

Large-Scale Transposon Mutagenesis of *Photobacterium profundum* SS9 Reveals New Genetic Loci Important for Growth at Low Temperature and High Pressure[∇]

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Microorganisms adapted to piezopsychrophilic growth dominate the majority of the biosphere that is at relatively constant low temperatures and high pressures, but the genetic bases for the adaptations are largely unknown. Here we report the use of transposon mutagenesis with the deep-sea bacterium *Photobacterium profundum* strain SS9 to isolate dozens of mutant strains whose growth is impaired at low temperature and/or whose growth is altered as a function of hydrostatic pressure. In many cases the gene mutation-growth phenotype relationship was verified by complementation analysis. The largest fraction of loci associated with temperature sensitivity were involved in the biosynthesis of the cell envelope, in particular the biosynthesis of extracellular polysaccharide. The largest fraction of loci associated with pressure sensitivity were involved in chromosomal structure and function. Genes for ribosome assembly and function were found to be important for both low-temperature and high-pressure growth. Likewise, both adaptation to temperature and adaptation to pressure were affected by mutations in a number of sensory and regulatory loci, suggesting the importance of signal transduction mechanisms in adaptation to either physical parameter. These analyses were the first global analyses of genes conditionally required for low-temperature or high-pressure growth in a deep-sea microorganism.

The bulk of deep-sea environments select for microorganisms which grow preferentially at low temperatures (psychrophiles) and high hydrostatic pressures (piezophiles) and which are capable of tolerating sporadic inputs of organic nutrients (9, 10).

Photobacterium profundum strain SS9 (69) is a psychrotolerant and moderately piezophilic bacterium. It was first isolated from an amphipod homogenate enrichment from the Sulu Sea (28). This microorganism, which is suitable for a variety of systems biology investigations, is capable of growth at temperatures of <2 to >20°C (optimal temperature, 15°C) and at pressures from 0.1 MPa to nearly 90 MPa (optimal pressure, 28 MPa). Analysis of the SS9 genome and transcriptome has suggested that two important aspects of the deep-sea adaptations of this organism are the nature of its pressure- and temperature-responsive genes and its high degree of metabolic diversity and redundancy (87).

Like the genes of other *Vibrionaceae*, the genes of *P. profundum* are partitioned onto two circular chromosomes (19, 87). 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 located on chromosome 2 (39, 87). In addition, *P. profundum* SS9 has a dispensable 80-kbp plasmid carrying

mostly genes with unknown functions (19, 87). Intraspecific gene variation between strains of *P. profundum* that differ in their degrees of piezoadaptation has been examined, and sequences acquired by lateral gene transfer or which could be important in high-pressure growth have been identified (19).

Genetic approaches have also been used to elucidate mechanisms of deep-sea adaptation in *P. profundum* SS9. During the course of investigation of factors that influence the pressure regulation of outer membrane protein abundance, the membrane-localized transcription factor ToxR was identified as a pressure sensor (92), and the *rseC* and *recD* genes have been found to be important for low-temperature and high-pressure growth and for 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 studies mentioned above included a global analysis of genes conditionally required for growth at high pressure or low temperature.

To complement and expand these studies, a collection of mini-Tn5 and mini-Tn10 transposon mutants was screened for high-pressure sensitivity and low-temperature sensitivity. In this paper we describe 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 0.75× 2216 medium (28 g/liter; Difco Laboratories) at 15°C. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani medium.

Antibiotics were used at the following final concentrations: rifampin (Rif), 100 µg/ml; kanamycin (Km), 100 µg/ml for *E. coli* and 200 µg/ml for *P. profundum*; and streptomycin (Sm), 50 µg/ml for *E. coli* and 150 µg/ml for *P. profundum*.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>P. profundum</i> SS9R	Rif ^r SS9 derivative	24
<i>E. coli</i> strains		
ED8654	Used for pRK2073 maintenance	67
DH5 α	<i>recA</i> strain used for cloning	37
XL1-Blue	<i>recA</i> strain used for cloning	Stratagene, La Jolla, CA
TOP10	<i>recA</i> strain used for cloning	Invitrogen, Carlsbad, CA
Plasmids		
pRK2073	Carries <i>tra</i> genes for conjugal transfer	14
pRK2013	Carries <i>tra</i> genes for conjugal transfer	14
pLOF	Mini-Tn10 transposon, Km ^r	40
pRL27	Mini-Tn5 transposon, Km ^r	52
pFL122	Broad-host-range vector, Sm ^r	54
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

5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added to solid medium at a concentration of 40 μ g/ml in *N,N*-dimethylformamide.

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were introduced into *P. profundum* strain SS9 by triparental conjugation using an *E. coli* strain containing the helper plasmid 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 (52) with *P. profundum* strain SS9R (a rifampin-resistant derivative of SS9) and screening for exconjugants growing with double selection (kanamycin plus rifampin) on 2216 marine agar plates (Difco laboratories, BD, Franklin Lakes, NJ).

Screening for auxotrophic mutants was performed by replica plating onto morpholinopropanesulfonic acid (MOPS)-glucose minimal marine medium (11). Colonies unable to grow on this medium were considered auxotrophs. Screening for mutants whose growth at low temperatures was impaired was performed by replica plating transposon mutants onto 2216 marine agar plates and incubating the plates at 15 and 4°C. The clones that grew poorly compared to the wild type after 120 h of incubation at 4°C were saved. They were subsequently rescreened in liquid medium, and growth plots were determined in triplicate. A cold sensitivity ratio was computed by dividing the low-temperature growth rate of each mutant by the maximum growth rate at 15°C. When 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 marine medium supplemented with 20 mM glucose, 10 mM HEPES (pH 7.5), 200 μ g/ml of kanamycin, and 50 mg/liter of phenol red as a color indicator for fermentative growth. Transposon mutants were individually inoculated into wells of a PCR plate with 96 elevated wells (Aygen Scientific, Union City, CA), and the wells were filled to the top with the high-pressure growth medium described above. The plate was sealed with a 96-well PCR Axymat (Aygen Scientific, Union City, CA), and we were 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). Screening for pressure sensitivity was done by replica plating transposon mutants, incubating them at high pressure (45 MPa) and low pressure (0.1 MPa) at 15°C, and looking for wells that retained the red color after 48 h, which indicated that no fermentative growth had occurred. The pressure-sensitive mutants were saved and rescreened by determining their growth plots in triplicate.

Pressure-dependent growth curves were obtained by using 1,000-fold dilutions of 48-h-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 and transferred to a glass tube, and the turbidity of the culture at 600 nm was measured with a Spectronic 20 spectrophotometer (Milton Roy, New York). The pressure sensitivity ratio of strains was assessed as previously described (23). Briefly, when the low-pressure culture entered early logarithmic phase (optical density at 600 nm [OD₆₀₀], 0.1 to 0.3), two values were recorded: the OD₆₀₀ of the 45-MPa culture (*a*) and the OD₆₀₀ of the 0.1-MPa culture (*b*). The pressure sensitivity ratio (*a/b*) was then computed. Under these conditions the pressure sensitivity ratio of the wild type was 1 to 1.1. Clones having a pressure sensitivity ratio of <0.5 were considered pressure sensitive. Clones having a pressure sensitivity ratio of >1.5 were considered pressure enhanced.

DNA extraction, purification, manipulation, and sequencing. Genomic DNA was extracted from 3 ml of 48-h-old *P. profundum* SS9 cultures by using a Wizard genomic kit (Promega, Madison, WI). The DNA was then further purified by extracting it once with a phenol-chloroform mixture and once with chloroform alone as described elsewhere (80).

Plasmid DNA was extracted using Qiagen (Valencia, CA) Miniprep (high-copy-number plasmids) and Midiprep (low-copy-number plasmids) kits and following the manufacturer's instructions. All enzymatic reactions were prepared using standard protocols (80). Enzymes were purchased from Invitrogen (Carlsbad, CA) or from New England Biolabs (Beverly, MA). DNA sequences were determined by thermal cycle fluorescent dideoxy sequencing with a MegaBACE 1000 (Amersham Biosciences, Piscataway, NJ) automated sequencer used as instructed by the manufacturer.

Arbitrary PCR amplification and transposon cloning. Rapid identification of the flanking sequences of the transposon insertions in *P. profundum* was accomplished by using a rapid arbitrary PCR method similar to the method used by Watnick and Kolter (90). This method involved two rounds of PCR. Briefly, during the first round genomic DNA from the mutant was PCR amplified with a primer unique to one end of mini-Tn10 (10extdx2 [5'-AGAGCATTACGCTGACTTG-3'] or 10extsx [5'-CACCCCTTGATTACTGTTTATGT-3']) or mini-Tn5 (pRL27Ext dx [5'-CCAGAAAGTGAGGGAGCCA-3'] or pRL27Ext sx [5'-GACAACAAGCCAGG GATG-3']) in combination with a degenerate primer (SS9arb1 [5'-GACCAC GAGACGCCACTNNNNNNNNNNCATGC-3'], SS9arb2-[5'-GACCACGAGA CGCCACTNNNNNNNNNNACTAG-3'], or SS9arb8 [5'-GACCACGAGACGC CACTNNNNNNNNNGATAT-3']) that was designed to hybridize to an arbitrary sequence on the chromosome and has a 5' GC clamp. The conditions used for the first round of amplification were five cycles of 94°C for 30 s, 30°C for 30 s, and 72°C for 60 s, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s. Two microliters of the PCR product was subjected to a second round of amplification (30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s) using a nested primer unique to the mini-Tn10 end (10int [5'-GTATGAGTCAGCAACACCTTCTTC-3']) or the mini-Tn5 end (pRL27Int dx [5'-GAGTCGACCTGCAGGCATGC-3'] or pRL27Int sx [5'-CGCACT GAGAAGCCCTTAGAGC-3']) and a primer with sequence identity to the 5' GC clamp of the arbitrary primer (arb3 [5'-GACCACGAGACGCCACT-3']). After the products of the second amplification were electrophoresed on a gel, the PCR products producing single bands were purified using an UltraClean PCR clean-up kit (MoBio Laboratories, Solana Beach, CA) and sequenced using a third transposon-specific nested primer.

The mini-Tn10 insertion points difficult to characterize by arbitrary PCR were determined by transposon cloning. Genomic DNA from the mutant was digested overnight with EcoRI, which did not cut inside the transposon. The fragments obtained were ligated into the EcoRI site of pUC18, transformed in *E. coli* TOP10 cells, and selected with kanamycin (100 μ g/ml) and ampicillin (100 μ g/ml). The transformants growing with 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 genomic DNA extraction, followed by complete digestion with BamHI, relegation and transformation into *E. coli* EC100D *pir*⁺ (Epicenter, Madison, WI), and plating onto Luria-Bertani agar plates containing kanamycin (50 μ g/ml).

The sequence flanking the transposon insertion was searched with BLASTN (7) using the nucleotide sequence of the *P. profundum* SS9 genome, which allowed retrieval of whole open reading frame (ORF) sequences. Downstream genes were analyzed using the genome browser available at <http://SS9.cribi.unipd.it>. The translated ORF sequence was classified using the COG database (84), the cellular localization was analyzed using SubLoc 1.0 (42), and the presence of signal peptides was analyzed using SignalP (13).

Complementation of selected mutants. Some of the mutants obtained were characterized further by reintroducing the wild-type copy of the disrupted gene. The ORF sequence, including the predicted promoter and additional upstream sequence, was PCR amplified using an Expand long-template PCR kit (Roche, Indiana) and ligated into the mobilizable broad-host-range vector pFL122 (54). The recombinant clones selected after blue-white screening were sequenced to determine the accuracy of the insert. These constructs were then conjugated into the appropriate mutants by triparental mating using previously described procedures (23). The cold sensitivity ratios and the pressure sensitivity ratios of the merodiploid mutants were then redetermined as described above and compared to those 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 preliminary screening, Southern blot and arbitrary PCR analysis of 12 auxotrophic mutants obtained by mini-Tn10 mutagenesis revealed the presence of hotspots for the mini-Tn10 transposition (4/12 unique insertions, located in PBPRA0199, PBPRA0269, PBPRA0280, and PBPRA0289). Tn10 has previously been found to exhibit strong sequence insertion bias (47). The same analysis performed with mutants obtained by using mini-Tn5 did not show the same bias (data not shown). Curiously, despite these limitations, the Tn10 mutants evaluated in this study did not provide a disproportionately low number of the cold-sensitive or pressure-sensitive mutants (see below).

Mutants were examined for defects in growth at a low temperature (4°C) at atmospheric pressure and at a high pressure (28 MPa) at 15°C. In the latter case a phenol red fermentation color screening method in pressurizable microtiter plates was used (Fig. 1). Putative cold-sensitive and pressure-sensitive mutants were rescreened, and bona fide cold-sensitive and pressure-sensitive mutants were evaluated further by generating a detailed growth curve; 1.5% (11/720) of the originally isolated mutants tested were auxotrophs. In contrast, only 0.13% (27/20,000) of the mutants screened were cold sensitive, and only 0.04% (8/20000) of the mutants screened were pressure sensitive. In addition, 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 had higher growth rates and the yields were higher at high pressure, indicating that high pressure partially compensated for their growth deficiencies. This is consistent with the view developed following transcriptome experiments, which indicates that *P. profundum* SS9 is under greater stress at atmospheric pressure than at elevated pressure, presumably reflecting its deep-sea origin (87).

When the cold-sensitive and pressure-affected mutants were considered together, 77% of the transposon insertions were located on chromosome 1, 23% were located on chromosome 2, and none were located on the plasmid. The gene disruptions and their locations are shown in Table 2 and Fig. 2. The finding that a larger percentage of loci required for growth at high pressure and/or low temperature were present on chromosome 1 was true even after normalization for the larger number of ORFs on this replicon. Transcriptome and codon adaptation index analyses indicated that the most actively transcribed

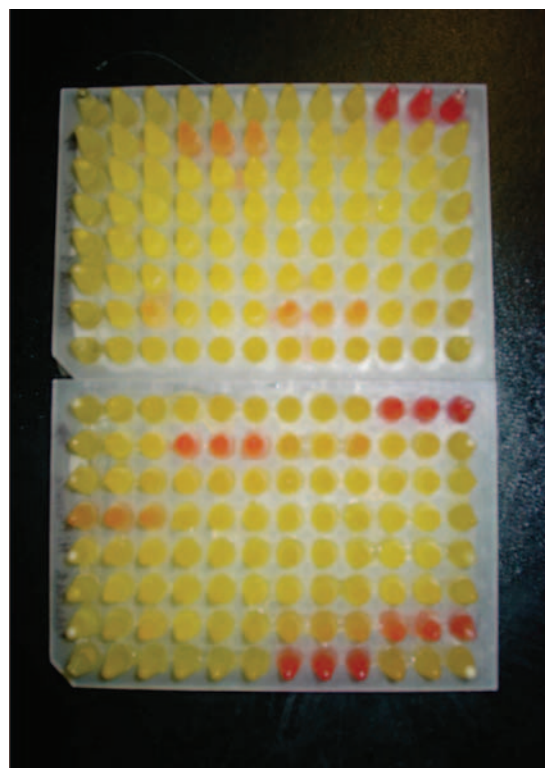


FIG. 1. Microtiter plate growth assay used in the first screening of the transposon mutants. Growth was detected by addition of phenol red to the growth medium, which changed color following the production of acid by the bacteria growing fermentatively. Yellow, growth; red, no growth.

genes are also located on chromosome 1 of *P. profundum* SS9 (87).

Although temperature and pressure are distinct thermodynamic parameters, in many cases the effect of pressure is synergistic with the effect of low temperature (28). In the case of *E. coli*, which is mesophilic with respect to both temperature and pressure, decreased temperature and increased pressure both perturb membrane structure and function and DNA replication, transcription, and translation (9). The ability of 16% of the mutants obtained here to grow was altered both at low temperature and at high pressure.

Isolation of cold-sensitive mutants. Mutants with mutations in 27 loci displayed a cold-sensitive phenotype (Fig. 3 and Table 2). Of these mutants, 21 were exclusively cold sensitive, 5 were also pressure sensitive, and one was also pressure enhanced. These mutants could be functionally divided into six categories based on the COG identity of the disrupted gene. Genes belonging to the poorly characterized COG functional classes (classes R and S) were clustered based on additional information provided by other analyses.

(i) Signal transduction mechanisms (class T) (strains FL1, FL4, FL11, FL13, and FL21). Microorganism sense and coordinate metabolic functions during cold stress (72). These responses occur through temperature-induced changes in DNA supercoiling (30, 49, 64), via effects on ribosomes (86), through the modulation of guanosine tetraphosphate (ppGpp) levels

TABLE 2. Mutants obtained in this study

Mutant	Gene ^a	Annotation	COG class(es)	Type of transposon insertions recovered in ORF	Phenotype ^b	Notes ^c
FL1	PBPRB2014	Transcriptional regulator; LuxR family	T	a-Tn5, b-Tn10	CS	
FL2	PBPRB0212	ATP-dependent DEAD box RNA helicase	L, K, J	Tn5	CS	Complementation analysis
FL3	PBPRA2678	Hypothetical protein	M	a-Tn5, b-Tn5	CS	Missing or divergent in <i>P. profundum</i> strains 3TCK and DSJ4
FL4	PBPRA3093	<i>rpoE</i> regulatory protein RseB	T	Tn5	CS, PS	Previously observed (23) suppressors; overexpressed at 28 MPa
FL5	PBPRA2681	Glycosyl transferase	M	a-Tn5, b-Tn5	CS	
FL6	PBPRA1774	ATP-dependent protease; LA related	O	Tn10	CS	Overexpressed at 28 MPa
FL7	PBPRA2684	Hypothetical protein involved in polysaccharide biosynthesis	R	Tn5	CS	Missing or divergent in <i>P. profundum</i> strains 3TCK and DSJ4
FL8	PBPRA0396	Hypothetical integral membrane protein	R	a-Tn5, b-Tn5	CS	Pfam00892 DUF6 domain
FL9	PBPRA2686	Tyrosine protein kinase	M	Tn5	CS	Missing or divergent in <i>P. profundum</i> strains 3TCK and DSJ4
FL10	PBPRA0428	Pyruvate kinase I	G	a-Tn10, b-Tn10	CS, PS	Suppressors; underexpressed at 28 MPa
FL11	PBPRA1082	DNA-binding protein H-NS	R	Tn5	CS, PE	Enhanced biofilm mutant
FL12	PBPRA0747	Suppressor protein SuhB	G	Tn10	CS, PS	Complementation analysis
FL13	PBPRA0189	Guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase SpoT	T, K	Tn5	CS, PS	
FL14	PBPRA2407	Periplasmic linker protein	M	Tn5	CS	
FL15	PBPRA3239	Hypothetical protein	S	Tn5	CS	Pfam06295; DUF1043 domain; upstream of <i>degQ/degS</i> orthologues; complementation analysis
FL16	PBPRA2282	Conserved hypothetical protein	G	Tn5	CS	pfam04074 DUF386 domain
FL17	PBPRB2009	Phosphotransferase cellobiose-specific component Iic	G	Tn10	CS	Possibly missing or divergent in <i>P. profundum</i> strain DSJ4
FL18	PBPRA0667	Conserved hypothetical protein	S	Tn10	CS	Missing or divergent in <i>P. profundum</i> strain 3TCK; pfam05943; DUF877
FL19	PBPRB1941	Hypothetical protein		Tn10	CS	Signal peptide
FL20	Unknown	Transposase	L	Tn10	CS	Multiple copies of the sequence: 8 copies on chromosome 2 and 5 copies on chromosome 1
FL21	PBPRB1757	Hypothetical protein	T	Tn10	CS	Signal peptide; missing or divergent in strain DSJ4; complementation analysis
FL22	PBPRB0828	Beta-hexosaminidase	G	Tn10	CS	
FL23	PBPRA3229	Phosphoheptose isomerase	G	Tn10	CS, PS	Probably <i>diaA</i> (44)
FL24	PBPRA0917	Polar flagellar protein FlaJ	N, U, O	Tn10	CS	Phenotype only on plates
FL25	PBPRA2700	Hypothetical protein	M	a-Tn5, b-Tn5	CS	pfam04464; phenotype only on plates
FL26	PBPRA0218	O-antigen ligase	M	Tn5	CS	Underexpressed at 4°C; phenotype only on plates
FL27	PBPRA0674	Conserved hypothetical protein	S	Tn5	CS	Missing or divergent in <i>P. profundum</i> strain 3TCK; pfam05638.3 (<i>hcp</i>); phenotype only on plates
FL28	PBPRA1039	SeqA protein	L	Tn10	PE	
FL29	PBPRA2596	L-Asparaginase	E, J	a-Tn5, b-Tn5	PS	Complementation analysis; possibly overexpressed at 45 MPa
FL30	PBPRA2658	3-Oxoacyl-(acyl carrier protein) synthase I	I, Q	Tn5	PS	Previously observed (3)
FL31	PBPRB0001	Chromosome II replication protein RctB		Tn5	PS	— ^d

^a The ORF numbers are those assigned by Vezzi et al. (87).

^b CS, cold sensitive; PS, pressure sensitive; PE, pressure enhanced.

^c The numbers in parentheses are reference numbers.

^d See reference 31.

(46), and by gene regulation through the activity of alternative sigma factors (74, 94).

The expression of genes required for low-temperature growth in SS9 could be modulated by specific regulators, such as the PBPRB2014 protein (strain FL1), a transcriptional regulator of the LuxR family, and the PBPRB1757 protein (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 possi-

ble role as a hybrid two-component system. Related regulatory systems have been observed in *Bacillus subtilis* and some cyanobacteria (79). In these organisms, the expression of cold-inducible genes, in particular the genes responsible for membrane unsaturation, is controlled by specific two-component systems.

(ii) Cell envelope biogenesis and outer membrane (class M) (strains FL3, FL5, FL7, FL9, FL14, FL25, FL26, and FL27). Workers have speculated about the involvement of capsular

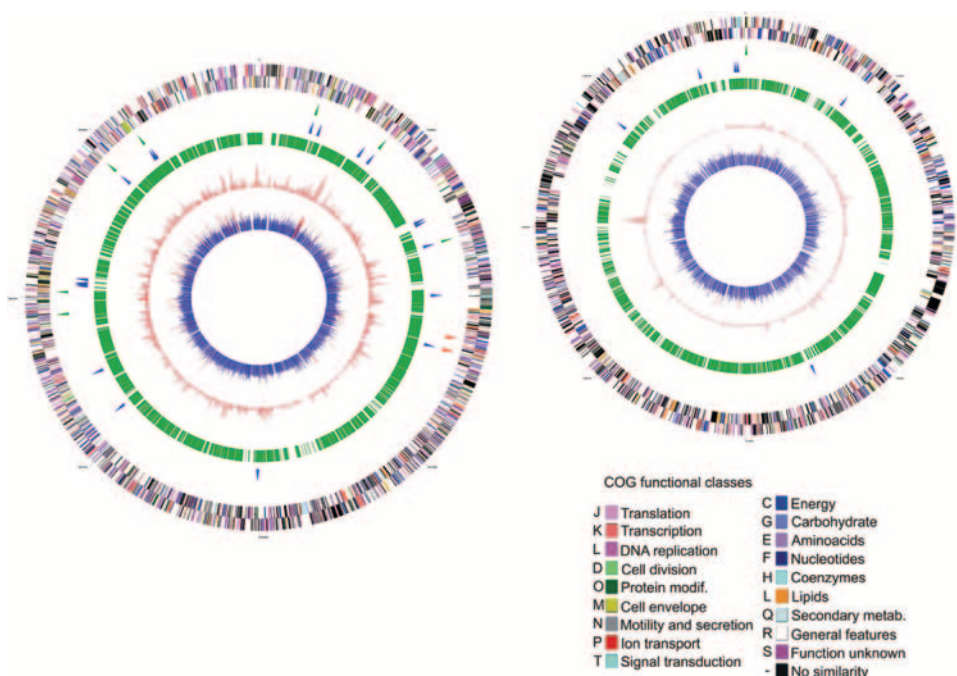


FIG. 2. Genomic localization of the transposon insertions in the two chromosomes of *P. profundum* SS9 (not to scale). (Left panel) Chromosome 1. (Right panel) Chromosome 2. From the outside in, the first two circles show the predicted protein coding on the two strands, with the colors indicating the COG functional classes. The third circle shows the locations of the pressure-sensitive (green) and pressure-enhanced (red) genes. The fourth circle shows the locations of the cold-sensitive (blue) genes. The fifth circle shows the syntheny with the draft genome of *P. profundum* 3TCK (www.venterininstitute.org). The sixth circle shows the mean fluorescence intensities obtained in the microarray experiments at 28 MPa, and the sixth circle shows the codon adaptation index, with scores of >0.5 indicated by red (87).

polysaccharide in adaptation to low temperature for some time (25, 60, 62).

FL26 contains a disruption in the gene encoding the PBPR0218 protein, a predicted O-antigen ligase for lipopolysaccharide (LPS) synthesis whose expression is reduced at low tempera-

ture (19, 87). The mutations in four of the remaining mutants are mutations in part of a large gene cluster that is highly divergent in the members of the species *P. profundum* (19). This cluster is approximately 35 kb long and contains many genes that are involved in various aspects of LPS O-antigen

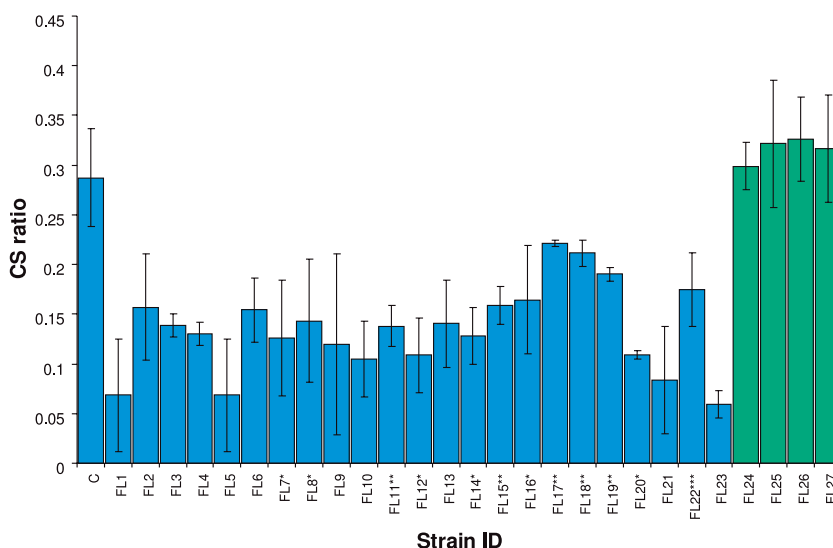


FIG. 3. Cold sensitivity (CS) ratios for the mutants isolated in this study. The values were computed as described in Materials and Methods. The green bars indicate the ratios for the mutants that displayed a reproducible phenotype only on plates. C, control strain. For some strains, the lag phase was 40 to 59 h (indicated by one asterisk), 60 to 79 h (two asterisks), or >79 h (three asterisks) longer than the lag phase for the control strain. The error bars indicate one standard deviation.

biosynthesis, extracellular polysaccharide (EPS) biosynthesis, and flagellar filament glycosylation (36). Some of these genes are also differentially expressed under different temperature and pressure conditions (PBPR2692, PBPR2701, and PBPR2710). Their functions are currently being characterized further (G. Ferguson et al., unpublished data). Strain FL7 (disruption of PBPR2684, hypothetical protein, COG functional class R) is included in this category of mutants based solely on the fact that its insertion is located within the predicted polysaccharide biosynthesis cluster. Using similar reasoning, we included strain FL27 among the envelope mutants. The mutated gene in strain FL27, PBPR0674, is homologous to the gene encoding hemolysin-coregulated protein (Hcp) of *Vibrio* species. While the function of Hcp has not been determined (95), Enos-Berlage et al. have shown that a mutation in *hcp* results in alterations in the formation of *Vibrio parahaemolyticus* biofilms, and thus Hcp could be associated with polysaccharide production (32).

Interestingly, strains FL25, FL26, and FL27 displayed the cold-sensitive phenotype when they were grown on agar plates but not when they were grown in liquid media (Fig. 3), suggesting that there are different EPS and LPS requirements for growth at low temperature under these two conditions. The basis for the distinction is not clear at this time. *E. coli* with a deletion of the *rfa* locus, which is responsible for assembly of the core oligosaccharide of LPS, is cold sensitive and nonmotile (70). The role of LPS in stabilizing the bacterial cell envelope is exerted both through its interaction with the EPS and through the fluidizing effects that its lipid components can have on the outer membrane (50).

Corsaro et al. (25), have suggested that the psychrophile *Pseudoalteromonas haloplanktis* TAC125 is unable to complete the biosynthesis of lipooligosaccharide at a suboptimal temperature as the phosphorylation of both lipooligosaccharide and EPS decreases with temperature. In these molecules, phosphate groups bind to divalent cations, such as Ca^{2+} and Mg^{2+} , stabilizing the extracellular leaflet of the outer membrane (50, 76) and modulating its permeability. LPS integrity is essential for correct incorporation of many proteins, such as porins, in the outer membrane (70). It is possible that changes in LPS and EPS, which change the surface properties of bacteria, are used in processes affected by low temperature, such as membrane fluidity and substrate transport (32).

(iii) Carbohydrate transport and metabolism (class G) (strains FL10, FL12, FL16, FL17, FL22, and FL23). Low-temperature growth was affected by mutations in a number of genes involved in the transport and the central metabolism of carbohydrates. These genes included the genes encoding the glycolytic enzyme pyruvate kinase I (PBPR0428), a component of a group translocation transporter specific for cellobiose (PBPR2009), and the conserved hypothetical protein PBPR2282, which may participate in the metabolism of *N*-acetylneuraminic acid and its derivatives (48).

Two genes deserve special attention. PBPR0747 is in this category because it is homologous to *suhB*, whose product was shown to possess inositol monophosphatase activity (68). However, the role of this product in the cell appears to involve 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). An *E. coli suhB* mutant is cold sensitive (43).

Strain FL23 has an insertion in PBPR3229, which was originally annotated as a phosphoheptose isomerase gene (87) but after closer inspection turned out to be orthologous to *diaA*, a gene required during the initiation of chromosome replication (44). Both PBPR0747 and PBPR3229 also affect the growth of *P. profundum* SS9 at high pressure.

(iv) Protein export (class U) (strains FL18, FL19, and FL24). Strain FL19 has a mutation in a gene encoding a hypothetical protein with a well-defined signal peptide (13). While it is possible that the product of the PBPR1941 gene is directly involved in some aspect of temperature adaptation, another hypothesis is that the insertion results in a blockage of the general secretory pathway (sec). This pathway is inherently cold sensitive in *E. coli* (73).

Other secretion routes could also be important for low-temperature growth. Strain FL18 has an insertion in PBPR0667, a homolog of *impC*, a gene of *Rhizobium leguminosarum* (16) that is part of a locus important for temperature-dependent secretion and establishment of the symbiotic interaction with the host plant roots. Strain FL24 has a mutation in PBPR0917, an orthologue of the flagellar chaperone gene *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 temperatures, when the activity of proteases involved in recycling of nonfunctional peptides is lower.

(v) Protein synthesis and turnover (classes J and O) (strains FL2, FL6, and FL15). Strain FL2 has a mutation in PBPR0212. This gene was annotated as a member of a family of genes encoding ATP-dependent helicases known as DEAD box helicases because of their characteristic amino acid 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* (56).

The insertion in strain FL6 is in PBPR1774, which codes for an LA-related protease. Similar ATP-dependent proteases have been shown to degrade nonfunctional proteins in the cytoplasm of *E. coli* (78) and to be important for cold acclimation in the marine cyanobacterium *Synechococcus* (75). Strain FL15 has an interruption in the gene for the hypothetical protein PBPR3239, which is upstream of and in the same transcriptional unit as the *degQ* and *degS* genes encoding the periplasmic serine proteases.

Cold shock can result in protein unfolding and aggregation (33). The aggregates are considered "dead ends," and accumulation of them may cause severe damage to the organism (78). It is possible that the protease mutations described above lead to accumulation of protein aggregates that affect growth.

(vi) Unknown (classes L and R) (strains FL8 and FL20). The mini-Tn10 insertion of mutant FL20 is in the region coding for a transposase. Transposable elements are very abundant in the genome of *P. profundum* SS9 (87). The basis for the cold sensitivity of this mutant is not clear at this time. The basis for the cold sensitivity of strain FL8 is also unknown. The interrupted gene, PBPR0396, codes for a hypothetical inte-

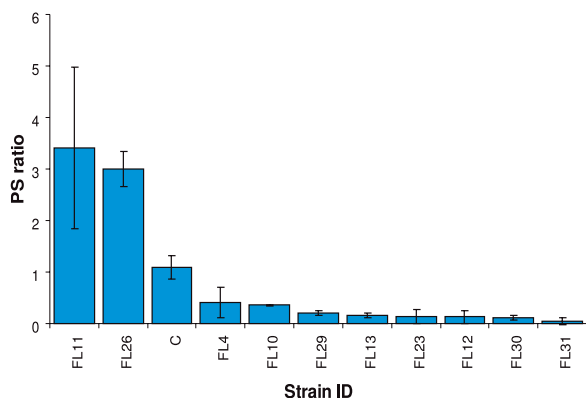


FIG. 4. Pressure sensitivity (PS) ratios for the mutants isolated in this study. The values were computed as described in Materials and Methods. C, control strain. The error bars indicate one standard deviation.

gral membrane protein and is part of an operon with genes encoding two ribosomal proteins, L21 and L27.

Isolation of pressure-altered mutants. Table 2 and Fig. 4 show the transposon mutants displaying a pressure-altered phenotype together with their pressure sensitivity ratios. The number of loci recovered in the pressure sensitivity screen was less than one-third the number of the cold-sensitive loci. 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 support one or both of the latter two hypotheses: (i) previously identified pressure-sensitive mutants with site-directed mutations were identified using the screen, thus demonstrating its sensitivity and arguing against the first hypothesis, and (ii) with the exception of PBPR2596 (L-asparaginase) and PBPR2658 [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, 58, 59, 65, 83), which is consistent with the last hypothesis. These mutants could be functionally divided into four categories based on prior research and the COG identities of the disrupted genes.

(i) Previously isolated genes (classes T and I) (strains FL4 and FL30). PBPR2658 (*fabB*) codes for the KASI enzyme (77) and has been linked to piezophilicity because of its role in the production of monounsaturated fatty acids which are essential for the proper membrane physical state at high pressure (3). Inactivation of *rseB* (PBPR3093), 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 not clear if the observed phenotype is determined by RseC involvement in the regulation of the alternative sigma factor RpoE (63) or by its role in other cellular functions, such as thiamine synthesis, as observed in *Salmonella enterica* serovar Typhimurium (12).

(ii) Metabolism (classes E and G) (strains FL10 and FL29). Strain FL29 contains an insertion in PBPR2596 encoding L-asparaginase. This enzyme is essential under conditions in which asparagine is the only nitrogen source (27). It is possible

that 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 contained only 15 μ M nitrate. The importance of an organic nitrogen source for metabolism is further reinforced by the upregulation at 45 MPa of PBPR1174 (periplasmic L-asparaginase), PBPR3391 (aspartate-ammonia lyase), and PBPR2173 (histidine-ammonia lyase), all of which contribute to the catabolism of amino acids.

Another target of pressure resulting in global alteration of metabolism was observed in strain FL10, which has a mutation in pyruvate kinase I (PBPR4028). Pyruvate kinase has previously been found to be a pressure-sensitive enzyme and to undergo adaptational changes in deep-sea animals (26, 57). The requirement for this isozyme could have been a reflection of the medium conditions employed (growth under glucose-fermenting conditions). Alternatively, because of the role of pyruvate kinase in glycolytic regulation (65), the mutant could be a regulatory mutant. Transcriptome studies indicated that five of the nine steps of glycolysis involve pressure- and temperature-regulated genes, one of which is PBPR4028. The growth of this mutant is also impaired at low temperature.

(iii) Chromosome replication (classes L, R, and G) (strains FL11, FL23, FL28, and FL31). Almost one-half of the pressure-altered mutants were associated with some aspect of chromosome structure and partitioning during cell division. Cell division and chromosome replication and segregation are among the most pressure-sensitive processes in a mesophilic bacterial cell (9, 96). Therefore, proteins underlying these cellular functions are expected to be under strong selective pressure to acquire functionality at depth. Bidle and Bartlett (15) reported impairment of growth at high pressure of a *recD* mutant of *P. profundum*. These workers were also able to show that high-pressure impairment of cell division in *E. coli* could be rescued by heterologous expression of the *recD* gene from *P. profundum* (15).

PBPR0001 is a gene that is considered essential for the replication of chromosome II in the family *Vibrionaceae* (31). Egan and Waldor (31) were unable to obtain a null mutant with a mutation in VCA0002, the orthologue of PBPR0001, in *Vibrio cholerae*. Because the insertion in strain FL31 is close to the 3' end of the ORF, we hypothesized that it results in only a partial loss of function. This further implies that the C terminus of PBPR0001 is required only under high-pressure conditions.

Two additional genes whose products are important for chromosome replication at high pressure were also discovered. The first gene, *seqA* (PBPR1039), is a negative regulator of the cell cycle, and a strain having a mutation in PBPR1039 is pressure enhanced. *E. coli* SeqA mutants have irregular growth caused by asynchronous patterns of replication (58). The stress imposed by growth at low pressure might exacerbate this phenotype in the case of SS9, but it is not clear if this is due to the role of SeqA as a cell cycle regulator (58), its importance for chromosome partitioning (55), or its effect on the structure of cell membranes (91).

While a *seqA* mutation resulted in a pressure-enhanced phenotype, pressure sensitivity and cold sensitivity were observed for strain FL23, which contains an insertion in *diaA*. This gene has recently been identified as a novel DNA-binding protein

involved in ensuring the initiation of chromosomal replication at the right time (44). Interestingly, studies with *E. coli* have shown that both *seqA* and *diaA* can suppress temperature-sensitive phenotypes associated with mutations in *dnaA*, which codes for the initiator of chromosome replication (44, 88).

The nucleoid-associated protein H-NS also influences growth at elevated pressure. Ishii et al. (45) observed that a Δhns strain of *E. coli* is at least 1,000-fold more sensitive to high hydrostatic pressure than the isogenic wild-type strain. In contrast, mutant strain FL11, which contains an insertion in the *hns* orthologue PBPR1082, grows better at high hydrostatic pressure than at atmospheric pressure. However, H-NS-deficient mutants of both *E. coli* (29) and *P. profundum* are cold sensitive, suggesting that the *hns* gene has different roles in the adaptation to temperature and in the adaptation to pressure and has evolved piezo-specific traits in *P. profundum*.

The cold sensitivity of FL11 could be caused by the role of H-NS in modulating the cold shock response (18, 45) or, alternatively, by an alteration in the EPS-LPS matrix. Enos-Berlage et al. (32) have reported that insertional inactivation of H-NS results in modifications of the capsular polysaccharide. FL11 produces a visibly thicker biofilm when it is grown in liquid media (data not shown).

(iv) Translation, ribosomal structure, and biogenesis (classes J, G, and T) (strains FL2, FL12, and FL13). Mutations in PBPR0747 (*suhB*), PBPR0212 (DEAD box helicase), and PBPR0189 (*spoT*) result in both cold and pressure sensitivity and are predicted to affect the structure or function of the ribosome.

Mesophilic ribosomes are extremely sensitive to high hydrostatic pressure (35, 51). *P. profundum* SS9 has the record number of ribosomal operons, and there is a large amount of intragenomic variation (53, 87). The intragenomic variation in the 23S rRNA of *P. profundum* SS9 is concentrated in helices 25 and 45. 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 PBPR0747.

Helix 25 is also the major site of interaction with ribosomal protein L13, a protein essential for assembly of the 50S subunit of the ribosome. In a correctly assembled ribosome, L13 is located within a few angstroms of loop 2475 (66). Two members of the DEAD box family of RNA helicases have been implicated in this step of ribosome biogenesis. The first enzyme, *DbpA*, was shown to interact with residues 2454 to 2606 of the 23S rRNA in vitro (85). The second enzyme, *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 PBPR0212 performs a similar function in the *P. profundum* cell, this possibly explains the cold-sensitive phenotype of the *P. profundum* mutant. The genome of *P. profundum* SS9 codes for at least nine DEAD box helicases (PBPR0562, PBPR1748, PBPR3542, PBPR0199, PBPR0212, PBPR0427, PBPR1008, PBPR1232, and PBPR1761), almost twice as many as the genome of *E. coli*, in which some of the proteins have been shown to have unique but partially overlapping functions in the cell (21). A similar expansion of genes encoding the DEAD box family of helicases has also

been observed in the psychrophilic gammaproteobacterial genomes of *Colwellia psychrerythraea* 34H (62) and *P. haloplanktis* TAC125 (61), suggesting that this phenomenon might be important for temperature adaptation.

In *P. profundum*, microarray analysis revealed differential expression of some of the DEAD box helicases under certain temperature and pressure conditions: the orthologue (PBPR0427 protein) most closely related to *E. coli* *DbpA* (NCBI accession no. P0A9P6) (17) is underexpressed at 28 or 45 MPa and 4°C (19, 87), the PBPR1232 protein is underexpressed at 45 MPa, and the PBPR1761 protein is underexpressed at 4°C. Notably, this pattern of expression, with the DEAD box helicases repressed at lower temperatures, is opposite the pattern that was observed in the Antarctic methanogen *Methanococoides burtonii* (56).

Another aspect of the pressure and temperature ribosome connection appears to be modulation of the stringent response. This phenomenon results in dramatic downregulation of ribosomal components following a variety of stresses and was first observed in *E. coli* cells subjected to amino acid starvation (81). A cell not undergoing the stringent response is said to be in a relaxed state. The effector molecule of the stringent response is ppGpp, which 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 decrease in ppGpp levels would result in the production of increased amounts 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 (93) and microarray analysis of high-pressure-shocked *Lactobacillus sanfranciscensis* (71).

In *P. profundum*, a mutant with a mutation in the *spoT* orthologue PBPR0189 is both cold and pressure sensitive. This phenotype might be caused by 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 (82).

An alternative hypothesis for the cold and pressure sensitivity of a *P. profundum* *spoT* mutant originated from the observation that in a *V. cholerae* mutant producing lower-than-normal levels of ppGpp *toxR* was transcriptionally repressed (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 (D. H. 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 (59), it would be interesting to analyze the pressure and cold sensitivity of a *P. profundum* *relA* mutant and a *spoT relA* double mutant.

Complementation of selected mutants. A subset of mutant strains (strains FL2, FL12, FL15, FL21, and FL29) was selected for complementation analysis. In most cases (strains FL2, FL12, and FL29) reintroduction of the wild-type copy of the gene resulted in a wild-type phenotype (Fig. 5), confirming the role of the disrupted ORF in growth at high hydrostatic pressure or low temperature. In one case (FL21, PBPR1757),

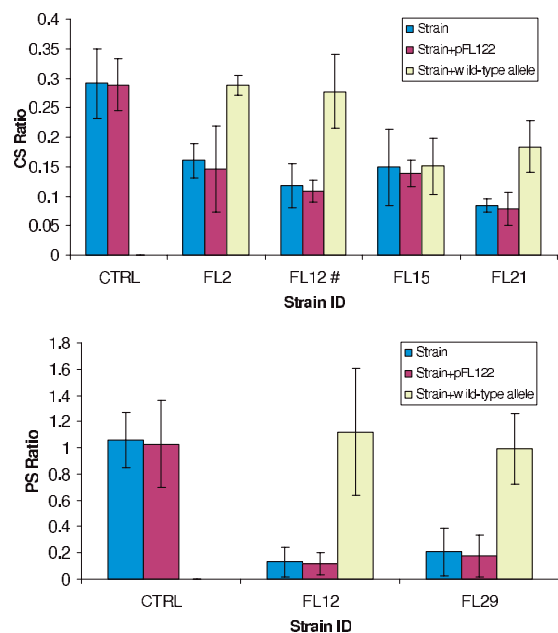


FIG. 5. Cold sensitivity (CS) and pressure sensitivity (PS) ratios for a selected subset of mutants after reintroduction of the wild-type copy of the allele on the plasmid vector pFL122. (Top panel) Complementation of cold-sensitive mutants. (Bottom panel) Complementation of pressure-sensitive mutants. The error bars indicate one standard deviation. Complementation of the mutation eliminated the increase in the lag phase observed in FL12 for the cold sensitivity ratio. CTRL, control.

the cold-sensitive phenotype was intermediate, suggesting the possibility of a partially dominant negative mutant or that a downstream gene might also have a role in low-temperature growth. In another case (FL15, PBPR3239) reintroduction of the wild-type copy did not complement the mutant phenotype. The insertion in PBPR3239 could exert a polar effect on transcription of the downstream genes PBPR3240 and PBPR3241 coding for orthologues of the DegQ and DegS serine proteases involved in recycling of nonfunctional proteins in the periplasmic space (89). 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 of *E. coli* is unable to respond to extracytoplasmic stress (2). Alternatively, it is possible that PBPR3239 functionally interacts with PBPR3240 in the periplasm. In fact, it has been predicted that PBPR3239 is localized in the periplasmic space (42), and the synteny conservation of the two ORFs in all the members of the family *Vibrionaceae* suggests that they are part of the same regulon.

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. The mesophile *Saccharomyces cerevisiae* was examined by these workers. This study expanded the number of *P. profundum* cold-sensitive mutants from 2 (6, 23) to 28 and the number of pressure-sensitive mutants from 4 (3, 4, 15, 23) to 10.

During the screening some transposon insertions were in genes previously implicated in low-temperature or high-pres-

sure growth (3, 23). However, not all the piezoadaptive genes obtained previously were recovered. This can be explained by the limited coverage of the transposon screen. Based upon Poisson statistics, we inferred that the minimal number of mutants that must 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 mini-Tn10 transposition, screening of 20,000 mutants was not likely to have been saturating. Indeed, of the 31 pressure-sensitive cold-sensitive loci discovered, only 7 were hit more than once. This study also highlights the fact that while there is some functional overlap between the adaptive responses to temperature and pressure, each condition affects microbial cells in a unique way. Most cold-sensitive mutants are not pressure sensitive, and most pressure-sensitive mutants are not cold sensitive. Almost all the cold-sensitive mutants discovered could be clustered into six COG functional classes (class T, signal transduction mechanisms; class M, cell envelope biogenesis and outer membrane; class G, carbohydrate transport and metabolism; class U, intracellular trafficking, secretion, and vesicular transport; class L, DNA replication, recombination, and repair; and class O, posttranslational modification, protein turnover, and chaperones). The fewer pressure-sensitive mutants were more diverse, although at least four of them could be associated with chromosome partitioning (classes L, R, and G) and three could be associated with ribosomal function (classes J, G, and T).

This is also the first study that provides direct genetic evidence for a crucial role of EPS genes in adaptation to low temperature (60). It has been hypothesized that one function of EPS at low temperature might be as a cryoprotectant under freezing conditions (60). This cannot be the only low-temperature function in the case of *P. profundum* as the cold-sensitive phenotype of the EPS mutants appears at temperatures well above the freezing point of water. Further studies are needed to evaluate the relationship between EPS structure and function and cold sensitivity.

Additionally, because of the wide variety of sensory and regulatory mutants with mutations affecting growth at high pressure and low temperature, it would be interesting to identify the genes under transcriptional control of each of the regulators. Keeping in mind that piezophilic bacteria are under stress at atmospheric pressure, it might also be interesting to embark upon a hunt for mutants with low-pressure sensitivity.

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