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Microbial Adaptations to the Psychrosphere/Piezosphere

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Abstract

Low temperature and high pressure deep-sea environments occupy the largest fraction of the biosphere. Nevertheless, the molecular adaptations that enable life to exist under these conditions remain poorly understood. This article will provide an overview of the current picture on high pressure adaptation in cold oceanic environments, with an emphasis on genetic experiments performed on *Photobacterium profundum*. Thus far genes which have been found or implicated as important for pressure-sensing or pressure-adaptation include genes required for fatty acid unsaturation, the membrane protein genes *toxR* and *rseC* and the DNA recombination gene *recD*. Many deep-sea bacteria possess genes for the production of omega-3 polyunsaturated fatty acids. These could be of biotechnological significance since these fatty acids reduce the risk of cardiovascular disease and certain cancers and are useful as dietary supplements.

Introduction

Low temperature and high pressure environments (the psychrosphere and the piezosphere, respectfully) occupy the largest fractions of the known biosphere in terms of volume. Within these environments psychrophiles exist which can grow at temperatures as low as -12 °C (Russell, 1990), and piezophiles (also termed barophiles) exist which can grow at pressures as high as 130 megapascal [Yayanos, 1998; 1 megapascal (MPa) = 10 bar ≅ 9.9 atmospheres]. The lower temperature limit is apparently set by the freezing temperature of intracellular water, whereas the upper pressure limit for life is not known. Although Archaea, particularly those belonging to the nonthermophilic branch within the Crenarchaeota kingdom, are highly abundant in low temperature and high pressure environments (Fuhrman *et al*., 1992; DeLong *et al*., 1994), all of the cultured psychrotolerant/psychrophilic piezophilic prokaryotes fall within well known lineages of the Bacterial domain. Results to date indicate that the adaptations that enable these deep-sea microorganisms to grow under low temperature and high pressure conditions entail subtle variations of certain regulatory processes and aspects of cellular architecture present in mesophiles. Here I will briefly review genetic and biochemical studies of pressure adaptation by psychrotolerant/psychrophilic piezophilic prokaryotes with an emphasis on results obtained from my lab with *Photobacterium profundum*. Other reviews covering high pressure adaptation by low temperature-adapted deep-sea organisms include those written by Bartlett (1992), DeLong (1992), Prieur (1992), Somero (1990; 1991; 1992), Bartlett *et al*., (1995), Yayanos (1995; 1998), Kato and Bartlett (1997), and within this miniseries symposium the article by Kato and Qureshi.

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Basis of Pressure Effects

The thermodynamic consequences of changes in pressure are straightforward. Elevated pressure promotes decreased system volume changes associated with the equilibria and rates of biochemical processes. Thus, life in the deep sea must entail life at low volume change, or other biochemical adjustments which compensate for the consequences of elevated pressure. The relationship of pressure to equilibrium and rate processes can be expressed mathematically as follows:

$$1) \quad K_p = K_1 \exp(-P\Delta V/RT)$$

$$\text{and } 2) \quad k_p = k_1 \exp(-P\Delta V^{\ddagger}/RT)$$

These two equations express the relationship of either the equilibrium constant (K) or rate constant (k) of a reaction to pressure, as determined by the size of the volume change that takes place during either the establishment of equilibrium (ΔV) or the formation of the activated complex ($\Delta V^{\ddagger}$). K_1 and k_1 are the equilibrium and rate constants, respectively, at atmospheric pressure; K_p and k_p are the constants at a higher pressure, R is the gas constant and T is absolute temperature. Because the equilibrium and rate constants are related to pressure in a logarithmic fashion, seemingly small changes in volume can lead to large changes in K or k. For example, a volume change of +50 cm^3mol^{-1} will lead to a 89% inhibition of a rate (or 89% change in K value) at 100 MPa pressure. A volume change of +100 cm^3mol^{-1} will lead to a 35% decrease in k or K at 10 MPa, and >99% decrease at 100 MPa. Volume changes accompanying many biological processes (both equilibria and reaction rates) are in the range of approximately 20-100 cm^3mol^{-1}. Therefore, pressure changes can lead to very substantial alteration (decrease or increase) of biological activities and structures in the absence of adaptation.

Because water itself is not highly compressible (about 4% per 100 MPa pressure), the effects of increased pressure on biochemical processes are not due to effects stemming from compression of the aqueous medium. However, water structure is very important in pressure sensitivity because changes in hydration of macromolecules and metabolites can lead to substantial alterations in system volume.

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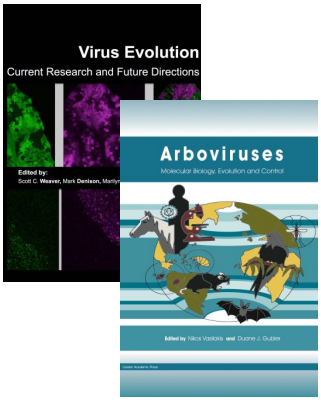

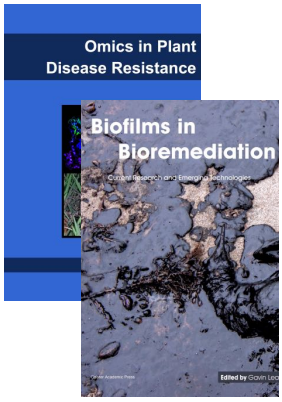

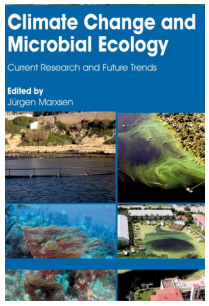

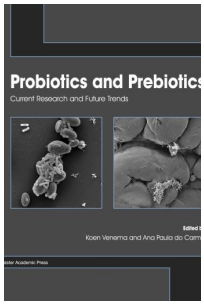

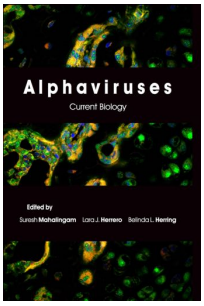

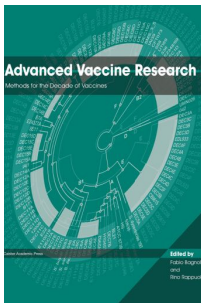

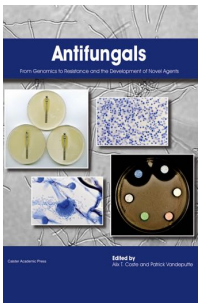

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Changes in hydrostatic pressure perturb the volume of a system without changing its internal energy (as occurs with temperature), or its solvent composition (as occurs with pH and osmolarity changes). Thus, pressure is both a unique and a fundamental thermodynamic parameter.

Identification of Pressure Sensitive Processes in Mesophilic Microorganisms

Although the basis of pressure effects on many simple chemical processes have been described, pinpointing the physical nature of pressure effects on biological systems is extremely complicated. Changes in pressure, as with changes in other physical parameters, can affect a variety of cellular processes. Experimentally, cell division, flagellar function, and more critically, DNA replication, protein synthesis, enzyme function, and membrane structure have all been found to be sensitive to elevated pressure in shallow-water bacteria (reviewed in Bartlett, 1992). Many of these studies have been undertaken using the moderately piezotolerant *Escherichia coli*, where it has been found that pressures higher than 10 MPa inhibit motility, 20 -50 MPa; cell division, 50 MPa; DNA replication, 58 MPa; protein synthesis, and 77 MPa; RNA synthesis.

When *Escherichia coli* was subjected to 55 MPa the number of polypeptides synthesized was greatly reduced, but many proteins exhibited elevated rates of synthesis relative to total protein synthesis (Welch *et al*., 1993). As with other stress responses, this pressure-inducible protein (PIP) response was transient. The protein exhibiting the greatest pressure inducibility was a small basic protein not induced by any other known stress conditions. Among the PIPs identified in *E. coli* many present an interesting paradox. High pressure induces more heat shock proteins (eleven) than most other conditions outside of those which precisely mimic a heat shock response, while also inducing more cold shock proteins (four) than most conditions outside of those which precisely mimic a cold shock response. The high pressure induction of heat shock proteins has been observed in many prokaryotic and eukaryotic microorganisms as well as human cells in tissue culture (reviewed by Bartlett *et al*., 1995).

Heat shock and cold shock proteins have inverse responses to a variety of conditions (VanBogelen and Neidhardt, 1990) so why should high pressure turn on both heat shock and cold shock proteins? Both cold temperature and high pressure inhibit an early step of protein synthesis. In addition, these conditions also both decrease membrane fluidity, increase nucleic acid secondary structure and either extremes of temperature as well as high pressure can reduce the stability of multimeric proteins (reviewed in Bartlett *et al*., 1995) . The high pressure induction of heat shock and cold shock proteins helps to underscore the unique and pleiotropic consequences of shifts in pressure on cell functions. The relationship between high pressure and high temperature stress is further extended by the observation that high temperature preincubation of the yeast *Saccharomyces cerevisiae* confers significant protection against lethal levels of hydrostatic pressure (Komatsu *et al*., 1991). How Hsps and other heat-inducible factors protect cells from high pressure stress is as yet unknown but could involve protecting membrane or protein stability.

Fatty Acid Regulation

Among the evolutionarily adaptive and transiently acclimatory changes thought to be important for growth at either low temperature or high pressure, membrane fatty acid alterations have received the most attention (Russell and Hamamoto, 1998; Yayanos, 1998). Both decreased temperature and increased pressure cause fatty acyl chains to pack together more tightly and influence gel state (L_β) to liquid crystalline (L_α) state transitions of membranes (reviewed by Bartlett and Bidle, 1999; Suutari and Laakso, 1994). For many phospholipids and natural membranes the transition slope in a temperature, pressure-phase diagram for the L_β - L_α main transition is approximately 20 °C/100 MPa at pressures lower than 100 MPa. Based on this relationship the combined effect of pressure and temperature on the phase state of a membrane from a deep-sea bacterium existing at 100 MPa and 2 °C is equivalent to an identical membrane at atmospheric pressure and -18 °C (lower than the low temperature limit for life). Decreased temperature or increased pressure have been found to result in increased unsaturation, chain length and the ratio of *anteiso* to *iso* branching, as well as decreased chain lengths. These changes may represent manifestations of "homeoviscous adaptation", the theory espoused by Sinensky (1974) whereby organisms adjust their membrane composition in response to changes in temperature in order to maintain a nearly constant membrane microviscosity. It has also been suggested that "homeophasic adaptation", maintaining a certain percentage of membrane phospholipids within a liquid crystalline state, is more physiologically relevant than the actual level of membrane fluidity (McElhaney, 1982).

Although once thought to be virtually nonexistent in bacteria, omega-3 polyunsaturated fatty acids (PUFAs) are also frequently present in the lipids of microbes from low temperature and high pressure environments. There is an increased proportion of docosahexaenoic acid (DHA; 22:6) and/or eicosapentaenoic acid (EPA; 20:5) found in microbes isolated from deep-sea versus shallow water animals (Yano *et al*., 1997). Higher percentages of EPA containing bacteria have also been noted in Antarctic versus temperate bacteria (Nichols *et al*., 1993).

***Photobacterium profundum* as a Model System for Studying Pressure Adaptation**

The only deep-sea isolate to yet be employed for genetic studies of pressure adaptation is *Photobacterium profundum* strain SS9. Members of this species have been isolated from amphipods in the Sulu Sea recovered from 2551 m and from sediment obtained from 5110 m in the Ryukyu Trench (DeLong, 1986; Nogi *et al*., 1998). SS9 has a relatively small genome size of 2 Mbp (Smorawinska and Kato; unpublished results), a temperature optimum of 15 °C and a pressure optimum of 20 - 30 MPa (DeLong, 1986). Why should this moderately piezophilic psychrotolerant species represent a useful model system for studies of life at high pressure and low temperature? It has a relatively rapid doubling time of 2 hours under optimal growth conditions and grows well over a wide temperature range (the 4 °C rate is nearly identical to the 15 °C rate) and pressure span (growth at 1 and 30 MPa are nearly identical and the upper pressure limit is 90 MPa).

Furthermore, it is possible to introduce a variety of broad host range plasmids into SS9 by conjugal transfer, and to perform transposon mutagenesis (Tn5 and mini-Mu), interposon mutagenesis and allelic exchange (Chi and Bartlett, 1993; 1995; Bartlett and Chi, 1994; Welch and Bartlett, 1996; 1998). Finally, SS9 responds to pressure shifts by modulating the abundance of a number of fatty acids and proteins as described below. As can be seen in Table 1, many of the genes obtained thus far from piezophilic bacteria have been isolated from strain SS9.

Fatty Acid Regulation in *P. profundum*

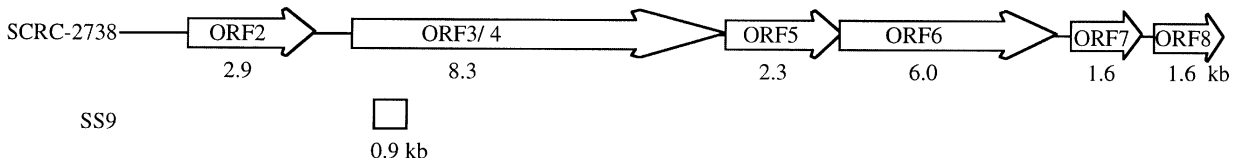
As with many other deep-sea bacteria, strain SS9 enhances the proportion of both monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) when grown at decreased temperature or elevated pressure. In order to assess the importance of unsaturated fatty acids (UFAs) to microbial growth at low temperature and high pressure, the effect of treatments and mutations altering UFA levels in strain SS9 was investigated (Allen *et al*., 1999). Treatment of cells with the ß-ketoacyl ACP synthase inhibitor cerulenin inhibited MUFA but not saturated fatty acid or PUFA synthesis, and led to decreased growth rate and yield at low temperature and high pressure in the absence of exogenous MUFA. The inspection of UFA auxotrophic mutants also indicated a role for MUFAs (particularly cis-vaccenic acid, 18:1) in growth of SS9 at low temperature and elevated pressure. One of these mutants, strain EA3, was deficient in the production of MUFAs and was sensitive to both low temperature and high pressure in the absence of exogenous 18:1 fatty acid. Another mutant, strain EA2, produced little MUFAs but elevated levels of the PUFA species eicosapentaenoic acid (EPA; 20:5n-3). This mutant grew slowly, but was not low temperature-sensitive or high pressure-sensitive. These results have provided the first evidence that UFAs are required for low temperature and high pressure adaptation, and have also suggested that overproduction of PUFAs might at least partially compensate for reduced MUFA levels. The fact that none of the MUFA mutants displayed reduced EPA levels further indicated that the production of these two types of UFAs are controlled by separate biosynthetic pathways.

The progress which has been made in recent years towards understanding the genetic basis of EPA synthesis has been used for directly testing the role of EPA in low temperature and high pressure growth of strain SS9. Much of the driving force for this research has been that omega-3 fatty acids such as EPA and DHA are considered important chemicals for biotechnology because of their activities in reducing the risk of cardiovascular disease and certain cancers, their use as dietary supplements in mariculture and poultry farming, and their application as substrates for the synthesis of certain hormone-like molecules (Simopoulos, 1991). The molecular biology of PUFA production began when a 38 kbp genomic DNA fragment was isolated from a *Shewanella putrefaciens* species (neither psychrophilic or piezophilic) which conferred EPA production ability to both *E. coli* and *Synechococcus* (Yazawa, 1996; Takeyama *et al*., 1997). The nucleotide sequence of this DNA was determined and based upon deletion analysis and deduced amino acid homology to other known enzymes involved in fatty acid synthesis, heterocyst glycolipid synthesis and polyketide antibiotic synthesis, six open reading frames were identified as important to EPA production (Yazawa, 1996). This genetic structure is very similar among diverse EPA and DHA producing bacteria (D. Facciotti, Calgene Inc.; personal communication). Because PUFA producing bacteria are present in many separate lineages, it is possible that the genes for PUFA biosynthesis have been acquired in many instances via horizontal gene transfer. Figure 1 shows a schematic of the *S. putrefaciens* SCRC-2738 genes involved in EPA biosynthesis and the relationship of their gene products to other bacterial proteins.

Based on the sequence of the SCRC-2738 EPA genes it was possible to employ the polymerase chain reaction to amplify a portion of the SS9 EPA biosynthesis gene cluster and to use this DNA fragment to construct an EPA mutant using reverse genetics (Allen *et al*., 1999). Much to our surprise this mutant displayed no growth defects whatsoever, including under conditions of low temperature and elevated pressure. Two explanations for this lack of altered phenotype could be that the increased MUFA content of the EPA mutant is capable of compensating for the absence of EPA or that EPA is only required under

Table 1. Genes Cloned from Deep-Sea Piezotolerant/Piezophilic Bacteria

Gene	Protein/function	Source organism(s)	Isolation Depth (m)	Reference
asd	aspartate ß-D-semialdehyde dehydrogenase	*Shewanella* DSS12 and DB6705	5110 and 6356	(Kato, *et al*., 1997)
cydD, *cydC*	cytochrome *bd* biosynthesis	Assorted *Shewanellas*	up to 11000	(Kato, *et al*., 1995; Kato, *et al*., 1996; Kato, *et al*., 1997; Kato *et al*., 1997; Kato *et al*., 1997; Li *et al*., 1998)
mdh	malate dehydrogenase	*P. profundum* SS9	2551	(Welch and Bartlett, 1997)
ompH, *ompL*	probable porins	*P. profundum* SS9	2551	(Bartlett *et al*., 1993; Welch and Bartlett, 1996)
recD	Exonuclease V subunit	*P. profundum* SS9	2551	(Bidle and Bartlett, 1999)
rpsD, *rpoA*, *rplQ*	RNA polymerase a subuni t and ribosomal proteins	Shewanella DB6705		(Nakasone *et al*., 1996)
rpoE operon	RNA polymerase s subunit and regulatory proteins	*P. profundum* SS9	2551	(Chi and Bartlett, 1995)
ssb	Single-stranded-DNA-binding protein	Various *Shewanella* strains	up to 8600	(Chilukuri and Bartlett, 1997)
toxRS operon	pressure sensors	*P. profundum* SS9	2551	(Welch and Bartlett, 1998)
ORF3/4 partial	EPA fatty acid synthase subunit	*P. profundum* SS9	2551	(Allen *et al*., 1999)



	ORF2	ORF3/4	ORF3/4	SS9 ORF3/4	ORF5	ORF6	ORF6
Comparison Protein	Vitamin B12 receptor	Glycolipid synthase	Polyketide synthase	ORF3/4	HglC	HglD	HglC
Organism	*Escherichia coli*	*Nostoc punctiforme*	*Amycolatopsis mediterranei*	*Shewanella* SCRC-2738	*Anabaena* sp.	*Anabaena* sp.	*Anabaena* sp.
Accession number	P06129	AF01680	AJ223012	U73935	U13677	u13677	U13677
Blast E	1e-12	0	1e-81	e-142	2e-36	3e-90	4e-63
Alignment	14.6	233	43	114	44	98	71

	ORF6	ORF6	ORF6	ORF7	ORF7	ORF8	ORF8
Comparison Protein	Glycolipid synthase	PksC Polyketide synthase	Polyketide synthase	Polyketide synthase?	PksE Polyketide synthase?	PleD	Response regulator
Organism	*Nostoc punctiforme*	*Mycobacterium leprae*	*Saccharopolyspora erythraea*	*Anabaena* sp.	*Bacillus subtilis*	*Synechocystis* sp.	*Aeromonas jandaei*
Accession number	AF016890	S73013	M63676	U04436	Z99112	D90902	U67070
Blast E	5e-60	6e-49	2e-45	1e-168	4e-86	1e-27	2e-13
Alignment	60	40	38	196	99	27	14

Figure 1. The *Shewanella* EPA Biosynthetic Genes
Similarity of deduced protein sequences from *Shewanella* SCRC-2738 ORFs and one partial SS9 ORF involved in EPA biosynthesis with additional deduced protein sequences available in GenBank. The position, orientation and size of the ORFs are indicated below. ORFs 2-8 are of *Shewanella* SCRC-2738 (indicated as ORFS 6, 10, 12, 14, 16, 18 in Genbank U73935; Yazawa, 1996). The significance of the similarities are presented as gapped Blast E values (Altschul *et al*., 1997) and alignment scores derived from optimized values of RDF2 (Lipman and Pearson, 1985).

certain physiological conditions not evaluated in our work.

Another possibility is that EPA is not required for psychrotolerant or piezotolerant bacterial growth, but as a nutritional source used by higher organisms with which EPA producing microoorganisms have established symbiotic associations. Many of the PUFA producing microorganisms that have been discovered have been isolated from vertebrate or invertebrate sources (Yayanos *et al*., 1979; Deming *et al*., 1984; DeLong, 1986; DeLong and Yayanos, 1986; Yazawa, 1996; Yano *et al*., 1997). Clearly there is a need to better define the roles and regulation of both MUFAs and PUFAs in deep-sea bacteria.

Pressure Regulation of Outer Membrane Proteins in *P. profundum*

Another pressure response of strain SS9 is to modulate outer membrane protein (OMP) abundance. When cells are shifted from atmospheric pressure to higher pressures the amount of the outer membrane porin-like protein OmpL is lessened and production of the porin-like protein OmpH is increased (Bartlett *et al*., 1989; Chi and Bartlett, 1993; Welch and Bartlett, 1996; 1998). Although *ompL* mutants are not impaired in growth at 1 atm and *ompH* mutants are not altered in growth at elevated pressure, physiological experiments with various mutants suggest that OmpH provides a larger channel than OmpL (Bartlett and Chi, 1994), a property which could be important in the deep sea where nutrients are frequently limiting. The *ompL* and *ompH* genes are transcriptionally regulated by the inner membrane-localized ToxR and ToxS proteins (Welch and Bartlett, 1998). These proteins were first discovered in *Vibrio cholerae* where they act both as environmental sensors and regulators of gene expression in response to changes in temperature, osmolarity, pH and the levels of certain extracellular amino acids (Miller and Mekalanos, 1988; Gardel and Mekalanos, 1994). ToxR is an oligomeric protein whose cytoplasmic domain binds to genes under its control (Dziejman and Mekalanos, 1994; Otteman and Mekalanos, 1995). ToxS modulates ToxR activity (DiRita and Mekalanos, 1991).

SS9 ToxR/S pressure sensing appears to depend on the physical state of the inner membrane. Treatment of SS9 with local anesthetics at concentrations which are known to increase membrane fluidity results in a low-pressure ToxR/S signaling phenotype (high OmpL, low OmpH abundance) even when the cells are grown at high pressure (Welch and Bartlett, 1998). We hypothesize that the low pressure mode of ToxR/S regulation represents ToxR/S in its active state, where it is capable of binding to its cognate regulatory sequences, resulting in either repression or activation of gene expression.

High pressure decreases the abundance as well as specific activity of ToxR/S. ToxR (and presumably ToxS) levels decrease with increasing pressure, but even if ToxR/S levels are artificially increased at high pressure by cloning the genes on a multiple copy plasmid, elevated pressure still produces a wild type OMP pattern. In contrast, at increased temperatures ToxR abundance drops, but OmpL/H levels do not substantially vary, presumably because ToxR specific activity increases with temperature. Thus, the SS9 ToxR/S system appears well tuned to function as a barometer (or piezometer) rather than a thermometer. In contrast, ToxR/S dependent pressure regulation has not been observed in any of the several mesophilic *Vibrio* spp examined (our unpublished results).

Genes Required for Piezoadaptation in *P. profundum*

In addition to the *toxRS* operon two addition loci have been uncovered whose products influence OMP abundance. These remaining loci are particularly interesting because mutations in them render the cells extremely sensitive to elevated pressure.

The first locus which was found to be required for both psychro- and piezo-adaptation in strain SS9 was identified among SS9 transposon mutants which possessed reduced OmpH levels. Transposon cloning and DNA sequence analysis indicated that insertions in the *rseB* gene of the *rpoE* operon were responsible for these pressure-sensitive mutants (Chi and Bartlett, 1995). This operon has been investigated in several gram-negative bacteria, most notably *E. coli* and *Pseudomonas aeruginosa* (Deretic *et al*., 1994; Lonetto, *et al*., 1994; Raina *et al*., 1995; Rouvière *et al*., 1995; Ohman *et al*., 1996; De Las Peñas *et al*., 1997; Missiakas *et al*., 1997). In these mesophiles the first gene in the operon, desginated *rpoE* in the case of *E. coli*, encodes an alternative sigma factor which activates the expression of a number of genes in response to damaging conditions in the extracytoplasmic compartment. The genes downstream from *rpoE* are designated *rseA*, *rseB* and *rseC* in *E. coli*. The *rseA* and *rseB* gene products regulate the activity of RpoE in response to the prevailing conditions within the membranes and periplasmic space. The last gene in the operon, *rseC*, is poorly understood. Mutations in *rseC* do not result in a dramatic change in RpoE activity in any of the mesophiles examined and *rseC* homologs have been found in several bacteria unlinked to *rpoE*. It is therefore believed that RseC functions independently of RpoE. Based on the phenotypes of *rseC* mutants in *Rhodobacter capsulatus* and *S. typhimurium* it has been suggested that RseC proteins function to promote the assembly of certain membrane-associated protein complexes (Beck *et al*., 1997).

The SS9 *rseB* insertion mutants were altered in the abundances of numerous outer membrane proteins in addition to OmpH. The basis for this phenotype was not determined, but could have resulted from excessively high RpoE acitivity resulting from the inactivation of the *rseB* gene. *E. coli* RpoE controls the expression of the periplasmic protease DegP (Raina *et al*., 1995; Rouvière *et al*., 1995), whose activity could control the turnover of many outer membrane proteins.

High pressure revertants of the SS9 *rseB* mutants were readily obtained. These strains were always concomittantly restored for low temperature growth ability. Most of these revertants possessed DNA rearrangements at the site of the transposon insertion, further demonstrating the importance of the *rpoE* locus to high pressure and low temperature growth. Complementation analyses indicated that *rseC* is required for piezo- and psychro-adaptation. *rseC* mutants in *E. coli* and *P. aeruginosa* are neither low temperature-sensitive nor increased in piezosensitivity. Thus, while the SS9 *rpoE* operon is closely related to its homolog in mesophiles and therefore must function using essentially the same mechanisms present in other bacteria, the functioning of SS9 RseC seems particularly attuned to low temperature and high pressure conditions. Clarification of these distinctions could provide new insight into the function of the *rpoE* operon gene products and their physiological significance in bacteria adapted to different temperature (and pressure) regimes. As with MUFAs and ToxR/S, RseC represents another membrane-localized component needed for pressure-adaptation or pressure-signal transduction.

The second gene discovered to be required for piezo-adaptation of SS9 was isolated by complementation of a pressure-sensitive mutant, EC1002, impaired in the expression of a *ompH*::*lacZ* transcriptional fusion (Chi and Bartlett, 1993; Bidle and Bartlett, 1999). At high pressure this strain develops into enlarged spherical cells which divide poorly . The gene responsible for complementation of high pressure growth and normal cell morphology was found to encode a protein, RecD, which is involved in homologous DNA recombination. Further study of mutant EC1002 confirmed that it possessed phenotypes consistent with those of a *recD* mutant, i.e. a deficency in plasmid stability, and that this strain harbored a stop codon within the *recD* gene. Two additional *recD* mutants were constructed by gene disruption and were also found to possess a pressure-sensitive growth phenotypes.

The connection between DNA recombination, *ompH* expression and growth and normal cell morphology at high pressure is still unknown. However, it may be noteworthy that mutants impaired in chromosome partitioning have previously been observed to possess defects in outer membrane proteins (Bahloul *et al*., 1996). RecD, along with RecB and RecC are subunits of Exonuclease V, which plays a major role in the homologous recombination pathway of bacteria*;* reviewed in Myers and Stahl (1994). RecBCD has been proposed to function as a destructive nuclease of linear DNA until it encounters the octameric sequence Chi (χ). At this sequence, RecD is hypothesized to dissociate from the complex, while the RecBC enzyme is proposed to convert to a χ-independent recombinogenic helicase, to produce single-stranded DNA that serves as a substrate for RecA-catalyzed recombination (Dixon *et al*., 1994).

Because *E. coli recD* mutants are hyper-recombinogenic (Biek and Cohen, 1986), we propose that excessive DNA recombination is at the heart of SS9 *recD* mutant pressure-sensitivity. If a *recD* mutant in SS9 exhibits an increased frequency of inter-chromosomal recombination and genome concatemerization, then elevated pressure could compromise cell growth by exacerbating this condition. This could be accomplished by further increasing the rate of chromosome multimerization, or by inhibiting the rate of resolution of recombined chromosomes. Chromosome multimers would not segregate properly and would thereby block subsequent steps leading to cell division. Thus, impaired chromosome segregation could explain the poor growth and enlarged swollen cells of SS9 *recD* mutants grown at high pressure. Figure 2 presents a schematic of this model.

Another intriguing aspect of SS9 *recD* is its effect on *E. coli* cell morphology. Among the earliest observations of the effects of moderate pressure on bacteria, is that stressful pressures induce the development of highly filamentous cells (ZoBell and Cobet, 1963; ZoBell, 1970). The introduction of the SS9 *recD* gene into an *E. coli recD* mutant prevented cell filamentation at elevated pressures (Bidle and Bartlett, 1999). This effect was recessive to wild type *E. coli recD*.

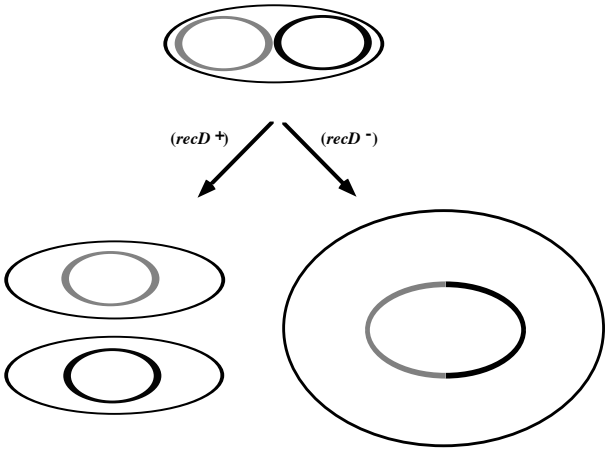


Figure 2. Schematic of the Possible Role of RecD in Preventing Interchromosomal Recombination in SS9 at Elevated Pressure

Protein Adaptation

Studies of pressure effects on proteins from shallow and deep-sea animals indicate that deep-sea fishes and invertebrates possess proteins with structural and functional adaptations to high pressure (Somero, 1990; 1991; 1992). Unfortunately similar comparisons have not been undertaken for proteins from shallow and deep-sea bacteria, and no definitive data exists for the types of amino acid changes favored at high pressure. However, one comparison has been made of homologous proteins from related bacteria differing in pressure adaptation. This was performed for the oligomeric single-stranded DNA-binding protein (SSB) from four closely related marine shewanellas whose pressure optima ranged from atmospheric pressure to 69 MPa (Chilukuri and Bartlett, 1997). The *Shewanella* SSBs could be divided into conserved amino- and carboxy-terminal regions and a highly variable central region. Analysis of the amino acid composition of the *Shewanella* SSBs revealed several features that could correlate with pressure adaptation. A decrease in the proline and glycine content of the *Shewanella* SSBs, particularly in the central variable region of the protein, correlated strongly with the increased pressure adaptation of the source organism. The reduction in helix breaking residues (proline) and helix destabilizing residues (glycine) in the more piezophilic shewanellas indicates a decrease in flexibility of the protein, rendering the protein less compressible (Gross and Jaenicke, 1994) and possibly increasing its stability under pressure. Engineered mutants of staphylococcal nuclease which reduce chain flexibility increase protein stability at high pressure (Royer *et al*., 1993).

In addition to intrinsic modifications of proteins from piezophilic bacteria, extrinsic factors could also be important in maintaining protein structure at high pressure. For example, small organic molecules act as thermostabilizers in some thermophiles (Hensel and König, 1988; Ciulla *et al*., 1994; Martins and Santos, 1995). Recently, the concentration of certain osmolyte species present in strain SS9 have been found to increase with hydrostatic pressure (Martin, Bartlett and Roberts; manuscript submitted). Yancey and coworkers have likewise found that the organic osmolyte TMAO occurs at elevated levels in deep-sea versus shallow-water fishes (Gillett *et al*., 1997). Furthermore, these investigators have found that TMAO reduces lactate dehydrogenase Km at high pressure. One possibility is that certain osmolytes which are excluded from the hydration layers of proteins could be used by deep-sea organisms to help counterbalance the protein hydrating influence of high pressure.

Concluding Remarks

There are opportunities for major discoveries to be made in deep-sea microbiology across a spectrum of levels of study extending from issues of biodiversity to the identification of genes required for piezoadaptation to membrane and protein biophysical studies at high pressure. With regard to the main focus of this review, the identification and elucidation of genes required for sensing and adapting to changes in hydrostatic pressure is still in its infancy. At this juncture it appears that genes which control membrane structure and DNA recombination are particularly important. But there are many possible pressure points in a bacterial cell, and many genetic modifications that must undoubtedly occur to enable life in the piezosphere.

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