO2R (5' – GCTCTAGAGCCACCCATTCATACGATGTGC – 3'), digested with *Eco*RI+*Xba*I and cloned in the expression vector pFL190 (33) cut with the same enzymes.

Motility. The swimming capabilities of the wild type *P. profundum* strains and the deletion mutants EAE1-EAE5 were assayed in the following way. First, tri-parental conjugations (7) were used to introduce in each strain pFL185 which expresses high levels of the *lacZ* gene under the constitutive promoter *pnptII*. The cells were inoculated with a needle at the center of heat sealable plastic bulbs that contained 100% strength 2216 Marine Broth, 0.04% (w/v) ferric ammonium citrate, 0.3% agar and 0.03% (w/v) of the chromogenic substrate S-Gal (Sigma Aldrich, St. Louis, MO, USA) which, when hydrolyzed by beta-galactosidase chelates with the added iron in the medium to form a black precipitate.

For high viscosity this medium was amended with 2.5% PVP-360. Swimming performance was quantitatively assessed from the distance traveled from the initial line of inoculation.

***In vivo* photoreactivation.** The effect of ultraviolet light on the survival *P. profundum* strains was tested as follows. Serial dilutions of late exponential cultures were plated on 75% strength 2216. For each strain a triplicate dilution series was prepared: the first one was left untreated, the second one was UV irradiated but not allowed to recover under blue light and the third one was UV irradiated, and allowed to recover under blue light.

The cells were UV stressed by irradiating them, uncovered, with a germicidal lamp (Philips G25 T8) for 10 seconds at a power of 220 $\mu W/cm^2$.

For the photoreactivation, the Petri dishes were then covered, to filter out the shorter wavelength radiation, and allowed to recover for 1 hour under black light (Philips TLD 15W/08) at an irradiance of 20 $\mu W/cm^2$. Irradiances were measured with

a Spectroline DM-365 XA digital radiometer (Spectronics corp., Westbury, NY, USA).

The plates were wrapped in foil and grown at 15°C for 5 days after which c.f.u. were counted and the number of colonies in the irradiated samples were compared with the unirradiated controls to calculate the percent survival.

All the experiments cell transfers and manipulations were performed under General Electric "gold" fluorescent light to prevent uncontrolled photorepair.

Results and discussion

General features of the genome of *Photobacterium profundum* strain 3TCK and comparisons with the genome of the previously sequenced strain SS9.

The genome of the shallow bathytype *Photobacterium profundum* 3TCK is divided in two chromosomes for a combined size of almost 6.2 Mbp (Table 5.2). This size and structure is comparable to that of the previously sequenced deep bathytytpe SS9 (67), but lacks an 80 kb dispensable plasmid present only in the deep bathytype. Chromosome 1 of strain 3TCK is approximately 5% smaller than chromosome 1 of strain SS9, whereas chromosome 2 is approximately 4% larger. Curiously *P. profundum* 3TCK has larger-than-average intergenic regions (~167bp), a feature shared with all the sequenced piezophiles (31), although the size of the intergenics is smaller than in the deep bathytype SS9 (~205bp).

ACT comparisons (6) between the nucleotide sequences of the two strains highlight the presence of a number of insertions/deletions, multiple inversions across the origin/terminus of both chromosomes, but only a limited number of translocations

across the chromosomes (Figure 5.1). The two genomes also differ in gene content. COG comparisons between the two strains revealed an over-representation in the shallow bathytype 3TCK of genes for transcription, energy production, carbohydrate and amino acid transport and metabolism (COG categories K, C, G, E) but a significant decrease in genes for motility and chemotaxis (N) and DNA replication, recombination and repair (L) (Figure 5.2).

The abundance of genes for category L (DNA replication, recombination and repair) in the deep bathytype SS9 can be explained by the large number of transposable elements that appear exclusively in the deep bathytype. This trend seems to be a distinctive feature of all deep-sea genomes thus sequenced (Lauro et al., unpublished) and has been observed in metagenomic surveys of different depths in the water column (11). Because of the wide-diversity of the identified transposases in the deep-sea samples that could not be accounted for by biases in community composition, DeLong et al. (11) have hypothesized that the over-representation of transposable elements relates to the slower growth and smaller effective population size of deep-sea microbial communities. Compatible with this hypothesis the 195 COG-cathegorized transposable elements found in SS9 belong to just 12 families, the most numerous of which has as many as 45 members. On the other hand, 3TCK encodes for just 3 COG-categorized transposable elements. Taken together these data support the hypothesis of intra-genomic amplification of transposable elements in the deep-sea due to habitat differences.

The over-representation in 3TCK of genes for carbohydrate (G) and amino acid (E) transport and metabolism is caused by the expansion of specific transporter

families such as the permeases of the major facilitator superfamily (COG0477; (35) and the permeases of the DMT superfamily (COG0697; (24) and the acquisition of genes for the degradation of urea and ethanolamine. These might be reflective of differences in the chemical environment of the sediments of San Diego Bay (where *P. profundum* 3TCK was isolated) versus those associated with deep-sea amphipods (from which *P. profundum* SS9 was isolated). The regulation of this large number of additional metabolic genes could also explain the expansion of the COG category for transcription (K) which is caused by a large number of transcriptional regulators unique to 3TCK.

Both strains contain a record number of ribosomal operons. These operons display intragenomic variation in *P. profundum* SS9, while they are almost identical in 3TCK.

Introduction of ribosomal operons from SS9 in 3TCK facilitates its growth at high hydrostatic pressure.

The fifteen ribosomal operons of the deep bathytype *P.profundum* SS9 show intra-genomic variation both at the level of the 16S rRNA gene and at the level of the 23S rRNA gene. Differences are concentrated in five loops, two on the 23S rRNA gene; helices 25 and 45, and three on the 16S rRNA gene; helices 10, 11 and 49 ((32) and the different operons contain various combinations of the short and long loops. The longer loops on the 16S of *P. profundum* SS9 have been hypothesized to be important for piezophilic growth (32).

Here I show that the introduction on a plasmid (pFL301) of a complete piezo-specific ribosomal RNA operon from the deep bathytype SS9 into the shallow bathytype 3TCK aids its growth at high pressure (Figure 5.3).

Members of the *Vibrionaceae* family are commonly regarded as r-strategists (12, 31) and the presence of multiple ribosomal operons has possibly evolved to allow for a quick response to sudden nutrient availability (27). This strategy could be particularly relevant in the deep sea, where nutrients arrive in pulses (73) and where being able to respond quickly to sudden carbon and energy influx could be fundamental to survival. Responses of this kind have been observed in deep-sea microbial communities (72).

Ribosomes are one of the cellular targets of high pressure inhibition in mesophilic bacteria (19) and translation (30) is among the most pressure-sensitive processes in a bacterial cell. This effect is thought to be caused by the dissociation of the ribosome under high pressure (19, 55) with the post-translocational complex being the most critical step (20). The effects of pressure on the structure/function of the mesophilic ribosomes have also been inferred indirectly by the overexpression of mRNA for the ribosomal proteins S2, L6 and L11 in high-pressure shocked *Lactobacillus sanfranciscensis* cells (48) and the overproduction of proteins L7/L12 and L6 in *E. coli* subjected to a sudden pressure upshift (70). The longer loops on helices 10 and 11 of the 16S could be involved in the interaction with ribosomal protein S20 (8) which might help stabilizing the ribosome at high pressure (18). Interestingly helix 10 was previously identified as a functional signature for discrimination between psychrotolerant and mesophilic species of *Bacillus* (49).

At this time it is not clear if the phenotype resulting from the introduction of pFL301 in the shallow bathytype 3TCK is due to the increased copy number of ribosomal operons or to the incorporation of the longer loops into the 3TCK ribosomes. Further improvement in 3TCK growth at high pressure might require replacement of all its rRNA operons with those from SS9, or perhaps replacement of particular ribosomal proteins or translation factors. Of course, many other cell functions could also limit further 3TCK high pressure growth ability.

Motility under high hydrostatic pressure.

One of the challenges that deep-sea bacteria face is maintaining motility under high hydrostatic pressure (40). Genes for motility and flagellar assembly in *P. profundum* SS9 are arranged in two large clusters (Table 5.3), one that is shared (SH) between the two bathytypes and a second one (PZ) that is unique to the piezophile SS9 as a result of a large contiguous deletion in the genome of 3TCK (Figure 5.4). This deletion accounts for the under-representation of genes from COG category N (motility and chemotaxis) presented in Figure 5.2. This second cluster is most similar to a lateral flagella gene cluster present in some *Vibrio* strains (37).

A comparison of the swimming abilities at high and low pressure has revealed that the deep bathytype *Photobacterium profundum* SS9 swims optimally at high hydrostatic pressure (30 MPa), but is partially inhibited in motility atmospheric pressure (0.1 MPa). In contrast, the shallow bathytype 3TCK swims optimally at atmospheric pressure but is inhibited at 30 MPa (Figure 5.5).

Initialy I hypothesized that the second motility gene cluster was responsible for the high-pressure swimming phenotype of SS9, however analysis of in-frame deletions of the flagellin and motor proteins in *P. profundum* SS9 suggests otherwise. Of the three flagellin structural genes, orthologs of the *E.coli fliC* gene, *flaA* (PBPRA0912) and *flaC* (PBPRA0913) belong to the SH cluster, while *flaB* (PBPRA0041) is part of the piezo (PZ) gene cluster. Only the mutants in *flaA* and *flaC* affect motility under all conditions tested while the *flaB* deletion results in loss of motility only at a combination of high pressure and high viscosity (Figure 5.6). Similarly, only the deletion in *motA2* (PBPRA0808) of the SH cluster abolishes motility at low viscosity while a *motA1* (PBPRA0048) mutant exhibits reduced motility only at high pressure and high viscosity (Figure 5.6).

The function of the two motors is predicted, by sequence similarity, to be driven by two different ion gradients: H^+ for MotA1 and Na^+ for MotA2. It is unclear, at this time, why the shallow bathytype would retain only Na^+-driven low-viscosity motility. Motility is an important adaptation for marine bacteria in particular to avoid grazing or in the continuous quest for food (21), and it is possible that increased selective pressure by these factors in the deep sea selects for more elaborate means of locomotion. In fact, McCarter (38) has suggested that the cost of carrying the additional genes of a dual-motility system is beneficial only in rapidly changing environments where optimal performance under different conditions is the key to survival.

The molecular basis of the swimming inhibition at different pressures can partially be explained by the positive volume changes associated with the

multimerization of proteins such as flagellin. The pressurization of flagellins *in-vitro* from the non-piezophilic *Salmonella enterica* serovar Typhimurium results in filament depolymerization (62). This, however, does not explain the partial loss of motility of *P. profundum* SS9 at atmospheric pressure which remains to be elucidated.

The conversion of the deep bathytype to UV resistance.

The absence of light (apart from chemiluminescence) in the deepest depths of the oceans argues against selection for genes associated with light tolerance. In fact the piezophilic strain CNPT3 has been shown to be extremely sensitive to UV radiation (34).

The two most common types of UV-induced lesions on DNA are the generation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone 6–4 photoproducts (6–4PPs) (76). This damage to DNA can be repaired by multiple pathways (76), but photoreactivation by deoxyribodipyrimidine photolyase, the product of the *phr* gene, is unique in that it requires blue light energy to split the CPDs or the 6-4PPs (66). Because of these features I predicted that the gene for the deoxyribodipyrimidine photolyase would be lost from exclusively deep-sea microbes. This hypothesis was bourne out in the genome sequence of SS9 (67). In addition, the recent analysis of the distribution of genes in a stratified water column (11) showed significant over-representation of *phr* genes from the euphotic region when compared to samples from deeper waters.

Unlike SS9, the shallow bathytype 3TCK contains a *phr* gene in a three gene cluster (ORF01427, ORF01428, ORF01429) with altered codon usage (Figure 5.7).

The *phr* gene (ORF01427) and the upstream hypothetical protein (ORF01428) are part of a predicted operon with a promoter upstream of ORF01428 driving their expression. A different promotor in the opposite direction is hypothesized to drive the transcription of the ECF sigma factor ORF01429.

Introduction of the *phr* gene cluster of 3TCK into SS9 (Figure 5.8; pFL303) resulted in approximately 1,000 fold increased survival after UV irradiation compared to the controls. This survival was dependant on blue-light incubation. A deletion encompassing the sigma factor (ORF01429) and most of the hypothetical protein (ORF01428) abolished photoreactivation (pFL304). If the promotor region is re-introduced in the right orientation into the deletion construct (pFL305), photoreactivation was partially restored, yielding approximately 100-fold more surviving c.f.u. than the untreated controls. The full restoration of the UV resistant phenotype could be obtained only by cloning, in the right orientation, both the promoter and the sigma factor (pFL306).

Based upon these results I hypothesized that the gene encoding the hypothetical protein is dispensable for photoreactivation activity and the UV resistance phenotype is solely dependant on the level of expression of the *phr* gene. To test both hypotheses the *phr* gene alone was cloned in a vector (pFL190) with an arabinose-inducible promotor (pFL307). The full UV resistant phenotype was observed only after induction with 0.1% arabinose (Figure 5.9) implying that high levels of expression of the *phr* gene alone are necessary and sufficient to confer UV resistance.

Members of the family *Vibrionaceae* have been shown to primarily rely on the activity of photolyases for the repair on UV-induced damage (56, 75). The genome of *Vibrio cholerae* N16961 (23) encodes for three different members of the cryptochrome/photolyase family (75). The first one (VCA0057) functions in repairing CPDs in dsDNA (75) the second one (VC1814) in repairing CPDs in ssDNA (56) while the function of the third one (VC1392) is still unknown. A similar array of photolyase-like ORFs can be seen in the genomes most other members of the *Vibrionaceae*, including the draft genome of *Photobacterium* sp. SKA34 (https://research.venterinstitute.org/moore/) encoding for orthologs to all three cryptochromes/photolyases of *V. cholerae*.

One notable exception can be observed in *Vibrio fischeri* ES114 (53). This genome contains only one photolyase (69) and the genes surrounding the *phr* gene (VFA0753) show an arrangement similar to the *phr* gene cluster in *P. profundum* 3TCK. VFA0753 is downstream and in the same transcriptional unit as VFA0752 (ortholog of ORF01428 from 3TCK). Upstream and transcribed in the opposite direction is a transcriptional regulator (VFA0751). However, this divergently transcribed regulator is a TyrR family regulator and not a sigma factor. These observations, associated with the altered codon usage of ORF1427 and ORF1429 led to the speculation that the *phr* gene cluster in *P. profundum* 3TCK might have been acquired by lateral gene transfer (LGT).

It has been suggested that CPD repair on ssDNA might aid in lateral gene transfer by repairing phage or free DNA that has been UV damaged. Recently, it has been shown that *V. cholerae* can be naturally transformed when grown on chitin (41).

Whether or not the diversity in number and arrangement of cryptochromes/photolyases within different members of the *Vibrionaceae* is reflective of different propensities for DNA uptake, at least in surface waters, is an open question.

Conclusions

Bacteria can be transported vertically through the water column, as a result of phenomena such as attachment to sinking particles (see, for example (61) or carried by upwelling currents. Growth and survival at different depths requires a number of adaptations to different chemo-physical parameters. Adaptations to high hydrostatic pressure require adjustments to membrane structure, DNA synthesis, translation and protein quaternary structure.

Some bathy-specific features can be acquired by LGT. The evidence presented here suggests that such is the case for the *phr* gene cluster in the shallow bathytype 3TCK. This would imply that 3TCK originated from deeper waters, an idea that is supported by its large intergenic regions and record numbers of ribosomal RNA operons (31). Another noteworthy feature of the *phr* gene cluster is that its expression in SS9 appears to benefit from the presence of a flanking sigma factor. There is a precedent for this type of observation. Sometimes acquired genes must be obtained as clusters of functional units to overcome, for example, the barrier caused by the incapacity to transcribe the LGT gene at the appropriate level (29). Genes providing marginal benefits like photolyase can also be readily lost from a population when the

increased metabolic cost for replication is not balanced by selective pressure as was observed between high- and low-ligth-adapted *Prochlorococcus* strains (52). All these processes generate and maintain the genomic diversity within bathytypes.

This is the first study employing intra-specific sequence comparisons in combination with genetics to address the molecular bases of phenotypic differences among bathytypes. Related studies could be performed using other genes or loci distinguishable between the strains described in this study, or involving other bathytype groups outside of the genus *Photobacterium*. Such in depth studies will be required for a more complete understanding of life at depth.

Table 5.1: Strains and Plasmids used in this study.

Strain/plasmid	Relevant genotype or description	Reference
P. profundum		
SS9	Wild type, deep bathytype of *P. profundum*	(10, 47)
3TCK	Wild type, shallow bathytype of *P. profundum*	(5)
SS9R	Rif^r SS9 derivative	(7)
3TCKR	Rif^r 3TCK derivative	This study
EAE1	SS9R derivative, Δ*flaA*, Rif^r	This study
EAE2	SS9R derivative, Δ*flaB*, Rif^r	This study
EAE3	SS9R derivative, Δ*flaC*, Rif^r	This study
EAE4	SS9R derivative, Δ*motA1*, Rif^r	This study
EAE5	SS9R derivative, Δ*motA2*, Rif^r	This study
E. coli strains		
ED8654	pRK2073 maintenance	(44)
DH5α	*recA*$^-$, used for cloning	(22)
XL1-Blue	*recA*$^-$, used for cloning	Stratagene
TOP10	*recA*$^-$, used for cloning	Invitrogen
Plasmids		
pRK2073	*tra* genes for conjugal transfer	(3)
pRL271	*sacB*-containing suicide plasmid, Em^r, Cm^r	(4)
pMB2190	pUC4K derived, Km^r cassette	P. Matsumura, U Illinois
pFL122	RSF1010 derived, broad host range cloning vector, Sm^r	(33)
pFL190	RSF1010 derived, expression vector, Sm^r	(33)
p519n*gfp*	RSF1010 derived, contains *pnptII* promoter before *gfp* gene, Km^r	(36)
pFL185	P519n*gfp*, with *gfp* replaced by *lacZ*	This study
pEAE1	PBPRA0912 (*flaA)* deletion construct in pRL271, Km^r	This study
pEAE2	PBPRA0041 (*flaB*) deletion construct in pRL271, Km^r	This study
pEAE3	PBPRA0913 (*flaC*) deletion construct in pRL271, Km^r	This study
pEAE4	PBPRA 0048 (*motA1*) deletion construct in pRL271, Km^r	This study
pEAE5	PBPRA0808 (*motA2*) deletion construct in pRL271, Km^r	This study
pFL301	SS9 long ribotype in pFL122, Sm^r	This study
pFL303	*phr* gene cluster in pFL122, Sm^r	This study
pFL304	Δ22 deletion of *phr* gene cluster in pFL122, Sm^r	This study
pFL305	pFL304 + *phr* promoter, Sm^r	This study
pFL306	pFL304 + *rpoD* + *phr* promoter, Sm^r	This study
pFL307	*phr* in pFL190, Sm^r	This study

Table 5.2: General Features of the *P. profundum* 3TCK genome.

* Assembly of March 2007, which still contained 6 gaps.

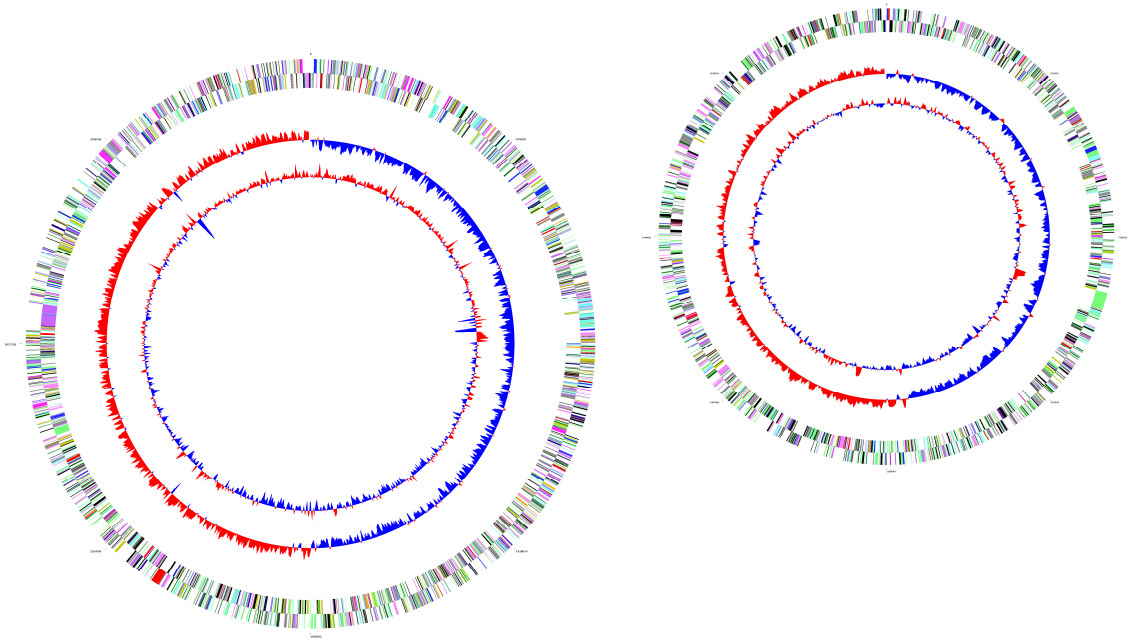
Δ Number obtained by PFGE analysis of *I-Ceu*I digested genomic DNA (65)

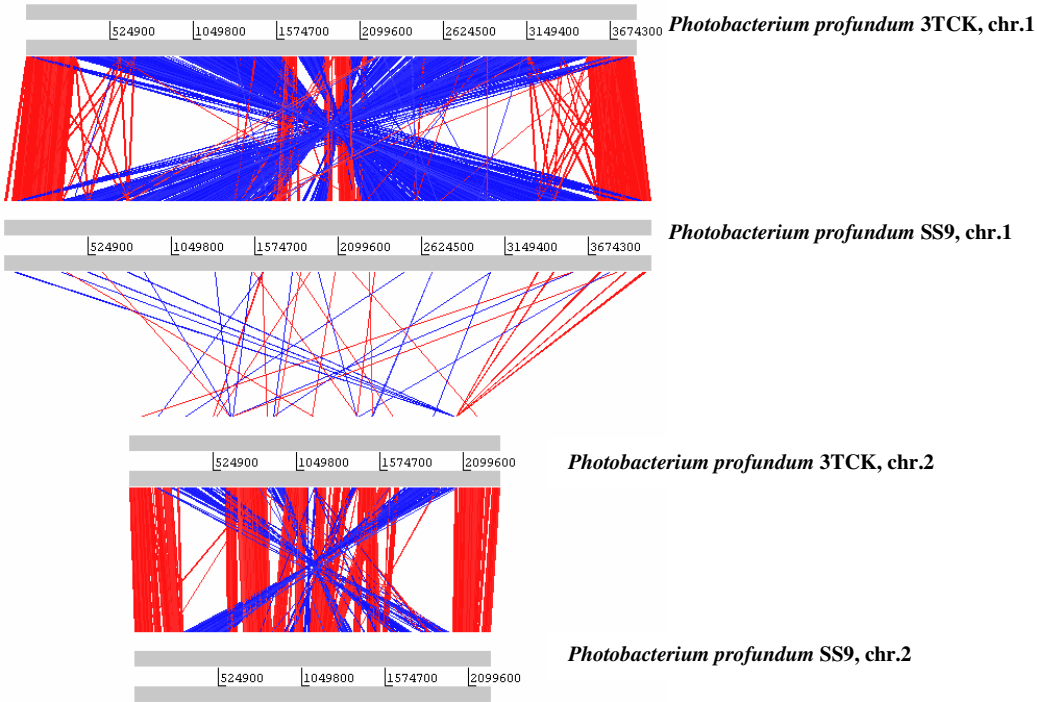
Feature	**Chromosome 1**	**Chromosome 2**
Size (bp)	3,836,305*	2,331,328
GC %	41.7	40.8
Percent coding	84.4	84.2
Number of ORFs	3474	2110
Average ORF size	931	930
Number of rRNA operons	14Δ	1
Number of tRNA	130	21

Table 5.3: Comparison of the ortgologous genes between the two flagellar gene clusters in *P. profundum* SS9. The PZ (piezo) gene cluster is absent from the shallow bathytype *P. profundum* 3TCK. Highlighted in yellow the ORFs upregulated at 0.1 MPa, in light blue those upregulated at 28 MPa (67).

***E. coli* gene name**	**PZ cluster ORF**	**SH cluster ORF**	**Predicted function**
fliD	PBPRA0042	PBPRA0915	Flagellar cap protein
fliC	**PBPRA0041**	**PBPRA0912 PBPRA0913**	**Flagellin**
flgL	PBPRA0039	PBPRA0911	HAP3
flgK	PBPRA0038	PBPRA0910	HAP1
flgE	PBPRA0033	PBPRA0904	Hook
fliK	PBPRA0045	PBPRA0928	Hook length control
flgD	PBPRA0032	PBPRA0903	Rod
flgF	PBPRA0034	PBPRA0905	Rod
flgG	PBPRA0035	PBPRA0906	Rod
flgH	gli36	PBPRA0907	L ring
flgI	PBPRA0036	PBPRA0908	P ring
fliE	PBPRA0021	PBPRA0922	Hook-basal-body component
flgB	PBPRA0030	PBPRA0901	Rod
flgC	PBPRA0031	PBPRA0902	Rod
motA	**PBPRA0048**	**PBPRA0808**	Motor component
motB	PBPRA0049	PBPRA0809	Motor component
fliF	PBPRA0022	PBPRA0923	M ring
flhA	PBPRA0012	PBPRA0936	Fla export and assembly
flhB	PBPRA0013	PBPRA0935	Fla export and assembly
fliG	PBPRA0023	PBPRA0924	Switch component
fliM	PBPRA0018	PBPRA0930	Switch component
fliNY	PBPRA0017	PBPRA0931	Switch component
fliH	PBPRA0024	PBPRA0925	Switch component
fliI	PBPRA0025	PBPRA0926	Fla export; ATP synthase
fliQ	PBPRA0015	PBPRA0933	Fla export and assembly
fliP	PBPRA0016	PBPRA0932	Fla export and assembly
fliR	PBPRA0014	PBPRA0934	Fla export and assembly
flgN	---	PBPRA0896	Potential chaperone
fliJ	PBPRA0026	PBPRA0927	Fla export and assembly
fliS	PBPRA0043	PBPRA0917	Fla export and assembly
flgA	PBPRA0029	PBPRA0898	Necessary for P ring addition
flgM	PBPRA0028	PBPRA0897	Anti sigma28 factor
fliA	PBPRA0047	PBPRA0939	RNA pol. Sigma28 factor

Figure 5.1: Chromosome organization in *P.profundum* strains. Above: genome structure of the two chromosomes of *P. profundum* 3TCK. From the outside inward: the first two circles represent the predicted protein-coding on the two strands, colored according to their TIGR role categories (legend below). The third represents GC skew and the fourth the percent G+C compared to the chromosome average. Below: ACT nucleotide comparison (6) between the chromosomes of *P. profundum* SS9 and those of *P. profundum* 3TCK.



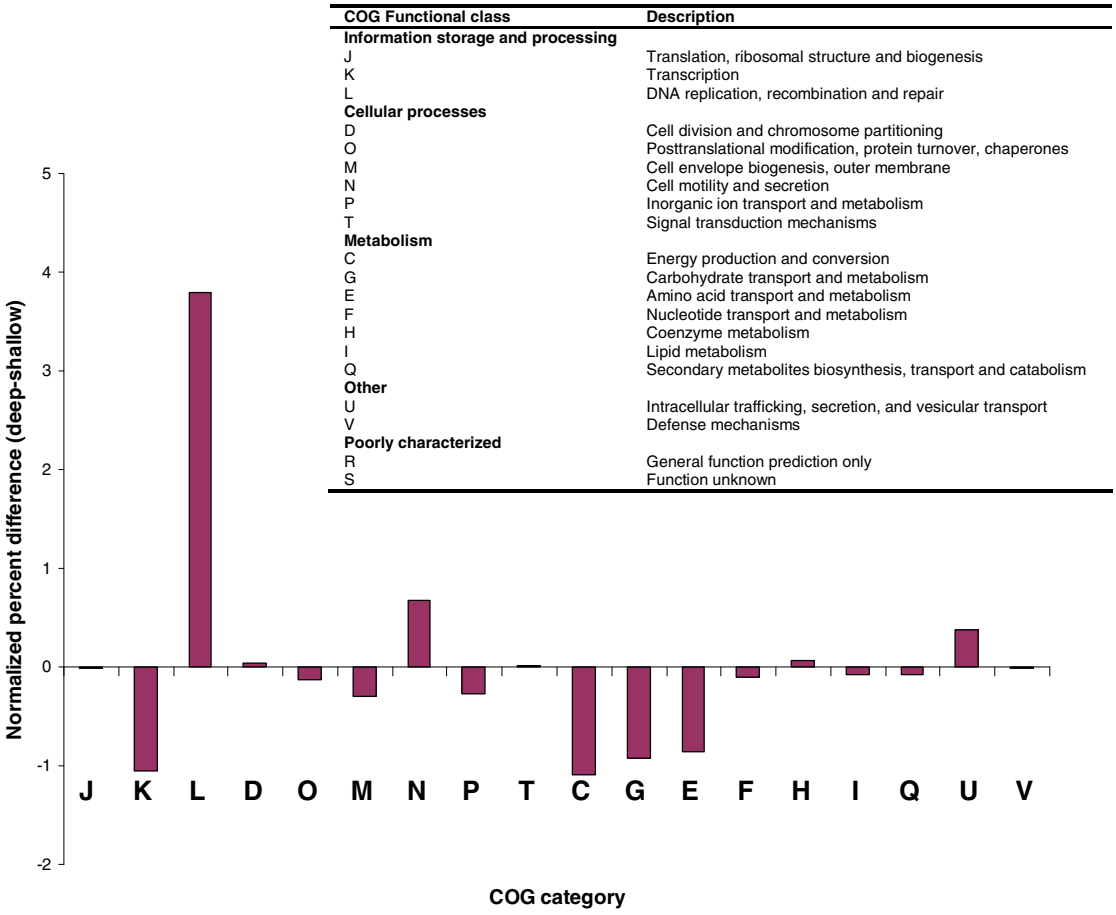


***Photobacterium profundum* 3TCK, chr.1**

***Photobacterium profundum* SS9, chr.1**

***Photobacterium profundum* 3TCK, chr.2**

***Photobacterium profundum* SS9, chr.2**



COG Functional class	Description
Information storage and processing	
J	Translation, ribosomal structure and biogenesis
K	Transcription
L	DNA replication, recombination and repair
Cellular processes	
D	Cell division and chromosome partitioning
O	Posttranslational modification, protein turnover, chaperones
M	Cell envelope biogenesis, outer membrane
N	Cell motility and secretion
P	Inorganic ion transport and metabolism
T	Signal transduction mechanisms
Metabolism	
C	Energy production and conversion
G	Carbohydrate transport and metabolism
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
H	Coenzyme metabolism
I	Lipid metabolism
Q	Secondary metabolites biosynthesis, transport and catabolism
Other	
U	Intracellular trafficking, secretion, and vesicular transport
V	Defense mechanisms
Poorly characterized	
R	General function prediction only
S	Function unknown

Figure 5.2: COGs (Cluster of Orthologous Groups) comparison between the deep bathytype SS9 and the shallow bathytype 3TCK. The shallow bathytype was enriched in genes for transcription (K), for energy production and conversion (C) and for metabolism and transport of carbohydrates (G) and aminoacids (E). The deep bathytype had higher percentages of genes involved in cell motility and secretion (N), intracellular trafficking and secretion (U) and DNA replication and repair (L).

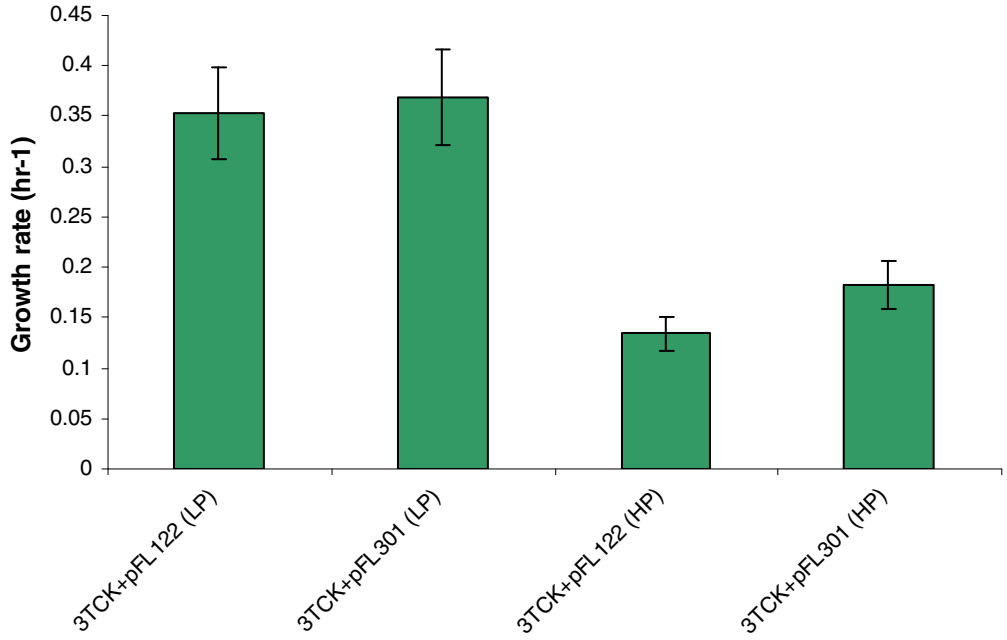


Figure 5.3: Growth rate comparison of *P. profundum* 3TCK grown at low (LP; 0.1 MPa) and high (HP; 30 MPa) pressure with a plasmid containing a complete ribosomal operon from the deep bathytype *P. profundum* SS9 (pFL301) compared to the vector alone (pFL122). Growth curves were performed in the presence of Sm selection at a 150 µg/ml.

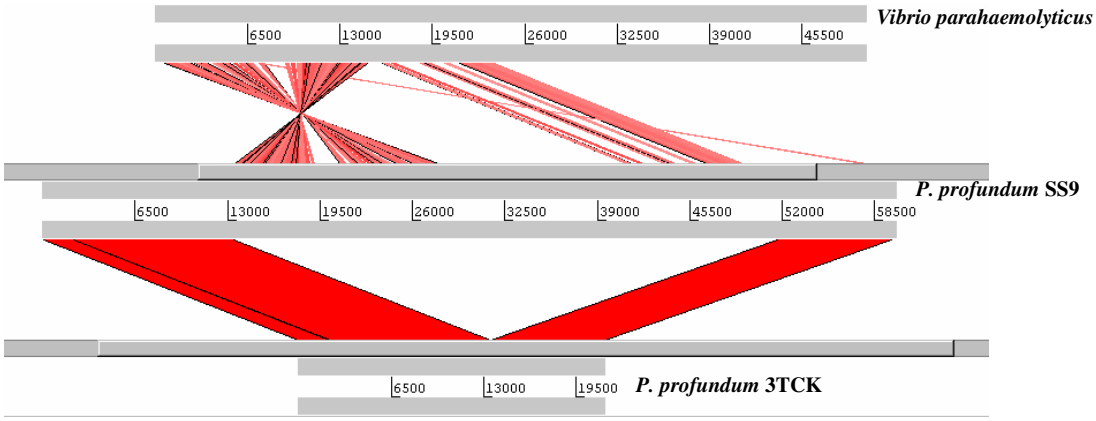
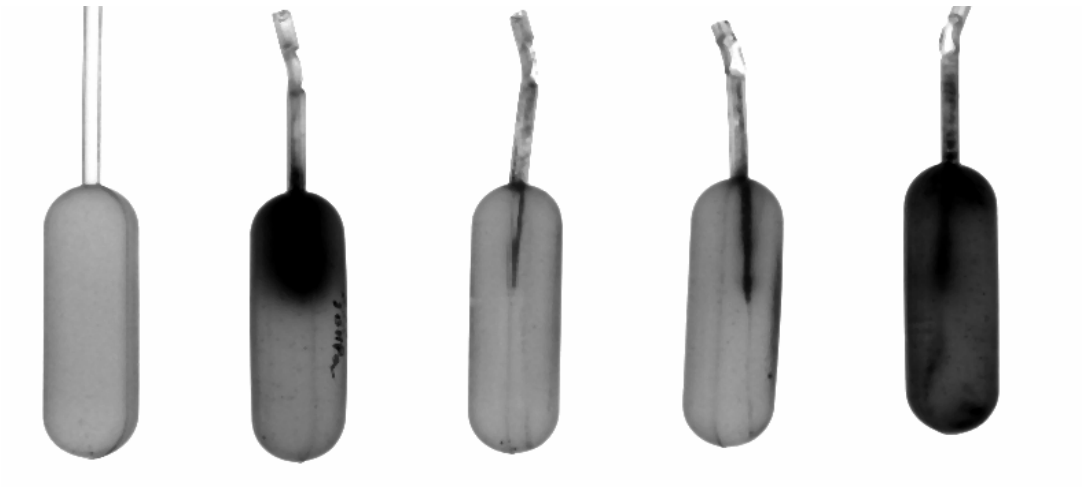


Figure 5.4: ACT comparison plot (6) between the lateral flagellar gene cluster in *Vibrio parahaemolyticus*, the second flagellar gene cluster of *P. profundum* SS9 and the corresponding region in *P. profundum* 3TCK.



a b c d e

Figure 5.5: Comparison of the motility of *P. profundum* SS9 and 3TCK in motility bulbs (0.3% Agar) prepared as described in materials and methods. From left to right: a) Blank, b) SS9 at 30 MPa, c) 3TCK at 30 MPa, d) SS9 at 0.1 MPa, e) 3TCK at 0.1 MPa.

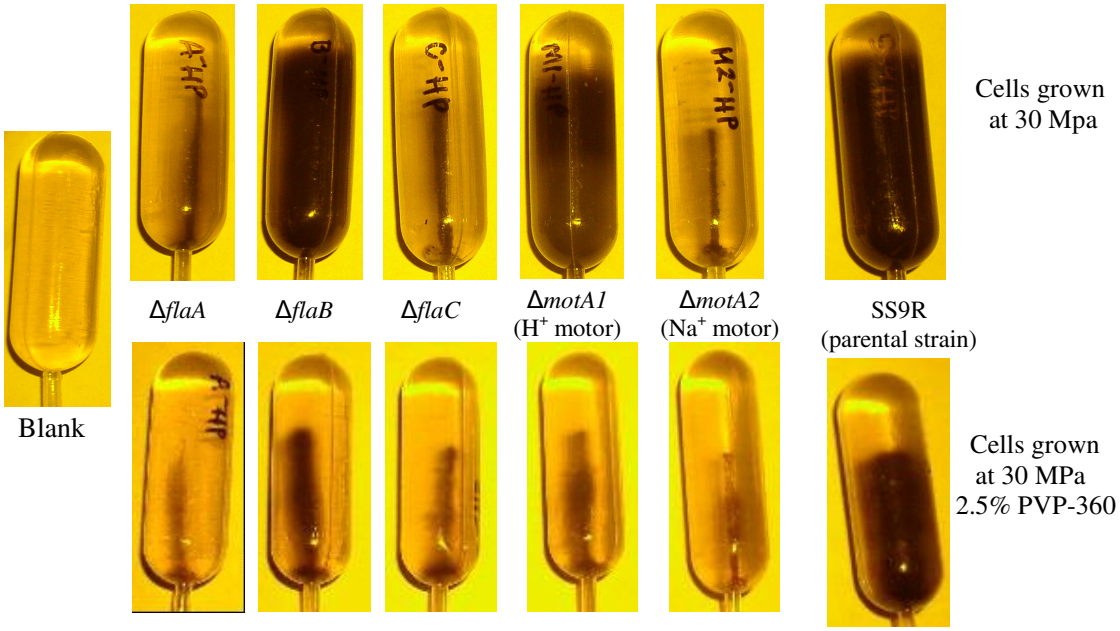


Figure 5.6: Comparison of the motility of mutant strains EAE1-EAE6 of *P. profundum* SS9 grown at 30 MPa at low (0.3% Agar) and high (0.3% Agar + 2.5% PVP-360) viscosity prepared as described in materials and methods. The flagellin A and C mutants as well as the sodium-motor (*motA2*) mutant are non-motile at high pressure compared to the parental strain. The flagellin B mutant and proton-motor (*motA1*) mutant however retain motility at low viscosity, but show only a weakly-motile phenotype when grown at increased viscosity.

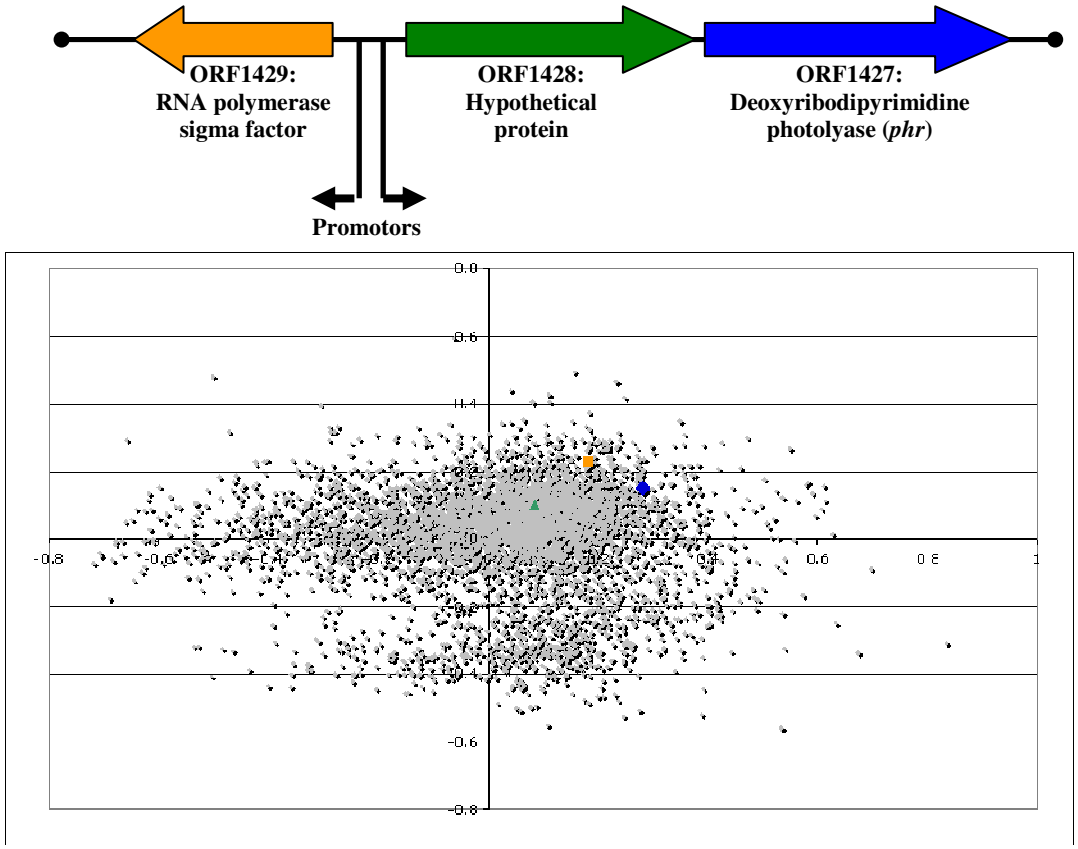


Figure 5.7: Correspondance analysis of the codon usage of the ORFs of *P. profundum* 3TCK. The ORFs belonging to the *phr* gene cluster (above) are color coded. Their position suggests recent acquisition by horizontal gene transfer.

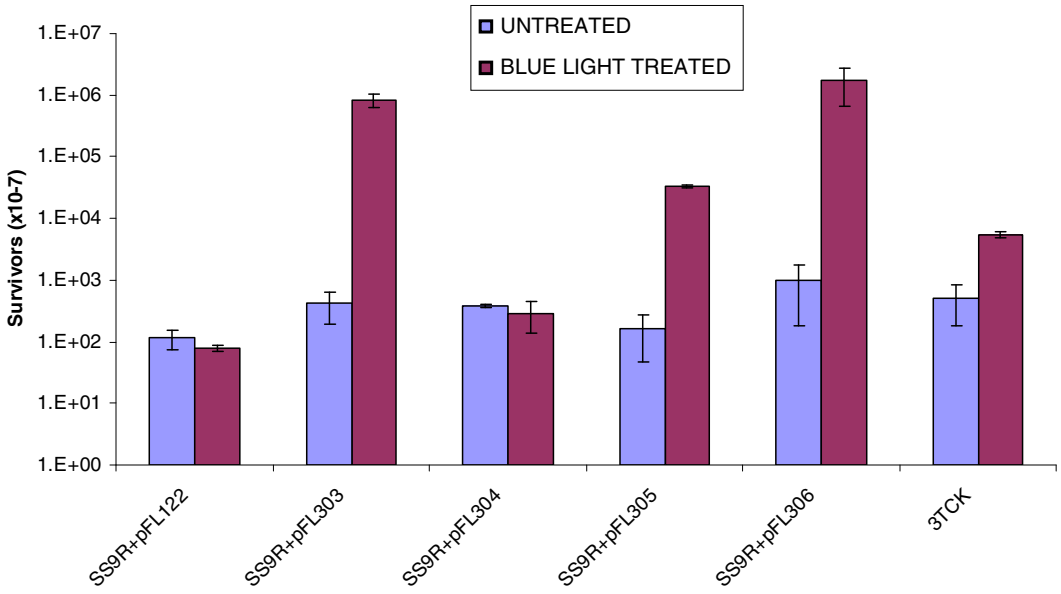


Figure 5.8: Introduction of the *phr* gene cluster from the shallow bathytype 3TCK into the deep bathytype SS9 confers UV resistance. This phenotype is not observed in the deletion construct that lacks the upstream hypothetical protein and sigma-70 factor (pFL304). The UV resistance phenotype can be partially restored by re-adding to pFL304 the promoter region of the cluster (pFL305) but is completely restored only when both the promoter and the sigma factor are added (pFL306). The absence of ORF1428 (Hypothetical Protein) does not affect UV sensitivity.

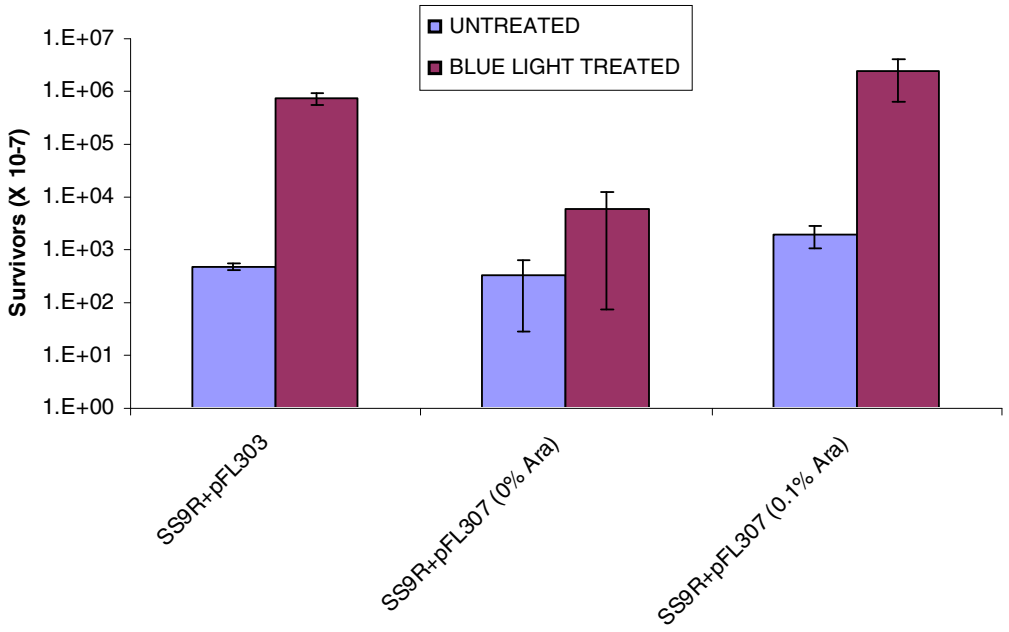


Figure 5.9: The UV resistant phenotype depends uniquely on the levels of expression of the *phr* gene (ORF1427). Cloning of the *phr* gene under the arabinose-inducible promoter of pFL190 confers UV-resistance to the cells only when grown with 0.1% L-arabinose.

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