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Synchronous Effects of Temperature, Hydrostatic Pressure, and Salinity on Growth, Phospholipid Profiles, and Protein Patterns of Four *Halomonas* Species Isolated from Deep-Sea Hydrothermal-Vent and Sea Surface Environments

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Four strains of euryhaline bacteria belonging to the genus *Halomonas* were tested for their response to a range of temperatures (2, 13, and 30°C), hydrostatic pressures (0.1, 7.5, 15, 25, 35, 45, and 55 MPa), and salinities (4, 11, and 17% total salts). The isolates were psychrotolerant, halophilic to moderately halophilic, and piezotolerant, growing fastest at 30°C, 0.1 MPa, and 4% total salts. Little or no growth occurred at the highest hydrostatic pressures tested, an effect that was more pronounced with decreasing temperatures. Growth curves suggested that the *Halomonas* strains tested would grow well in cool to warm hydrothermal-vent and associated subseafloor habitats, but poorly or not at all under cold deep-sea conditions. The intermediate salinity tested enhanced growth under certain high-hydrostatic-pressure and low-temperature conditions, highlighting a synergistic effect on growth for these combined stresses. Phospholipid profiles obtained at 30°C indicated that hydrostatic pressure exerted the dominant control on the degree of lipid saturation, although elevated salinity slightly mitigated the increased degree of lipid unsaturation caused by increased hydrostatic pressure. Profiles of cytosolic and membrane proteins of *Halomonas axialensis* and *H. hydrothermalis* performed at 30°C under various salinities and hydrostatic pressure conditions indicated several hydrostatic pressure and salinity effects, including proteins whose expression was induced by either an elevated salinity or hydrostatic pressure, but not by a combination of the two. The interplay between salinity and hydrostatic pressure on microbial growth and physiology suggests that adaptations to hydrostatic pressure and possibly other stresses may partially explain the euryhaline phenotype of members of the genus *Halomonas* living in deep-sea environments.

Euryhaline bacteria, which can grow over an extremely wide salt range, are nearly ubiquitous in marine environments and are often cultured from deep-sea sediments and hydrothermal vents (32, 52, 70, 76). These microorganisms are also abundant; bacteria capable of growth on media with 17% total salts, including primarily members of the genera *Halomonas* and *Marinobacter*, were found to comprise a remarkably high percentage (up to >10%) of the total microbial community in hydrothermal-vent habitats and the overlying water column in the North and South Pacific ocean (32). Several recently characterized *Halomonas* strains isolated from low-temperature hydrothermal fluids, hydrothermal plumes, and sulfide rock, including those from 1:50 to 1:500 fluid dilution enrichments, were found to have a minimum growth temperature of −1 to 2°C at 0.1 MPa and 4% total salts (33), similar to Antarctic *Halomonas* isolates (38, 58, 76). This low minimum temperature for growth closely matches the temperature of the deep sea below 1,500 m (and shallower towards the poles) and leaves open the question of whether *Halomonas* strains collected from the deep sea, despite their numerical significance, are able to grow in situ in deep regions of the ocean outside of cool to warm hydrothermal-vent habitats.

Piezotolerant microorganisms grow more slowly as hydrostatic pressure increases above the sea surface pressure of 0.1 MPa, whereas the growth rate of piezophilic microorganisms is fastest at hydrostatic pressures of >0.1 MPa (15, 82). For both groups of microorganisms, hydrostatic pressure and temperature act synergistically, such that low temperatures and high hydrostatic pressures have analogous effects on cellular proteins and membranes (2, 13, 14, 83). Hydrostatic pressures equivalent to mid-ocean-ridge or abyssal depths have been shown to increase the cardinal growth temperatures of hyperthermophiles isolated from hydrothermal vents (7, 26, 28, 55), the optimum growth temperature of a mesophilic *Pseudomonas* strain obtained from deep-sea sediments (31), and the ability of psychropiezophiles isolated from the cold deep sea to tolerate (or prefer [16]) slightly warmer temperatures (81, 83). Stated another way, the higher the hydrostatic pressure, the higher the temperature allowed (T_{max}) or required (T_{min}) to permit growth. Given that the minimum temperature for growth typically increases with increasing hydrostatic pressures, we hypothesized that *Halomonas* spp. with a T_{min} of −1 to 2°C at 0.1 MPa would not be able to multiply in cold deep-sea habitats.

Cardinal growth temperatures and salinities may also vary with respect to each other, just as lag times and maximum cell yields may also depend on the temperature-salinity regimen. Growth curves have shown that the optimum and maximum salt concentrations for growth of the moderate halophiles *Halomonas halophila* and *Vibrio anguillarum* (reclassified as *Listonella anguillarum* [35]) increased with increasing temperatures (22, 57). Similarly, "*Pseudomonas halosaccharolytica*" (proposed name) grew faster with elevated salinities at elevated temperatures (51). Growth curves of a facultatively halophilic coccoid bacterium isolated

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from salted mackerel (ultimately classified as *Marinococcus halophilus* [23, 49]) likewise showed that the T_{opt} was 35°C with 1.0 M NaCl but that the T_{max} with 0.1 M NaCl was only 30°C (48). This salinity-temperature relationship, however, is sometimes observed only for a single cardinal growth parameter or not at all (8, 21, 29, 47, 75, 78).

Moderately halophilic and euryhaline bacteria isolated from marine environments provide an opportunity to examine the combined effects of salinity, hydrostatic pressure, and temperature on microbial growth, phospholipid profiles, and protein expression. Very few studies examining the interactions of all three of these stresses have been performed thus far. Euryhaline and moderately halophilic bacteria, including *Salinisphaera shabanensis* and *Halanaerobium* spp., have been isolated from the brine-seawater interface above the Shaban and Kebrit brine deeps (22 to 25°C), at 1,325 and 1,466-m seawater depths, respectively, in the Red Sea (1, 17). At 30°C, *S. shabanensis* grew with 1 to 28% NaCl at 0.1 MPa and with 5 to 20% NaCl at 15 MPa, but it did not grow at ≥20 MPa (1). At 0.1 MPa, this bacterium accumulated up to 4 M concentrations of compatible solutes under salt stress (17), although the production of compatible solutes under hydrostatic pressure has not yet been reported. Physiological experiments monitoring compatible solutes in the less haloversatile psychropiezophile *Photobacterium profundum* strain SS9 revealed an additive effect of hydrostatic pressure and salinity on the production of compatible solutes whereby the combination of the two stresses resulted in a disproportionate increase in the accumulation of these compounds (37).

The present set of experiments utilized four *Halomonas* strains, three of which were isolated from deep-sea hydrothermal vents and one of which was isolated from surface seawater, and explored several facets of the synchronous effects of temperature, hydrostatic pressure, and salinity on microbial growth. The selected parameters were modeled in part on the conditions found at deep-sea hydrothermal vents and the surrounding cold deep sea in order to delineate the marine habitats that (assuming adequate nutrients and energy sources) permit the growth of *Halomonas* species. *H. axialensis* and *H. hydrothermalis*, both of which were isolated from hydrothermal vents, were further investigated by the use of lipid profiles and protein expression patterns because preliminary experiments at a near-optimal temperature and salinity indicated a surprisingly robust growth response at abyssal hydrostatic pressures.

MATERIALS AND METHODS

Growth curves. Three deep-sea *Halomonas* strains that were isolated from hydrothermal vents, namely *H. axialensis* ATCC BAA-802^T, *H. meridiana* strain Slthf1 (ATCC BAA-801^T), and *H. hydrothermalis* ATCC BAA-800^T, and one *Halomonas* strain isolated from surface seawater on the Hawaiian coast, *H. pacifica* ATCC 27122 (3), were grown under a range of conditions of temperature (2, 13, and 30°C), hydrostatic pressure (0.1, 7.5, 15, 25, 35, 45, and 55 MPa), and salinity (4, 11, and 17% total salts). A growth medium (4% total salts, pH 8.0) described by Kaye and Baross (32) was augmented with NaCl to achieve higher salt concentrations. Cells were inoculated into fresh medium and kept at the relevant incubation temperature for 3 to 12.5 h (for 30°C experiments), 22.25 to 27.5 h (for 13°C experiments), or 2 to 5 days (for 2°C experiments) to allow cells to acclimate to the temperature and salinity conditions before pressurization. The inoculated medium was then used to completely fill 4.6-ml polyethylene transfer pipettes (Samco Scientific) (9) that were sealed by melting the tips in a Bunsen burner flame and crimping them with needle-nosed pliers. The pipettes were placed into stainless steel pressure vessels (Tem-Pres Division, LECO Corp.), topped off with distilled water at the appropriate temperature, manually pressurized with an Enerpac 11-400 pump outfitted with a quick-connect coupling device (84), using distilled water as the hydraulic fluid, and placed into an incubator. The hydrostatic pressure inside the vessels was checked after final equilibration to the incubation temperature and again before depressurization. The vessels remained within ±1 MPa of the target hydrostatic pressure at 7.5 and 15 MPa and within ±3 MPa of the target hydrostatic pressure at ≥25 MPa. The vessels were sequentially sacrificed at five time intervals and slowly depressurized (30 to 60 s), and a triplicate set of pipettes was harvested for each time point and each organism. Growth was assessed at 0.1 MPa by using the same general protocol, with pipettes that were submerged in distilled water.

Growth at 13 and 30°C was monitored by measuring the optical density at 600 nm with a Lambda UV/VIS spectrophotometer and that at 2°C was monitored by direct counts after filtering and staining of the cells with DAPI (4′,6-diamidino-2-phenylindole) (56). The growth rate was calculated by taking the slope of the log of the exponential portion of the growth curve. If the exponential portion of the growth curve could not be clearly ascertained, a region of maximal slope on a log plot from the beginning of the growth curve was used. For growth curves measured by optical density, growth rates were converted into increases in cell numbers over time by calculating the ratios of growth rates determined simultaneously by cell counts and optical turbidities at 20°C, 4% total salts, and 0.1 MPa. The 95% confidence intervals of the slope of the regression through the data points were also calculated.

PLFA analysis. *H. axialensis* and *H. hydrothermalis* were grown at 30°C and 0.1 or 45 MPa in 23-ml transfer pipettes (Samco Scientific) in medium with either 4 or 11% total salts. The cultures were grown in pressure vessels as described above and were harvested during early- to mid-exponential-phase growth. The cells were centrifuged at 10,000 × *g* at 26 to 29°C immediately after decompression; the pellets were resuspended in 50 mM Tris buffer (pH 8.0) and frozen at −69°C. A subsample of each pellet was sent to Microbial Insights Inc. (Rockford, Tenn.) for standard phospholipid fatty acid (PLFA) analysis by a modified Bligh and Dyer method (79). Lipids were extracted in a one-phase chloroform-methanol buffer solution, recovered and dissolved in chloroform, and separated into neutral, glyco-, and polar lipid pools. Polar lipids were additionally transesterified by exposure to mild alkali. PLFA were identified and quantified by gas chromatography-mass spectrometry (limit of detection, 7 pmol).

Protein analysis. Subsamples of the same cell pellets used for PLFA analysis were delivered to Kendrick Laboratories Inc. (Madison, Wis.) for fractionation into cytosolic and membrane-bound proteins and subsequent one-dimensional gel electrophoresis. The samples were lysed with an osmotic lysis buffer containing nuclease and protease inhibitors and then centrifuged for 30 min, after which the supernatants were removed and the entire procedure was repeated with the addition of 100 μl of fresh lysis buffer and 100 μl of deionized water. A volume of 100 μl of sodium dodecyl sulfate (SDS) boiling buffer without β-mercaptoethanol was added to the remaining pellet, which was mixed by vortexing and held in a boiling water bath for 5 min. The protein concentration for each fraction was then determined with a bicinchoninic acid assay (68). The samples were then lyophilized, resuspended in a buffer solution comprised of 5.0% SDS, 10% glycerol, 5% β-mercaptoethanol, and 63 mM Tris (pH 6.8) to achieve a final concentration of 5 mg of protein ml^{-1}, and placed in a boiling water bath for 5 min. SDS slab gel electrophoresis (34, 50) was run for each protein fraction in triplicate by use of a 10% acrylamide slab gel (125 mm long by 150 mm wide by 0.75 mm thick) overlaid with a 25-mm-long stacking gel at 15 mA for 3.5 h or until the bromophenol blue front migrated to the end of the slab gel. Gels were stained with Coomassie blue, destained in 10% acetic acid until the background clarified, and dried between cellophane sheets. Lastly, the gels were digitized with a laser densitometer (Molecular Dynamics), and the stain density of individual protein bands was quantified as a fraction of the total stain density per lane by the use of Nonlinear Dynamics 1D Advanced software (version 5.0). Triplicate runs were used to ensure the consistency of protein band patterns and to calculate standard deviations of band intensities. The images provided here were obtained by rerunning gels with a single lane devoted to each sample and each protein fraction.

RESULTS

Growth curves. In previous studies, *H. axialensis*, *H. meridiana* strain Slthf1, *H. hydrothermalis*, and *H. pacifica* exhibited psychrotolerant and euryhaline growth at 0.1 MPa (Table 1). The three strains isolated from hydrothermal vents were able to grow at temperatures as low as −1 to 2°C and grew signif-

TABLE 1. Source of *Halomonas* strains used in hydrostatic pressure experiments and their basic growth features at sea surface pressure

Strain (reference)	Sample characteristics			Growth temperature (°C) at 0.1 MPa and 4% total salts			Growth salinity (% total salts) at 0.1 MPa and 30°C		
	Sample type	Depth (m)	Temp (°C)	T_{min}	T_{opt}	T_{max}	S_{min}	S_{opt}	S_{max}
H. axialensis (32, 33)	Low-temperature hydrothermal fluid[a]	1,533	27	−1	30	35	0.5	4	24
H. meridiana strain Slthf1 (32,33)	Low-temperature hydrothermal fluid[a]	2,580	9	−1	20–35	40	0.5	2–7	22
H. hydrothermalis (32,33)	Low-temperature hydrothermal fluid[a]	2,580	9	2	30	40	0.5	4–7	22
H. pacifica (3, 38)	Tropical seawater	0	ND[b]	2–4[c]	ND[b]	45	0	0.5–3	20

[a] The salt content of hydrothermal fluids (measured as chlorinity) was ~90% that of ambient seawater levels (33).
[b] ND, not determined.
[c] Growth at 2°C may depend on the medium. The T_{min} is reported as 2 or 4°C (32, 38).

icantly better at 2°C than *H. pacifica*. The previously reported T_{min} at 0.1 MPa for *H. pacifica* was 4°C, although slight growth was found at 2°C with the growth medium employed in this study and a previous study (32).

Temperature-hydrostatic pressure-salinity growth curves (Fig. 1 to 3) showed that temperature had a dominant effect on the growth rate, whereby the fastest growth (0.17 to 0.74 h^{-1}) occurred at 30°C for each *Halomonas* strain, regardless of the tested combination of hydrostatic pressure and salinity. (Note that the color coding on the growth plots was reset for each bacterium such that, for example, red indicates the fastest growth for each organism even though the corresponding growth rate value is different for each strain.) In addition, increased hydrostatic pressures generally resulted in decreased growth rates for each organism at any given salinity and temperature combination, indicating piezotolerant growth for each

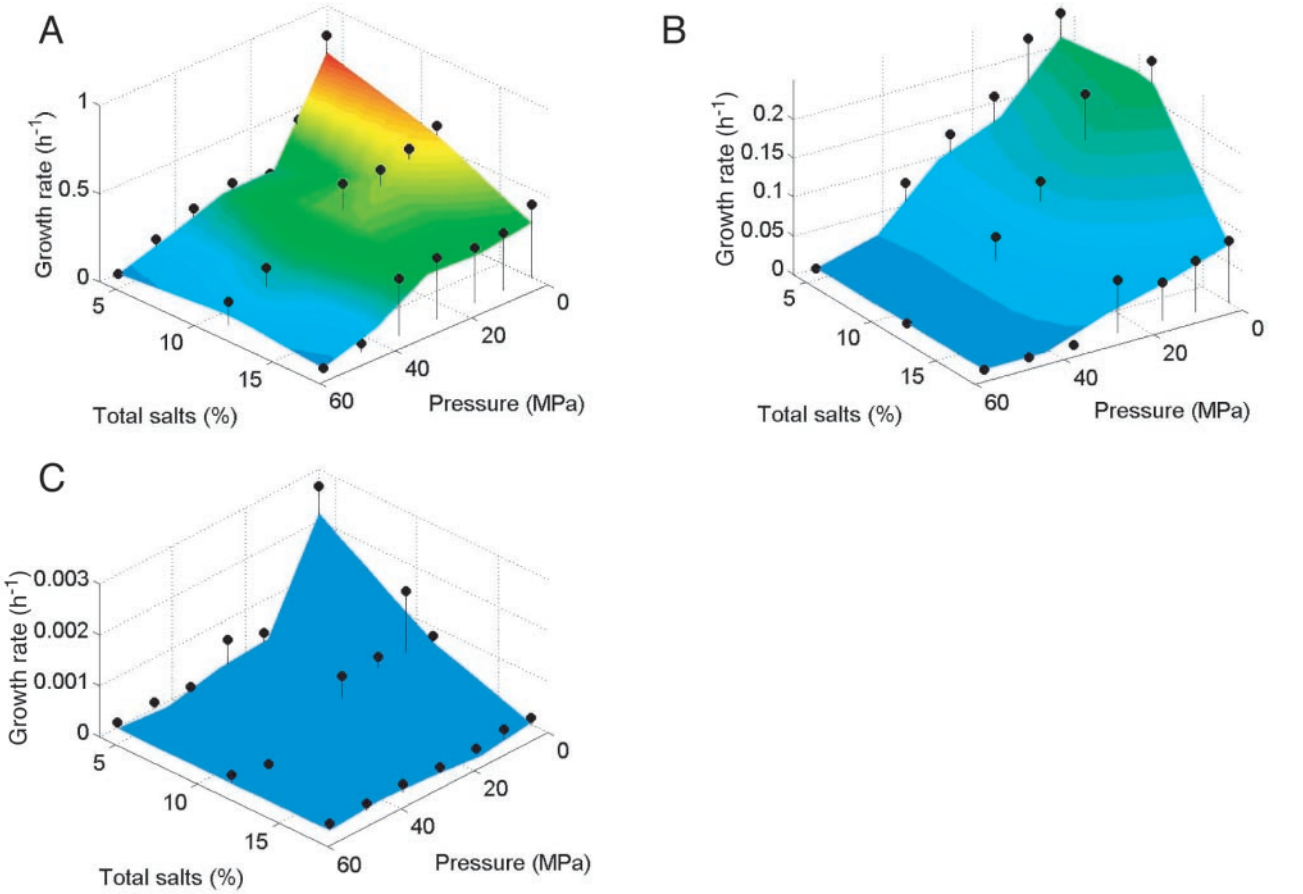

FIG. 1. Growth of *H. axialensis* at 30°C (A), 13°C (B), and 2°C (C), with 4, 11, and 17% total salts, and under 0.1, 7.5, 15, 25, 35, 45, and 55 MPa of hydrostatic pressure. Warm colors (red) indicate faster growth. The 95% confidence intervals are approximated by the distance from the contoured plot surface to the black dots. Note the differences in the *z*-axis scale among Fig. 1 to 3.

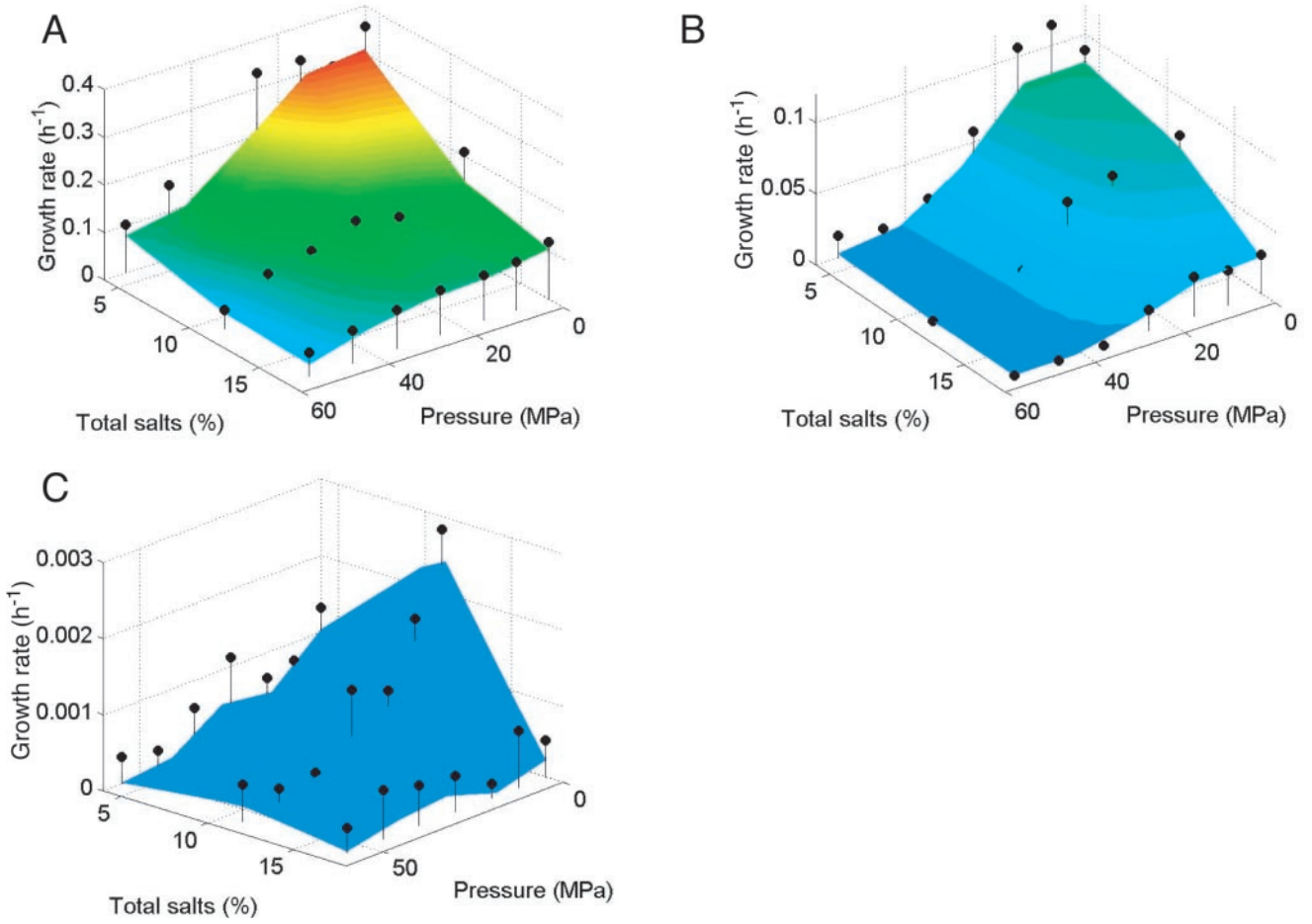

FIG. 2. Growth of *H. meridiana* strain Slthf1 at 30°C (A), 13°C (B), and 2°C (C). Other features are as described for Fig. 1.

strain. Growth was very slow or did not occur above 25 MPa at 2°C at all salinities tested. Similarly, decreased temperatures generally resulted in decreased maximum hydrostatic pressures for growth. For example, at 30°C and 4% total salts, the highest hydrostatic pressures that permitted growth for *H. axialensis* (Fig. 1A) and *H. meridiana* strain Slthf1 (Fig. 2A) were 45 and >55 MPa, respectively, but at 2°C these values dropped to 25 and 45 MPa, respectively (Fig. 1C and 2C).

The response to salinity in combination with different temperatures and hydrostatic pressures provided some unexpected growth patterns. At 0.1 MPa, *H. axialensis* grew fastest with 4% total salts versus higher salinities at both 30°C (Fig. 1A) and 2°C (Fig. 1C), but it grew equally well with 4 and 11% total salts at 13°C (Fig. 1B). Growth was favored in 11% total salts at 30°C at the higher hydrostatic pressures of 7.5 to 55 MPa (Fig. 1A). Salt-enhanced growth was not seen at 13°C (Fig. 1B) or 2°C (Fig. 1C). At 13°C, the growth rates were roughly equal at 4 and 11% total salts between 7.5 and 55 MPa (Fig. 1B), whereas at 2°C the growth rates declined with increasing hydrostatic pressures and/or salinities (Fig. 1C).

H. meridiana strain Slthf1 showed a consistently piezotolerant and psychrotolerant response at 30°C (Fig. 2A) and 13°C (Fig. 2B), with the fastest growth always occurring with 4% total salts. In contrast, its growth at 2°C was markedly enhanced with 11% total salts over the hydrostatic pressure range that yielded growth (0.1 to >35 MPa) (Fig. 2C). Similarly, culturing with 11% total salts increased the growth rate of *H. hydrothermalis* at 2°C over the hydrostatic pressure range of 0.1 to >35 MPa (Fig. 3A). The growth curves of *H. hydrothermalis* at 13 and 30°C had large 95% confidence intervals, masking any growth trends (data not shown).

H. pacifica also showed salinity-enhanced growth, but only at 30°C and >45 MPa (Fig. 3B). Growth was faster with 11 and 17% total salts under these conditions than with 4% total salts. (The local peak in growth rate observed at 30°C, 25 MPa, and 17% total salts was associated with a large 95% confidence interval.) At 13°C, the growth rate decreased with both increasing salinities and hydrostatic pressures (Fig. 3C). No significant growth occurred at 2°C (data not shown).

Salt-enhanced growth with increased hydrostatic pressures and low temperatures did not appear to correlate with whether the organism was halophilic (*H. axialensis* and *H. pacifica*) or moderately halophilic (*H. meridiana* strain Slthf1 and *H. hydrothermalis*).

PLFA analysis. Under the conditions tested (30°C, 0.1 or 45 MPa, 4 or 11% total salts), the predominant phospholipids for both strains examined, *H. axialensis* and *H. hydrothermalis*, were 18:1ω7c, 16:0, and 16:1ω7c, comprising 94.2 to 96.3% of the total (Fig. 4; Table 2). *H. hydrothermalis* contained slightly more of the minor components 18:1ω9c and 18:1ω7t than

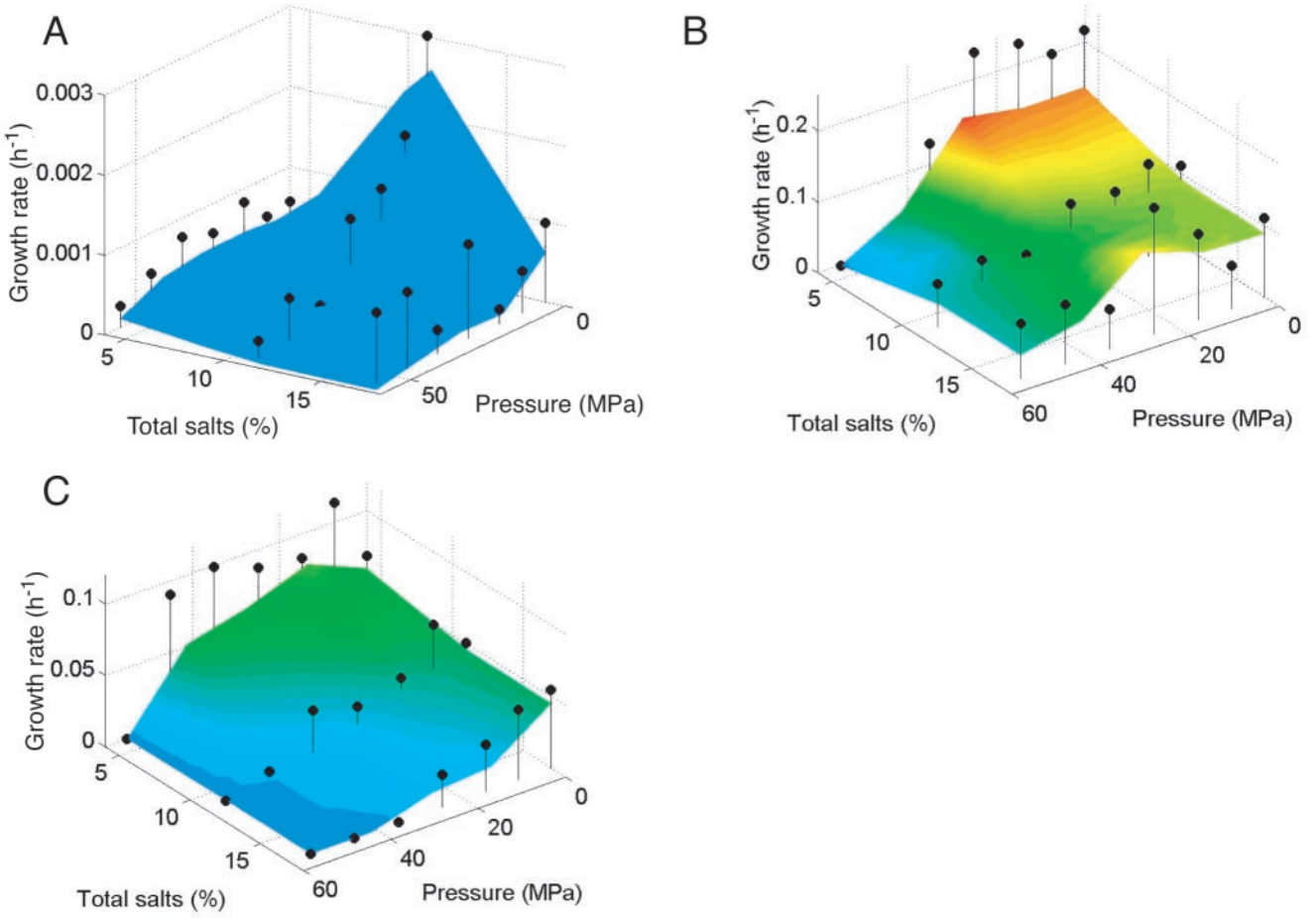

FIG. 3. Growth of *H. hydrothermalis* at 2°C (A) and growth of *H. pacifica* at 30°C (B) and 13°C (C). Other features are as described for Fig. 1.

H. axialensis, and these lipids decreased in concentration with increased salinity in *H. hydrothermalis*. *H. axialensis* contained slightly more cy19:0 (0.6 to 1.0%) than *H. hydrothermalis* (0.0 to 0.2%). At 0.1 MPa, the degree of lipid saturation in *H. hydrothermalis* increased with increased salinity, from 20.0 to 23.4%. The monounsaturated fatty acids (MUFA) 18:1ω7c and 16:1ω7c comprised 71.4 to 76.7% and 84.5 to 85.7% of the total at 0.1 and 45 MPa, respectively, while the saturated fatty acid 16:0 decreased in concentration, from 17.5 to 24.1% to 10.3 to 11.8%, simultaneously as the hydrostatic pressure and the proportion of MUFA increased. The medium used to grow the cells contained negligible quantities of lipids (data not shown).

Protein patterns. The cytosolic and membrane protein fractions of the same samples of *H. axialensis* and *H. hydrothermalis* as those analyzed for lipids contained 1.2 to 6.7 and 0.94 to 14.5 mg of protein ml^{-1}, respectively. Forty micrograms of protein was run in each gel lane in triplicate, with computerized band matching (data not shown), and the results are summarized in Fig. 5.

When *H. axialensis* was grown at 30°C with 4% total salts at both 0.1 and 45 MPa, the overall protein patterns were similar within the respective cytosolic and membrane protein fractions (Fig. 5), although a suite of proteins was slightly induced or inhibited by hydrostatic pressure (Table 3). Two proteins were significantly induced by hydrostatic pressure and are considered hydrostatic-pressure-induced proteins (PIP; cytosolic band *f* and membrane band *c* [Table 3]).

Overall, the protein profiles within each protein fraction for *H. hydrothermalis* at 30°C were also similar to each other (Fig. 5) whether the strain was grown at approximate seawater salinity (4% total salts) or an elevated salinity (11% total salts) and at a sea surface (0.1 MPa) or deep-sea (45 MPa) hydrostatic pressure. However, comparisons of individual bands did reveal a variety of interactions between salinity and hydrostatic pressure (Table 4). Some proteins were induced only by elevated salinity, including strongly enhanced salt-induced proteins (SIP) (membrane bands *g* and *k*), and some proteins were PIP (cytosolic bands *l*, *m*, *n*, *o*, *p*, *v*, and *w*). However, in several instances hydrostatic pressure and salinity had opposing effects—mostly on membrane protein production (cytosolic band *q* and membrane bands *j*, *l*, *m*, *n*, and *o*)—resulting in no apparent net effect on protein expression with the combination of hydrostatic pressure and salinity. Similarly, the induction of certain cytosolic proteins by hydrostatic pressure was mitigated by an elevated salinity (cytosolic bands *l*, *m*, *n*, *o*, *p*, *v*, and *w*). The expression of other cytosolic proteins was inhibited only with a combination of elevated salinity and hydrostatic pressure (cytosolic bands *r*, *s*, and *t*). The production of one mem-

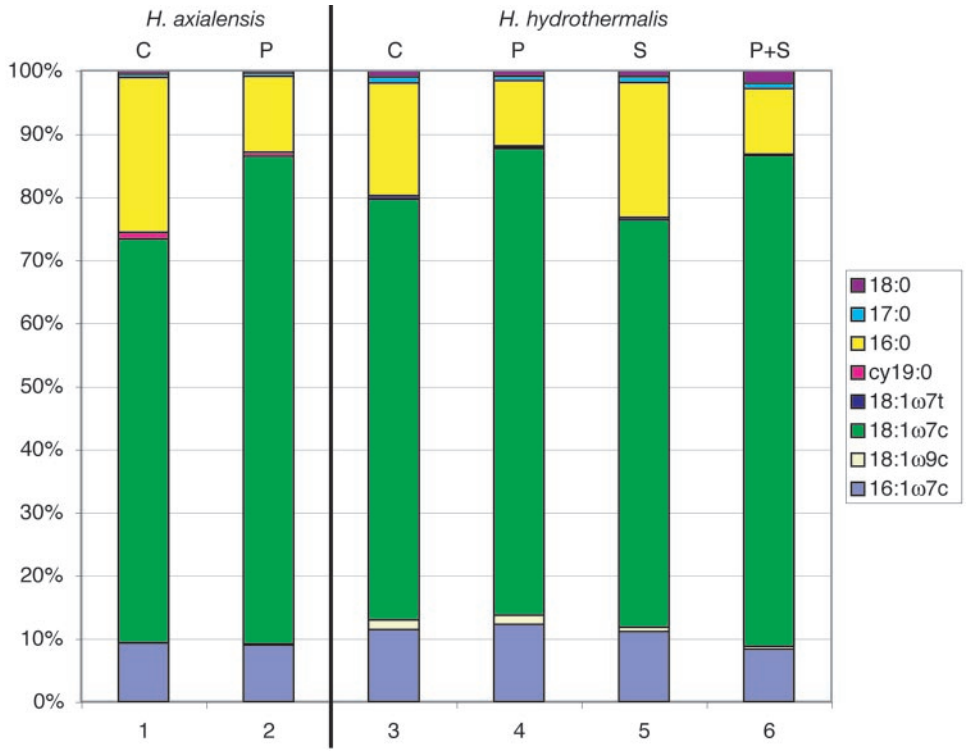

FIG. 4. Phospholipid fatty acid profiles of *H. axialensis* (columns 1 and 2) and *H. hydrothermalis* (columns 3 to 6) grown at 30°C under different conditions of hydrostatic pressure and salinity. C, growth under control conditions (4% total salts and 0.1 MPa); P, growth with elevated hydrostatic pressure (45 MPa and 4% total salts); S, growth with elevated salinity (11% total salts and 0.1 MPa); P+S, growth with both elevated hydrostatic pressure and salinity (45 MPa and 11% total salts). Only lipids that comprised ≥0.5% of the total are shown.

brane protein was significantly inhibited by hydrostatic pressure but was enhanced with an increased salt concentration; when the two stresses were jointly imposed, the inhibition by hydrostatic pressure was retained as the dominant control (membrane band *i*). Intriguingly, some proteins that were induced by either elevated salinity or hydrostatic pressure were not induced by the combination of the two (cytosolic band *u* and membrane band *h*).

DISCUSSION

The *Halomonas* strains tested were psychrotolerant and piezotolerant, as they were able to grow only slowly or undetectably under cold deep-sea conditions (2°C and >15 MPa) with the medium employed. The growth rates for all of the *Halomonas* strains were higher under the cool to warm conditions that characterize deep-sea low-temperature hydrothermal fluids and associated subseafloor environments (13 to 30°C, >15 MPa) than at 2°C. *Halomonas* spp. are therefore candidates as subseafloor heterotrophs, and their growth may be restricted to these habitats in the deep sea. (Cool deep basins, such as the Mediterranean Sea, which is 10 to 15°C at its depth, may also be conducive to the growth of *Halomonas* spp.) Indeed, molecular phylogenetic analyses of low-temperature hydrothermal fluids and nearby deep seawater at Axial Seamount on the Juan de Fuca Ridge in the northeast Pacific Ocean indicated that *H. axialensis* is a subseafloor resident atop this volcano (J. Z. Kaye and J. A. Baross, unpublished data). These growth rate data additionally suggest that certain *Halomonas* spp. will grow or thrive in cold, cool, and warm hypersaline deep-sea environments, such as the brine-seawater interface found above brine pools located in the Gulf of Mexico (65, 66), the eastern Mediterranean Sea (5, 6, 11, 12, 19, 30, 39, 64), and the Red Sea (10, 24). In fact, *H. aquamarina* was one of only three taxa cultured from the brine-seawater interface above the Urania Basin at an ~3,500-m depth in the eastern Mediterranean Sea (62). The brine pools themselves are anoxic, however, and anaerobic growth at elevated salinities and hydrostatic pressures was not tested in this study.

An elevated salinity appeared to compensate partially for the depression of the growth rate caused by low temperatures. *Halomonas* isolates from hydrothermal vents showed increasingly halophilic responses at low temperatures and >7.5 MPa. It is possible that an enhancement of growth at low temperatures by increased salinity would be seen in *H. pacifica* if the cells were tested at a slightly warmer temperature of a few degrees above its T_{min} of 2 to 4°C (32, 38). Low temperature is analogous to elevated hydrostatic pressure in certain effects on proteins and membranes (2), and indeed the same salt compensation effect was seen at 30°C, but only at >7.5 MPa for *H. axialensis* and >45 MPa for *H. pacifica*.

Mechanistically, hydrostatic and osmotic pressures exert opposing influences on protein hydration and thus tend to cancel the deleterious effects of each other (37, 80). Hydrostatic pressure favors hydrated protein surfaces due to volume considerations, but osmotic pressure due to solutes like trimethylamine *N*-oxide, glutamate, and betaine favors folded proteins via

TABLE 2. PLFA profiles of *H. axialensis* and *H. hydrothermalis* grown at 30°C under different salinity and hydrostatic pressure conditions

PLFA category and identity[a]	Total PLFA (%) at indicated pressure[b]					
	H. axialensis		*H. hydrothermalis*			
	4% total salts		4% total salts		11% total salts	
	0.1 MPa (1)	45 MPa (2)	0.1 MPa (3)	45 MPa (4)	0.1 MPa (5)	45 MPa (6)
Terminally branched saturates						
i15:0	0.0	0.4	0.1	0.0	0.0	0.0
i17:0	0.1	0.0	0.1	0.0	0.0	0.0
a17:0	0.2	0.3	0.3	0.3	0.3	0.4
Monoenoics						
16:1ω9c	0.0	0.1	0.0	0.0	0.0	0.0
16:1ω7c	9.1	8.8	11.2	12.2	10.9	8.2
16:1ω7t	0.1	0.1	0.1	0.0	0.1	0.0
16:1ω5c	0.1	0.2	0.1	0.0	0.1	0.1
17:1ω6c	0.1	0.2	0.0	0.0	0.0	0.0
cy17:0	0.4	0.2	0.2	0.2	0.2	0.4
18:1ω9c	0.1	0.2	1.5	1.4	0.7	0.4
18:1ω7c	62.8	75.7	65.5	73.5	63.5	76.7
18:1ω7t	0.0	0.0	0.5	0.3	0.4	0.0
18:1ω5c	0.1	0.2	0.1	0.0	0.2	0.4
cy19:0	1.0	0.6	0.0	0.2	0.0	0.2
Normal saturates						
14:0	0.4	0.2	0.3	0.0	0.3	0.0
15:0	0.2	0.2	0.2	0.0	0.2	0.0
16:0	24.1	11.8	17.5	10.3	21.1	10.3
17:0	0.6	0.5	1.0	0.7	0.9	0.9
18:0	0.5	0.4	1.0	0.9	1.0	2.0
20:1ω7c	0.1	0.1	0.0	0.0	0.0	0.2
Polyenoic						
18:2ω6	0.0	0.0	0.1	0.0	0.1	0.0
Totals						
Terminally branched saturates	0.4	0.8	0.6	0.3	0.3	0.4
Monoenoics	73.8	86.2	79.4	87.8	76.2	86.4
Normal saturates	25.8	13.1	20.0	11.9	23.4	13.2
Polyenoic	0.0	0.0	0.1	0.0	0.1	0.0

[a] The lipid a15:0 was not detected. No branched monoenoic or mid-chain branched lipids were detected.
[b] Corresponding columns in Fig. 4 are noted in parentheses after the pressures.

preferential exclusion and the minimization of bound water (37, 61, 80). It is important to bear in mind that within *Halomonas* cells the concentration of compatible solutes, not salt, increases in response to osmotic stress induced by salt ions or organic solutes (20, 76).

Antagonistic interactions between hydrostatic pressure and increased solute concentrations have been observed in a variety of disparate experiments. An elevated salinity was previously shown to increase the maximum hydrostatic pressure for growth in the psychrophiles *Moritella marina* (54, 73) and *Streptococcus faecalis* (reclassified as *Enterococcus faecalis* [63]) (36) and to decrease the piezosensitivity of *Escherichia coli* to an extremely high hydrostatic pressure of 270 MPa (25). Likewise, 15% NaCl (compared with 3% NaCl) dramatically enhanced the survival of a halophilic euryhaline marine isolate of *Micrococcus roseus* (reclassified as *Kocuria rosea* [69]) under a very high hydrostatic pressure of 138 MPa (72). Osmotic pressure (created with low concentrations of ethanol) also mitigated the hydrostatic-pressure-induced inhibition of cell division in *E. coli*, enabling a reversion of cell morphology from an elongated form to the canonical rod shape (71). Additional in vitro studies showed similar counterbalances between hydrostatic and osmotic pressures. The dissociation of the Arc repressor protein from DNA, its substrate, decreased linearly with increased glycerol concentrations (53). Hydrostatic pressure was also seen to reverse the effects of osmotic pressure (created with organic compounds) on DNA site selection by the restriction endonucleases EcoRI, BamHI, PvuII, and EcoRV, highlighting the opposing interplay that these two stresses have on the hydration of proteins and protein-substrate complexes (59, 60). A salt-induced enhancement of growth with elevated hydrostatic pressures (at cold and warm temperatures) is consistent with the opposing influences of salinity and hydrostatic pressure observed in a variety of systems.

In this study, hydrostatic pressure exerted the dominant control on phospholipid profiles. Consistent with all previous hydrostatic pressure studies, the degree of lipid saturation decreased with increased hydrostatic pressure (2, 82), presumably as a means to maintain membrane fluidity. Consistent with previous studies of *Halomonas* strains and other moderately halophilic and euryhaline species (41, 42, 44, 51, 74, 77), the proportion of MUFA decreased in concentration (and the proportion of saturated fatty acids concomitantly increased in concentration) with increased salinity, as was observed with *H. hydrothermalis* at 0.1 MPa and 4 or 11% total salts. An elevated salinity slightly decreased the degree to which hydrostatic pressure caused an increase in membrane unsaturation. Cyclopropane fatty acids (CFA) were in very low abundance (0.0 to 1.0%), and changes in their proportions were not apparent, as previously documented with *Halomonas* and other moderately halophilic and euryhaline bacteria (27, 41, 42, 45, 51, 74). While changes in the proportion of CFA with salinity are not always observed among *Halomonas* spp. (46), the CFA cy17:0 and cy19:0 typically comprise a much larger proportion of

TABLE 3. Impact of elevated hydrostatic pressure (45 versus 0.1 MPa) at 30°C on proteins of *H. axialensis*

Protein fraction	Protein		Effect of pressure[a]	Comment[b]
	Band label	Approximate mass (kDa)		
Cytosolic (see Fig. 5A)	*a*	100	−−	P repression
	b	90	−−	P repression
	c	60	+	P enhancement
	d	50	+	P enhancement
	e	43	+	P enhancement
	f	42	+++	PIP
	g	41	−−	P repression
	h	38	−	P repression
	i	36	−	P repression
	j	20	+	P enhancement
	k	15	−	P repression
Membrane (see Fig. 5B)	*a*	160	++	P enhancement
	b	150	−−	P repression
	c	60	+++	PIP
	d	33	+	P enhancement
	e	26	−	P repression
	f	25	+	P enhancement

[a] The symbols indicate the relative degrees of change in the intensities of protein bands.
[b] P, pressure.

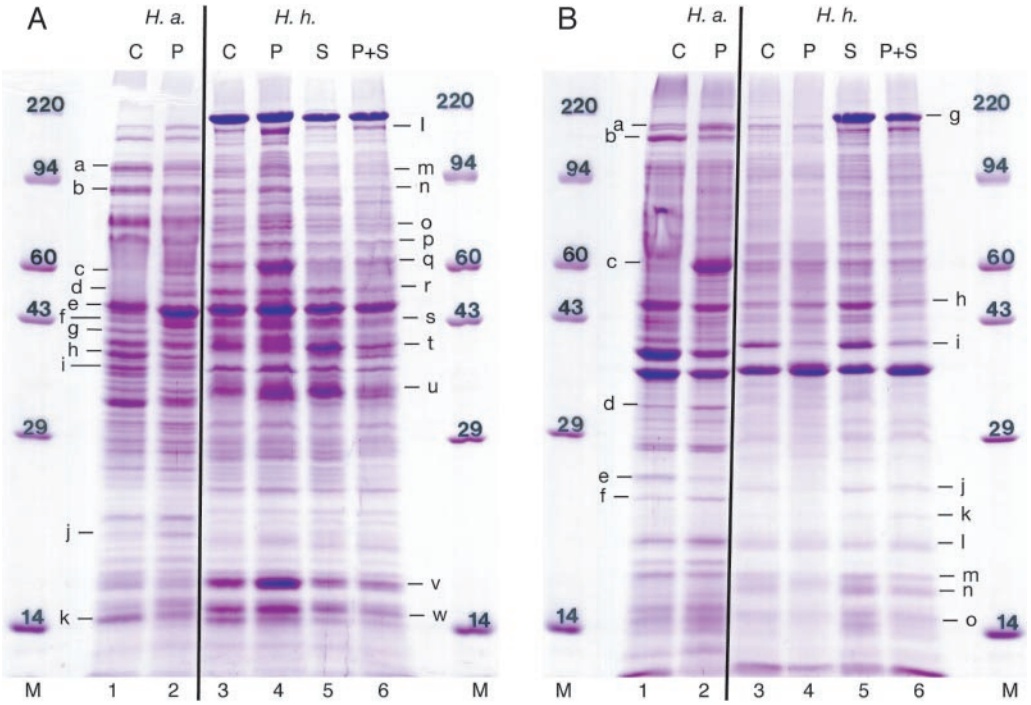

FIG. 5. Cytosolic (A) and membrane (B) protein patterns of *H. axialensis* (*H.a.*; lanes 1 and 2) and *H. hydrothermalis* (*H.h.*; lanes 3 to 6) obtained at 30°C. Lanes M, molecular weight markers; other lanes are numbered identically to the columns in Fig. 4. Protein bands with changes in intensity are noted with letters.

the phospholipids present (up to 37%) (18, 40, 67). It should be noted as well that lipid profiles vary significantly with the growth phase and between members of the family *Halomonadaceae* (4, 43). In this study, cells were grown to early to mid-exponential phase, whereas for the other lipid analyses cited, cells were usually harvested during the late exponential or stationary phase. This difference may explain the very low concentration of CFA observed in this experiment.

TABLE 4. Impact of elevated hydrostatic pressure (45 versus 0.1 MPa) and salinity (11 versus 4% total salts) at 30°C on proteins of *H. hydrothermalis*

Protein fraction	Protein		Effect of stressor[a]			Comment
	Band label	Approximate mass (kDa)	P	S	P and S	
Cytosolic (see Fig. 5A)	*l*	190	+			S cancels P enhancement
	m	100	+			S cancels P enhancement
	n	90	+			S cancels P enhancement
	o	70	+			S cancels P enhancement
	p	65	+			S cancels P enhancement
	q	60	+	−		S and P cancel each other
	r	50			−	Repression by combination of S and P
	s	43			−	Repression by combination of S and P
	t	40			−	Repression by combination of S and P
	u	35	+	+		S and P combined cancel protein induction by either stress alone
	v	16	+			S cancels P enhancement
	w	15	+			S cancels P enhancement
Membrane (see Fig. 5B)	*g*	200		+++	+++	SIP only[b]
	h	47	+	++		S and P combined cancel protein induction by either stress alone
	i	40	−−	+	−−	P repression dominates over S enhancement
	j	25	−	+		S and P cancel each other
	k	23		+	+	SIP only[b]
	l	21	−	+		S and P cancel each other
	m	19	−	+		S and P cancel each other
	n	18	−	+		S and P cancel each other
	o	15	−	+		S and P cancel each other

[a] The symbols indicate the relative degrees of change in the intensities of protein bands; a lack of a symbol indicates no significant change. P, pressure; S, salinity.
[b] SIP, salt-induced protein.

The cytosolic and membrane protein patterns determined at 30°C showed an overarching consistency at low and high hydrostatic pressures and salinities, indicating that cellular functions are generally consistent between warm, shallow and deep, marine, and hypersaline habitats. On a finer scale, however, the protein patterns revealed a variety of hydrostatic pressure and hydrostatic pressure-salinity effects. *H. axialensis* was grown at 0.1 and 45 MPa, and several proteins were stimulated (including two PIP) or repressed by hydrostatic pressure. *H. hydrothermalis* was grown under four hydrostatic pressure-salinity regimens, and while some proteins appeared to be salt or hydrostatic pressure specific and likely performed osmoregulatory or hydrostatic pressure adaptation functions, frequently the combination of elevated salinity and hydrostatic pressure illustrated that the two stresses mitigate the effects of the other. Salinity and hydrostatic pressure were thus competitive, not synergistic, for these proteins. These proteins may be involved in the adaptation of phospholipid membranes given that salinity and hydrostatic pressure exerted opposing influences on lipid saturation. A link between a salt- or hydrostatic-pressure-induced general stress response and/or salt- or hydrostatic-pressure-adapted growth is conceivable given the behavior of the interaction of these stresses. The augmentation of the production of compatible solutes in *Photobacterium profundum* strain SS9 by the combination of increased salinity and hydrostatic pressure (37) highlights one aspect of this connection. If there is a link, it is possible that the euryhaline phenotype of deep-sea *Halomonas* spp. and other bacteria may partially reflect an adaptation for growth in pressurized, in addition to hypersaline, marine environments. The protein patterns also specifically indicate that deep-sea brine-seawater interface environments may not induce the expression of certain genes during growth. Both the protein expression and growth rate data thus suggest that deep-sea hypersaline regions, in addition to cool and warm hydrothermal-vent environments, are amenable to the growth and proliferation of members of the genus *Halomonas*.

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