Ecology, phylogeny and physiological adaptations of euryhaline and moderately halophilic bacteria from deep-sea and hydrothermal-vent environments

Jonathan Zachary Kaye

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

Doctor of Philosophy

University of Washington

2003

Program Authorized to Offer Degree: School of Oceanography

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to Proquest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature Jonathan Z Kaye

Date November 24, 2003

University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Jonathan Z. Kaye

and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the final examining committee have been made.

Chair of Supervisory Committee: John A. Baross

John A. Baross

Reading Committee: John A. Baross

John A. Baross

Jody W. Deming

Jody W. Deming

Deborah S. Kelley

Date: November 24, 2003

University of Washington

ABSTRACT

Ecology, phylogeny and physiological adaptations of euryhaline and moderately halophilic bacteria from deep-sea and hydrothermal-vent environments

Jonathan Zachary Kaye

Chair of the Supervisory Committee: Professor John A. Baross
School of Oceanography

The γ-proteobacterial genus *Halomonas* is among the most abundant groups of microorganisms in hydrothermal-vent and pelagic habitats. Characterization of six novel *Halomonas* strains, including four new species isolated from hydrothermal plumes, low-temperature seafloor vents and sulfide rock, revealed traits consistent with other members of the genus, such as versatile heterotrophy, strong resistance to Cd^{2+}, and growth with 0.5 to ≥22% total salts. The strains shared more phenotypic traits with each other than with other *Halomonas* spp., including cold-shifted cardinal growth temperatures. Selected *Halomonas* spp. were also strongly resistant to Co^{2+}, Cu^{2+} and Zn^{2+}. The strains tested precipitated 55–98% of Cd^{2+} from solution, possibly as CdS or $CdHPO_4$, but only during static (versus agitated) growth. Amplification of *Halomonas* and *Marinobacter* 16S rRNA genes from low-temperature hydrothermal-vent and deep-sea samples delineated a distinct biogeography for four clades within these genera and revealed that certain cultured representatives cluster with environmentally relevant groups. The distribution patterns revealed that *Halomonas* sub-group 2A comprised a subseafloor population at Axial Seamount on the Juan de Fuca Ridge and suggested that other clades may include members that are cold-adapted or associated with metal-sulfide deposits. Growth curves produced using a range of hydrostatic pressures, temperatures and salinities confirmed 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. Elevated salinity enhanced growth under certain high-hydrostatic-pressure and low-temperature conditions, highlighting a synergistic effect on growth for these combined stresses. Profiles of cytosolic and membrane proteins of *H. hydrothermalis* obtained at 30°C under high and low salinity and hydrostatic-pressure conditions indicated a variety of hydrostatic-pressure-salinity effects, including proteins whose expression was induced by either elevated salinity or hydrostatic pressure, but not by the combination of the two. The interplay between salinity and hydrostatic pressure on microbial growth and physiology suggests that a hydrostatic-pressure adaptation, not a salt adaptation, may explain the euryhaline phenotype of members of the genus *Halomonas* living in deep-sea environments. These versatile psychrotolerant bacteria may flourish in subseafloor habitats from which they could seed the overlying water column.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

Even a dissertation is a snap-shot event in life. I, along with everyone else, am cemented in the present tense; the past exists only as long as the duration of memory processing in the mind — in staccato millisecond bursts. Have I *really* been in graduate school for eight years?

Well, there are good reasons. I have accrued a rewarding set of experiences during my time here, for which there are many people to thank, in many ways. Jim, Byron and Melanie, my first lab mates, not only taught me how to maneuver in the laboratory and at sea but also were a constant source of scientific engagement and, ultimately, friendship. The same can be said of numerous other Oceanography folks, past and current, especially Yves-Alain, Jill, Giora, Andy and Julie. I want to acknowledge Shelly for continuous aid in the laboratory, both emergency and routine, and, most importantly, for her contagious laugh. I am very appreciative of Susanne's programming help with Matlab as well. A tremendous thank-you is also extended to the Baross family who joined the group after I did: Sheryl, Julie, Matt, Mausmi, Billy, Robin and Joe. I thoroughly enjoyed our time together and am cheered by the maracas and hebephrenia at lab meetings. Joe and Sheryl: I owe you untold quantities of beer and coffee for the tower of data you generated. For friends outside of school, it is truly impossible to convey how happy you have made my time in Seattle — I am choked.

I would also like thank chief scientists John, Deb, Marv and Bob for leading expeditions to remote seafloor vents in the North and South Pacific Oceans. Time spent in the field, both at sea and exploring the ophiolites in Oman and Cyprus, placed oceanography into a tangible context and has been an unbeatable learning experience. These adventures have catapulted my appreciation and understanding of the Earth and its smallest critters.

In many ways, I feel as if I have had a second bar mitzvah (though this time I got drunk at the party afterwards). In addition to the language of the dissertation seeming as peculiar to some as ancient Hebrew, this manuscript represents a transfer of scientific passion and modes of thought, from generation to generation. I would thus like to thank my family for continuous heartfelt support and encouragement: the scientific influences are very clear; my earliest memories include science fair projects, creating ecosystems for pet lizards, and trips to the La Brea tar pits. Now I can see how my family's enthusiasm for science and art (and their connectivity) has molded me. My committee members, Jody, Dave, Deb and Woody — part of my scientific family — have also been a scientific inspiration and a continuous source of learning about hydrothermal-vent and deep-sea science. I am very grateful for the constructive criticism they have provided over the years; this manuscript has been vastly improved by their comments as well.

Lastly, I sincerely want to thank my Northern-Italian-Catholic-Native-American-Czechoslovakian rabbi advisor, John. He has taught me the most about how to do science and has been unendingly generous with his ideas, time and support. His creativity, optimism, excitement and depth of knowledge are inspirational. John has been a great friend too, invested and engaged, and, fortunately for me, he is just as much of an oddball.

Thank you all for sharing this moment.

INTRODUCTION

Wherefore "stress"?

A microorganism endures stress when it encounters environmental conditions which are near the periphery or outside of its genetically programmed *n*-dimensional region of growth. The boundaries of this complex space that delineates when, and where, a cell can persist or grow are measured on scales of temperature, water activity, hydrostatic pressure, pH, radiation, reduction potential, and concentrations of nutrients, heavy metals, antimicrobial compounds and oxygen. There exists a geometric point boxed inside of this multifaceted zone that we define as "optimum," or fastest, growth. However, the probability that a microorganism experiences the conditions that permit it to divide at its maximum empirical growth rate is low. For example, no known microorganism experiences its maximum growth rate under the temperature and hydrostatic-pressure conditions of the cold (<5°C) deep sea (658), thus excluding most of the marine environment as a location in which a maximum growth rate can be achieved. In the remainder of the marine ecosystem, maximum growth rates may occur sporadically during colonization of particles, when pathogens initially invade animal tissues (despite an uncertain nutrient supply and constant attack by host defense systems), and during unusual warming of coastal surface waters, for example. Additional exceptions may arise, especially given that most marine microorganisms have yet to be cultured in the laboratory. But the current data strongly suggest that the majority of microorganisms rarely divides at its fastest, or so-called "optimum," laboratory-determined growth rate in the environment.

The alternative, and arguably more informative approach to understanding microbial behavior *in situ*, is to conceptualize microorganisms in marine environments as existing in a sub-optimal, if not stressed, state. The conditions found in coastal oceans with relatively high concentrations of nutrients (despite the potential for ultraviolet irradiation at the very surface) are relatively benign — with different thermal (and light-adapted) classes of microorganisms preferring high-latitude, temperate or tropical waters. In the open ocean, however, nutrients (NO_3^-, PO_4^{3-}, trace metals and organic carbon) are typically limiting, and those which are regenerated in surface waters are rapidly consumed (28, 296, 392, 402, 403). With depth below the mixed layer, organic nutrients become increasingly depleted; heterotrophic microorganisms mine sinking particles and fecal pellets of the most nutritious organic-carbon compounds first (27, 403, 553). Hydrostatic pressure increases linearly and temperature decreases asymptotically to less than –1 to 3°C with depth in most ocean basins, and organic nutrients are typically in low concentration (0.2–2.0 mg C l^{-1}) and/or recalcitrant to consumption in abyssal regions (403). The chemosynthetic communities at hydrothermal vents along mid-ocean ridges create oases of organic carbon, but these environments also typically expose microorganisms to ionizing radiation, heavy metals, and extreme fluctuations in oxygen concentration and temperature. Subseafloor habitats associated with mid-ocean ridges also host microbial communities, though the full extent (including the stressful extremes) of the physicochemical gradients where life exists beneath the seafloor is unknown (36, 148, 570). There are also deep-sea brine environments — cold, cool, warm and extremely hot — associated with dissolution of buried evaporite deposits or freshly produced by hydrothermal and magmatic processes beneath the seafloor at mid-ocean ridges, but little is known about the microbial communities which may (or may not) inhabit them.

The laboratory-based notion of fastest growth as "optimal growth" retains utility in microbial ecology, primarily as a reference value or starting point for further scrutiny of microbial physiology and potential behavior in the environment. In many instances, linking "optimal growth" to the environment is in fact sensible: extreme halophiles thrive in briny habitats and psychrophiles populate polar regions (Table 0.1). Further, deviations from the fastest growth rate measured in the laboratory illustrate how certain factors, such as temperature, hydrostatic pressure, salinity and oxygen concentration, only act as stressors within the context of the physiology and evolutionary inheritance of the microorganism in question. In other words, some like it hot; 90°C is well within normal physiological parameters for a hyperthermophile but would completely destabilize the essential macromolecules of a mesophile. Other stressors, however, consistently inhibit or eliminate microbial growth. No known microbe is positively tactic towards harmful levels of radiation, antibiotics that harm it, a total lack of nutrients, or high concentrations of heavy metals (though an ability to cope with these stresses can convey significant competitive advantage).

But the sum of the traits that comprise fastest "optimal growth" can be misleading if simplistically applied to the environment in an attempt to pigeonhole a microorganism into a habitat. Microbial ecologists must be prudent with the broadly (and overly?) swept tenet that the results of physiology and growth-rate experiments executed in the laboratory can be used directly to characterize microbial behavior in the environment and, in some cases, the environment itself; examples permeate the literature. While this belief is based, in part, on the reasonable assumption that natural selection causes necessary genes to be retained in proper working order, we must be careful when divining the driving evolutionary force — lest we fall into the adaptationist trap and metaphorically (and incorrectly) ascribe function to Gould and Lewontin's majestic, but structurally irrelevant, spandrels tucked between the arches that support the dome of the *Basilica di San Marco* in Venice (224). The word "optimum" subversively implies evolutionary fitness.

The seeming simplicity of the deterministic nature of microorganisms is an added pitfall. Stimuli reproducibly induce distinct phenotypic responses in these microscopic sacs of tightly regulated enzymes — how much more complicated can it be? The trick is to determine which environmental stimuli are relevant and in combination with which other environmental factors. The full complement of the genome-encoded capabilities of a microorganism as probed in the laboratory dangerously — for ecologists — subsumes which traits are actually expressed *in situ*.

One might ask: Why are there mesophilic bacteria in cold deep-sea sediments? Why can a microorganism isolated 2000 km from a polluted or hydrothermal-vent site tolerate millimolar levels of cadmium? Why are bacteria from marine environments "optimized" to any salinity but that of seawater, and why can some marine species grow well on media with 20% total salts while others cannot? Why are many piezophilic bacteria "optimized" to a hydrostatic pressure more shallow than that of the depth of capture? Moreover, why are these microorganisms typically "optimized" to hydrostatic-pressure-temperature conditions not found in their immediate habitat (or typically anywhere in the deep Pacific, Atlantic, Indian, Arctic or Southern Oceans outside of hydrothermal-vent and subseafloor habitats)? Why are heavy-metal-resistant microorganisms also frequently found to be resistant to antibiotics?

We do have solid, ecologically grounded answers to some of these questions — for example, sea-surface mesophiles hitch a ride to the seafloor on sinking particles — and there is of course some fundamental phenotypic plasticity. But to solve other questions we must envision novel habitats or modify our understanding of what a given phenotype means in an evolutionary and ecophysiological context, or both.

As a first step, growth-rate data can be used to construct curves that standardize for a given environmental factor (417), an adaptation of an approach used by Svante Arrhenius to analyze rates of chemical reactions. The broad plateaus, rather than single peaks, characteristic of these graphs (of growth rate versus the inverse of a given physiological parameter on an absolute scale) reveal where, once the effect of temperature, for example, is quantitatively removed from the growth rate, a microorganism is dividing under presumably equal physiological conditions. The increase in growth rate that is merely due to an increase in temperature — and thus an increase in molecular vibrations and rates of chemical reactions — which gives traditional curves (of growth rate versus temperature, for example) a higher-temperature-leaning lopsidedness and peak, is erased. These Arrhenius graphs essentially account for "Q_{10}" values and highlight that a physical effect, not evolution, is driving the response to temperature *within* the range that permits growth; the range itself is, however, a consequence of natural selection. Arrhenius plots clarify why the fastest growth rate does not suggest adaptation to a singular, precise temperature in the environment and indicate that microorganisms grow at their *evolutionary optimum* over a range of temperatures, which in some cases may span 20–40°C. Thus, for example, we can understand why a psychrophile is evolutionarily adapted to permanently cold environments yet still grows faster at warmer temperatures that it may never experience. Arrhenius plots can theoretically also be constructed for growth over a range of salinities, hydrostatic pressures, or any other environmental parameter.

But microorganisms in natural systems must cope simultaneously with an array of environmental factors. At what point does an evolutionary optimum for growth cede to evolutionary sub-optimal growth and stress? A stress response likely ensues beyond the edges of the plateau of an Arrhenius plot, but it may also occur within the plateau given that a single Arrhenius graph only takes one of many growth factors into account. It is not possible to tease apart such complexities with a single curve, especially given that a growth rate represents the summation of an incredibly large number of reactions and processes.

And it gets even more complicated. While Arrhenius plots help clarify conceptions of microbial ecology by mitigating misleading inferences about microbial behavior in — and characteristics of — the environment, microbial physiology can be sufficiently cryptic to warrant attribution of growth-rate patterns (both traditional peaks and Arrhenius plateaus) collected with respect to one environmental parameter to an entirely different environmental factor. For example, the extreme radiation resistance of *Deinococcus radiodurans* evolved to repair extensive DNA damage suffered during desiccation, an adaptation that also enables cells to withstand high levels of ionizing radiation (41). In this case, growth-rate data plotted against water activity, not radiation levels, best illuminate the ecology of this bacterium.

This thesis explores these two occasionally inconsistent aspects of microbial ecology, here termed as "environmental" and "physiological" approaches. With the preceding example, the "environmental" hypothesis would suggest that *Deinococcus radiodurans* evolved highly efficient DNA-repair systems to cope with ionizing radiation present in its natural niche (say, nuclear waste — hypothetically). The "physiological" hypothesis would conjecture (correctly, in this case) that its potent DNA-repair mechanisms evolved for a purpose other than for the radiation resistance first observed in the laboratory: that is, desiccation resistance. In this thesis, the model system used to investigate the "environmental"-"physiological" dichotomy includes moderately halophilic and euryhaline bacteria (defined in Table 0.1) and focuses on the genus *Halomonas*. These mesophilic bacteria were very abundant (sometimes culturable in numbers >10% of the total microbial community) in hydrothermal-vent and pelagic environments (304). The genus *Halomonas* contains species that are remarkably resistant to salt and heavy metals, unusual phenotypic characteristics that make understanding their distribution, diversity, stress response, survival and success in marine habitats so compelling. What exactly do euryhaline growth and strong metal resistance mean for the ecology of *Halomonas*? Are they reflections of the environment or some alternative cellular physiology, or both?

The remainder of the introduction is divided between descriptions of the geology, chemistry and microbiology of hypersaline habitats (known and hypothesized) on the one hand and, on the other, an exploration of the known physiological response of cells to solitary and combined stresses of environmental relevance (temperature, hydrostatic pressure, salinity and heavy metals, among others). Each route helps explain the evolution of the salt- and metal-tolerance hallmarks of the genus *Halomonas*. For ease of comparison with the literature, I use the traditional definition of "optimum" growth as fastest growth in the following sections (except in Table 0.1 and where otherwise noted). The chapters which follow include (i) characterization of novel species of *Halomonas* isolated from hydrothermal-vent habitats that introduces the genus and its currently known ecology; (ii) 16S rRNA gene sequence phylogenies of populations of moderately halophilic and euryhaline bacteria present in low-temperature hydrothermal fluids and deep seawater that illustrate their diversity and the environmental relevance of the isolates characterized in Chapter 1; (iii) growth curves, protein patterns and phospholipid profiles obtained under selected hydrostatic-pressure, temperature and salinity conditions that help constrain where in the ocean members of the genus *Halomonas* might grow and if there may be an underlying stress response induced by elevated salinity and/or hydrostatic pressure; and (iv) heavy-metal studies of selected *Halomonas* and other hydrothermal-vent mesophiles that aim to understand how they cope with and detoxify Cd^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}, thus further constraining permissive habitats and investigating additional possible cross-regulated stress-response systems.

ENVIRONMENTS

The most accessible, and consequently the best studied, hypersaline habitats are located along coast lines, on land, and in terrestrial subsurface environments that are tapped by drill holes and wells (4, 5, 53, 67, 225, 278, 564, 628). Indeed, the majority of research with moderately and extremely halophilic bacteria and archaea has transpired in hypersaline lakes and salterns (278). The deep sea is far less explored, though it too contains an array of brine environments that span a wide spectrum of temperature and chemical characteristics.

Sea surface and lacustrine hypersaline habitats

Hypersaline habitats dot coastlines where evaporation causes salinity to rise in waters partially or completely isolated from the sea. These environments include lagoons, sabkhas and artificial solar salterns from which humans harvest salt. Landlocked thalassohaline (derived from seawater) and athalassohaline (not derived from seawater) lakes, such as the Dead Sea, Great Salt Lake, hypersaline Antarctic lakes and African soda rift lakes, can reach saturation with respect to various salts including halite ($NaCl$) and gypsum ($CaSO_4 \cdot 2H_2O$). Typically these sites are located in low-latitude deserts and thus the waters and associated sediments are usually warm. Antarctic hypersaline lakes each have a unique chemistry and hydrography and are of course very cold. Deep Lake, for example, was measured at –18°C in winter; this lake, along with numerous other Antarctic hypersaline lakes, only attains a few degrees above zero during summer (376).

Sea ice accumulates interstitial brine as ice crystals grow and exclude seawater-derived salt ions from the crystalline matrix (588). Growth of ice crystals similarly concentrates microorganisms and dissolved and particulate organic material (325). These brines are also very cold, liquid down to at least –30°C (128), and harbor diverse communities of psychrophiles (147, 323).

Cold and cool deep-sea brines

Locally extensive, cold and cool seafloor brine pools, each deriving from dissolution of buried evaporites, have been detected at the bottom of the Gulf of Mexico and Mediterranean Sea (Table 0.2; 200, 377, 612, 645). While the geology and chemistry of these environments have been explored in detail, very few microbiological studies have been performed on samples from these systems.

The eastern Mediterranean region is characterized as a convergent plate boundary where the African oceanic crust is actively subducting beneath the Eurasian continent (645). The region is geologically complex, in part because the continental shelf of Africa has begun colliding with an accretionary complex located atop the subduction zone (645). Evaporite beds several kilometers thick were deposited in the late Miocene when sea level in the Mediterranean Sea was several kilometers lower than today (the Messinian salinity crisis), and these salt deposits are currently embedded in the accretionary prism at a modern water depth of ~3 km (581). Numerous transform faults, which locally transect the accretionary complex, have exposed buried evaporite layers to the deep sea (98, 337, 338) and in some cases act as conduits to brine-spewing mud volcanoes enriched with biogenic methane (109).

Five anoxic brine pools have been discovered in the eastern Mediterranean (98, 634), and the sediment record from other deeps indicates that some contained brine in the past (285). The brine deeps typically cover <50 km^2 and are located along the Mediterranean Ridge accretionary complex (133, 200, 285, 377, 535, 645). The brine-seawater interface lies at a unique depth for each basin, indicating a different source aquifer for each (65, 200, 377, 634). The temperature of the Tyro and Atalante Basins was found to be similar to equivalent depths nearby in the Mediterranean Sea (13–14°C), whereas the temperature in the Urania Basin was measured to increase from 17 to 28°C with depth (200, 377). The chemistry of the Urania Basin brine further suggested that reactions at up to 45°C have occurred at >500 m below the seafloor (109). The Tyro Basin has accumulated sapropelic organic-rich sediments (>2% organic carbon), and brine chemistry indicates that the salt derived from halite dissolution of a Messinian (5–6 Ma) salt deposit, as is inferred for all of the deeps (133, 285, 612). Differences in brine chemistry among the deeps are highlighted by varying ion ratios, $\delta^{11}B$ values and concentrations of Mg^{2+} and K^+, as well as the unique presence in the Bannock Basin of interlocking gypsum crystal masses up to 0.5 m in width that encrust the seafloor; these differences are indicators of distinct evaporite beds for the salt in each brine pool (74, 132, 200, 377, 535, 612). The deeps are relatively young as well. The Discovery Basin is estimated to have begun filling with brine only 700–2000 years ago (634). An extrapolation of the extent of brine lakes in the eastern Mediterranean Sea predicts a total coverage of ~1000 km^2 (200, 377).

The brine-seawater interface of the Urania Basin was examined microbiologically (524). High percentages of culturability (4–54% of the total microbial community) using two heterotrophic media incubated aerobically or anaerobically were obtained from above and in the brine-seawater interface (one sample each), resulting in the exclusive isolation of flavobacteria, *Alteromonas macleodii* and *Halomonas aquamarina* (524). Culturability success from brine inocula was <0.03% (524).

The serendipitously discovered Orca Basin in the Gulf of Mexico also contains an anoxic brine (541). It is 400 km^2 in area, located on the continental slope, and slightly warmer than the overlying water column (5.6°C versus 4.2°C) (541). Regionally expansive salt diapirs have both forged the basin bathymetry and fed it with brine (541). Hypersaline fluids began accumulating 7900 years ago by dissolution of a nearby deformed Jurassic halite deposit, seepage onto the seafloor, and pooling into a depression (2, 540).

Several characteristics of these cold and cool deep-sea brine pools are consistent between the eastern Mediterranean Sea and Gulf of Mexico. For example, particles become trapped at the brine-seawater interface of all of these deep-sea brine pools, a potential site of enrichment for heterotrophic microorganisms (336, 540). The brines are anoxic because density stratification prevents further supply of oxygen after microorganisms have consumed it during respiration of organic matter (540). In the Orca basin, microbial biomass and ^{3}H-uridine uptake rates were found to be lower at and below the brine-seawater interface as compared with the overlying water column (336). Also in the Orca Basin, enrichments in particulate ATP in the brine-seawater interface were attributed to the accumulation of trapped cells, increased ATP production upon exposure to elevated salinity, and decreased ATP degradation in the brine — not *in situ* growth (601). Depressed nutrient uptake rates and ATP enrichments should also characterize the brine-seawater interface above the eastern Mediterranean Sea brine pools. A striking difference, however, is that H_2S, while indeed found in measurable concentrations in the brine-seawater interface of the Orca Basin brine, is generally not detected within brine itself (540, 649), whereas H_2S is found at mM concentrations in the eastern Mediterranean Sea brine pools in which it has been measured (200). A lack of H_2S is frequently attributed to inhibition of microbial anaerobic sulfate reduction due to the high salt concentration and/or rapid precipitation of H_2S with Fe^{2+} as iron-sulfide minerals (2, 540, 649).

The transient nature of these brines bears significantly on the microbial ecology since most deeps appear to have filled with brine during the Holocene. The first denizens could include euryhaline and moderately halophilic bacteria derived from the overlying seawater. Increasing salinity would further restrict the habitat to microorganisms capable of growth at the highest salt concentrations. As oxygen was depleted during organic-matter respiration (and not refreshed due to intense density stratification), only facultatively or obligately anaerobic microorganisms could subsequently survive. It is more difficult to envision extremely halophilic microorganisms (which require >10–15% total salts for growth and have unknown or limited abilities to survive in seawater) colonizing deep-sea brines given that they would have to navigate through a potentially hostile seawater environment. Possible inocula for these habitats include sinking particles containing cells that originally derived from hypersaline environments on the Mediterranean Sea or Gulf of Mexico coastlines, or other nearby deep-sea anoxic brines. Extremely halophilic microorganisms, as compared with moderate halophiles, may have a lower probability of inoculating deep brines over short time scales because the habitat from which they are known to derive is significantly more distant than the adjacent overlying seawater in which moderate halophiles can grow. Alternatively, there may be hypersaline subseafloor environments which can seed both the deep-sea brine pools and deep seawater with high-salt-adapted microorganisms.

We only can observe brines that have accumulated in seafloor depressions. Other brines emanating from the seafloor, which are not trapped by bathymetry, may constitute small 'streams' flowing down-slope over sediment, locally affecting sediment microbial communities, benthic animals and fish. The benthos may colonize solid substrates within a few cm of a brine pool and salt-tolerant microorganisms (including H_2S-oxidizing bacteria) may thrive within the brine-seawater interface, as was observed around and along the overflow of Gollum Lake (East Flower Garden brine seep), a small, shallow brine lake located at 72-m depth in the Gulf of Mexico (78, 216, 335).

Warm deep-sea brines

There are twenty-five known warm brine pools beneath the Red Sea (137, 165, 356, 465). As in the Mediterranean Sea, these brines derived from Miocene evaporite deposits, accumulated in depressions in the seafloor and may exist only periodically (55, 285). Unlike the eastern Mediterranean Sea, however, the Red Sea is an active rift valley that began undergoing a transformation less than 5 Ma from a continental to oceanic rift (from south to north) with an axial trough (66). The deeps in the central Red Sea (when oriented north-south) are mostly arranged *en echelon* in relation to the nascent rift and are geothermally heated from below, often raising the temperature of the brines — up to 67°C — well above that of the overlying water column at 22°C (66, 239). In the Atlantis II Deep, brine chemistry, sediment composition, Sr, S and O isotopes, and anhydrite fluid-inclusion analyses indicated overlapping signatures of brine boiling, hydrothermal circulation through basaltic (or proto-basaltic) rock, and dissolution of evaporites exposed during tectonic extension of the seafloor (84, 120, 124, 479, 530, 670). It should be noted, however, that not all of the deeps contain chemical signatures of hydrothermal circulation and high-temperature brine-rock reactions (670). These cooler deeps are therefore more similar to those located beneath the eastern Mediterranean Sea.

Early explorations of the Red Sea brines focused on the Discovery Deep (which bears the same name as the Mediterranean Sea brine basin), Atlantis II Deep and Chain Deep, all located at ~2000 m depth (Table 0.2; 76). Fluids within the deeps are typically highly enriched in heavy metals, and the Atlantis II Deep was modeled to be continuously precipitating metal oxides and sulfides (512), leaving billions of dollar's worth of Zn, Cu, Pb, Ag and Au on the seafloor (56, 57, 530, 539, 635, 671). The metalliferous sediments contain 4% organic carbon (when scaled according to the ratio of the density of metalliferous to typical pelagic sediments) (639). Nephels peaks in and just below the brine-seawater interface likely indicate layers of trapped organic particles, as well as human garbage (512). A recent hydrographic survey revealed a constant temperature-salinity structure in the anoxic Shaban, Kebrit and Discovery deeps since 1965 (Table 0.2; 238, 239). The Atlantis II Deep, however, has steadily increased in temperature (from 56 to 67°C) and has increased in volume since sampling began forty years ago (238, 239).

Hydrothermal circulation through buried evaporites occurs beneath the Red Sea deeps, providing heat, salt and metals to the brine pools (120, 479, 530). The increase in temperature in the Atlantis II Deep over the past several decades is associated with increased hydrothermal activity and increased concentrations of Mn and Fe in the brine (though Cu slightly decreased in concentration) (530). Active hydrothermal vents have yet to be filmed or photographed there, but hydrothermal mineral precipitates and inactive massive sulfides comprised of pyrite, marcasite, sphalerite and galena (but nothing with significant Cu) that formed at 110–450°C are present in sediments and poking out of the seafloor (530, 671). Pb dating constrained the age of the deposits to 5000–28,000 years (530). The Atlantis II Deep is essentially a bath of briny hydrothermal fluid (76, 77, 389, 530). H_2S is absent because metals are in excess molar concentrations, and thus metals remain in solution in the brine until diffusing into upper, more oxidized brine layers where they oxidize and precipitate (530).

A handful of microbiological studies — all too few — have been performed on samples from the Red Sea brine pools, including cultivation of a few novel euryhaline and extremely halophilic mesophilic bacteria and community 16S rRNA gene fingerprints (see later section on the combined effect of hydrostatic and osmotic pressures). There are no reports of high-temperature-adapted microorganisms, yet there is clearly an enormous potential for discovering novel thermophiles and hyperthermophiles from these environments that grow with elevated levels of salt. Isoprenoid analysis of sediments indeed indicated a contribution from methanogenic and thermophilic archaea (387). One limiting factor, however, may be the availability of oxidants in the majority of the brine pool, but fermentation of sediment-derived organic compounds, reduction of precipitated Fe oxides, and other novel metabolisms are entirely possible. These hot, salty environments resoundingly contradict the assertion (130) that euryhaline and moderately to extremely halophilic hyperthermophiles do not exist based on a lack of niche.

Hot subseafloor brines

Extremely hot brines are created during subseafloor hydrothermal circulation in association with active mid-ocean ridges or from cooling magmas in ridge or other plutonic settings. The global reach of the mid-ocean-ridge system highlights the importance of these brine-generating processes, but at the same time their extremely high temperatures require that they be conductively cooled in order to be relevant to the currently known — but ever expanding (298) — temperature spectrum within which life exists.

Phase separation is a widespread phenomenon believed to occur continuously at depth in active, hot mid-ocean-ridge environments as fluids rise and decompress. When the hydrostatic-pressure-temperature conditions for this decompression are below the critical point of seawater — 407°C and 29.85 MPa for a 3.2% NaCl solution — sub-critical phase separation (boiling) results in production of large quantities of fresh vapor; the salinity of the remaining fluid increases only slightly over short time scales (61, 623). Boiling is known to occur at the relatively shallow ASHES vent field atop Axial Volcano (94, 371) and adjacent to shallowly emplaced dikes (351, 625).

In contrast, super-critical phase separation (condensation) generates briny fluid as a discontinuous phase in a two-phase emulsion (58). Due to differing physical properties of the vapor and brine (for example, density, viscosity and surface tension), the brine is assumed to be sequestered within the oceanic crust and may accumulate during prolonged periods of condensation (59, 62, 140, 188, 189, 218, 310). For example, brines are believed to be retained at depth beneath the Endeavour Segment of the Juan de Fuca Ridge (95, 138). Chemistry of vigorously venting sulfide structures in the Main Endeavour Field shows evidence for prolonged super-critical phase separation, driven by heat extracted from a crystallized magma chamber (95, 138). Fresh fluids along the Endeavour Segment are common in the highest temperature vents, while fluids with salinities elevated relative to seawater are common in less vigorously venting, lower temperature (~300°C) vents at the Salty Dawg and Mothra vent fields (95, 138). Similarly, at the Lucky Strike vent field on the Mid-Atlantic Ridge, relatively shallow vents above a deep reaction zone are characterized by the flushing of evolved waters with a moderate condensation signature (622). Eruptive events (as compared with cooled or cooling magma) may eventually lead to the release of even saltier fluids when brines finally flush from the seafloor (92). For example, fluids with a chloride content well above seawater have emanated from vents on the Cleft Segment of the Juan de Fuca Ridge for at least seven years as the system ages and cools (92, 93, 621).

In certain volcanically active mid-ocean-ridge environments, as in equivalent terrestrial systems (117, 488), extremely saline fluids will exsolve from cooling magmas during the final stages of melt crystallization (310, 312, 416). In ophiolites such as Troodos, Cyprus, magmatic brines contain 40–60% NaCl at 450–600°C in the upper plutonic gabbros and plagiogranites (310, 313, 314). Here, when the system is cooled to 200–400°C, progressively deeper cracking allowed the penetration of evolved seawater (2–7% NaCl) into the upper plutonic sequence; brines with >20% NaCl were not found in pillow lavas or the stockwork beneath the fossil vent system (310, 313, 314). Fluid inclusion analyses of mid-ocean-ridge gabbros similarly reveal deep, exsolved magmatic brines of >50% NaCl at >700°C followed by penetration of seawater-derived fluids of 1–7% NaCl circulating at 300–400°C (312). Lastly, the presence of cooler, more saline fluids derived from magmas and/or seawater containing up to 20% NaCl at 275–350°C were more recently discovered in gabbros from the Mid-Atlantic Ridge (309).

Acidity generated by water-rock reactions in addition to elevated temperatures mobilize heavy metals from minerals, resulting in metal-enriched fluids (538). Subseafloor fluids decompress as they rise through the stockwork beneath a hydrothermal vent field, and when this decompression crosses the two-phase curve of seawater (in hydrostatic-pressure-temperature space), it results in fluids separating into distinct brine and vapor-rich phases (58). Metals preferentially partition into brines, further increasing their concentration (60, 155, 507, 598).

These brines, while extremely salty, are also extremely hot. Conductive cooling is a possible mechanism to cool these brines without diluting the salt, thus creating novel hypersaline habitats for microorganisms within the currently known temperature range for life. These brines may remain for millions of years deep within the crust in gabbroic rocks, which eventually transit to off-axis regions and cool conductively. But again, there is little evidence of brine-rock reactions in the oceanic crust (despite the occurrence of hypersaline fluid inclusions), so the extent of these hypothesized brine habitats remains difficult to constrain.

Sulfide structures

There is a possibility that sulfide structures contain microenvironments of elevated ionic strength. Polymetallic sulfides precipitate from solution when reduced, acidic hydrothermal fluid mixes with oxygenated, alkaline seawater. Sulfide structures grow where hot fluids vent into the deep-sea water column through seafloor cracks and fissures. They can grow rapidly and tall (494, 593), and the mineralogy changes as the temperature and fluid chemistry drift or rapidly fluctuate over various time scales (592). The interior pore spaces and cracks are characterized by a spectrum of gradients in temperature, Eh, pH, metal concentration, and oxygen content (594). Centimeter-scale chemical reaction and transport models show that pore fluid concentrations of Cl^- and Na^+ in interior zones may be less than in both seawater and hydrothermal-fluid end members (592, 594). Hypothetically, however, the ion content of fluids in micrometer-scale cracks may increase as pore spaces re-heat or fill during mineral precipitation and hydration reactions. Newly discovered minerals, including the Na- and Cl-bearing zinc sulfate mineral, gordaite ($NaZn_4[SO_4][OH]_6Cl \cdot 6H_2O$) (3, 75, 272, 415), also indicate possible localized enrichments of seawater solutes during precipitation and dissolution reactions.

Moderately halophilic microorganisms have been cultured from sulfide-rock samples (304, 577), but, more compellingly, a phylogeny based on 16S rRNA gene sequences revealed the presence of numerous obligately halophilic haloarchaea in one sulfide structure studied (577). Haloarchaea require >10–15% total salts in order to grow, thus indicating a permanent brine habitat within this sulfide structure. This putative hypersaline environment could additionally harbor moderately halophilic bacteria, such as members of the genus *Halomonas*. These tantalizing results clearly require pursuit.

Habitat summary

Moderately to extremely halophilic microorganisms are found in hypersaline environments located along coastlines, in sea ice, and in other sea-surface locations, but the significance of deep-sea brine habitats to these organisms is poorly understood. Geographically disparate, anoxic cold, cool and warm brine pools exist beneath the Gulf of Mexico, the eastern Mediterranean Sea and the Red Sea, though the number of microbiological studies are incredibly few. These locations undoubtedly will eventually provide novel microorganisms that are adapted to, or at least grow under, high-hydrostatic-pressure and high-salt conditions. The possibility of isolating extremely halophilic thermophiles and hyperthermophiles from the Red Sea brines and associated subseafloor habitats is irresistible. The impact of these isolated brine environments on the microbial ecology of the greater ocean ecosystem is unclear, however. Could these brine pools serve as a source of moderately halophilic bacteria to the rest of the sea? Anoxia is one mitigating factor, but, more importantly, the comparatively miniscule volume of brine and the few locations in which it is found do not convincingly explain the high abundance of salt-tolerant bacteria isolated in the Pacific Ocean (304).

Similarly, hot brines located along mid-ocean ridges, which exist to an extent proportional to the ridge system itself, are insufficient to account for the high concentrations of mesophilic moderate halophiles isolated from hydrothermal-vent and pelagic environments because of the inaccessibility of the deepest crustal fluids and the extremely hot temperatures. If the presence of moderately halophilic bacteria in hydrothermal-vent habitats did reflect a subseafloor brine, one would predict the existence of a metabolic array of moderately to extremely halophilic hyperthermophiles emanating from similar systems. Bearing in mind the caveat of low culturability success, hyperthermophiles have not yet been cultured at salinities above ~10–11% total salts (221, 303). The uncertainties and dynamic complexities of pore-fluid chemistry within sulfide structures, which contain a temperature gradient from 2 to >350°C, indicate that this niche may still yield some clues about salt-adapted microorganisms in hydrothermal-vent environments.

DISPERSAL

The notion of passive dispersal of microorganisms from niches of enrichment through seawater has been invoked to explain why various physiologies — for example, metal resistance, salt tolerance and thermophily — are occasionally found in unexpected locales (177). Dispersal in the direction of current flow, by rising within hydrothermal plumes or megaplumes, or via sinking in association with particles (149) — not to mention by wind at and above the sea surface — undoubtedly occurs, and one would predict that the abundance of a given type of microorganism that was enriched at a certain location would decrease with distance from that site due to predation, lysis and cell degradation. Indeed, for example, metal-resistant bacteria are typically more prevalent in metal-contaminated sites, with decreasing abundance correlated with kilometer-scale distances (136, 258, 259, 391, 591)

The time scale of dispersal is an open question, however; clues are revealed in the following examples. *Halomonas aquamarina* survived ninety-two-day starvation experiments at 5 and 20°C (286). Similarly, hyperthermophiles can survive in cold, oxygenated seawater, at least over month time scales (276, 569). And contrary to the prediction that psychrophilic microorganisms from Arctic and Antarctic environments would be highly phylogenetically divergent (561), it is now known that closely related strains are found in both polar environments (81, 85), presumably transported via cold deep-sea currents over hundred-year or greater time scales during which time they could be growing *in situ*. Another example involves a report of aerobic thermophilic microbial activity at 52°C in a cold deep-sea sediment sample obtained at a distance of several hundred kilometers from both hydrothermal vents of the East Pacific Rise and the nearest islands; it was unclear, however, which organisms were responding to the warm incubation, impossible to pinpoint their true origin, and unknown how long the microorganisms responsible for the activity signal had been present (156). Lastly, sulfate reduction at up to 60°C was reported for a sediment collected from a permanently cold (0–15°C) coastal site off the coast of Denmark; a thermophilic, endospore-forming bacterium was also isolated from the sample (267). Indeed, spore-forming microorganisms may have a much higher probability of being dispersed to habitats in which they cannot grow given their evolved long-term survival strategies.

Dispersal of microorganisms in a direction other than that mediated by deep-sea currents, rising plumes or sinking particles is more difficult to envision. An upward flux of particulate organic matter was hypothesized as a means of transporting positively buoyant materials from sediments and the deep sea to the surface of the ocean (662). This phenomenon, though rarely studied, has been documented solely for lipid-rich, animal-associated particles, including eggs, larvae and gelatinous materials (127, 498, 555, 633). Microorganisms that attached to positively buoyant particles could theoretically hitch a ride towards the sea surface, but even the ascending lipid-rich particle flux associated with hydrothermal plumes contained only zooplankton, not bacterial or archaeal, biosignatures (127, 633).

I find the dispersal hypothesis unsatisfying — primarily for microorganisms that do not form endospores — because a corollary hypothesis is that all microorganisms that can survive in seawater would be found in all ocean environments. On land, comparisons of multiple gene loci in hot-spring cyanobacteria suggested endemism and limited genetic exchange between environments of appropriate conditions for growth (462). And in marine ecosystems, hyperthermophiles are not cultured from all ocean samples, for example. In addition, invoking dispersal would predict that metal-resistant bacteria and hyperthermophiles should both be culturable from hydrothermal plumes given that plumes entrain low-temperature hydrothermal fluids from seafloor vents (340). Hyperthermophiles and metal-resistant mesophiles thrive in warm to hot seafloor and subseafloor hydrothermal-vent habitats, but only metal-resistant mesophiles, not hyperthermophiles, are cultured from 2°C chronic hydrothermal plumes located several hundred meters above the seafloor (257, 304, 569).

While certain data strongly suggest dispersal in particular cases (again, especially for endospore-forming microorganisms), cross-regulated stress-response systems detailed in the following sections, as well as those yet to be discovered, can also account for unexpected physiologies. This idea rests at the conceptual core of my thesis: a given laboratory-determined phenotype may actually reflect a physiological adaptation not intended by evolution to manage the given stress. Among *Halomonas* spp., salt and metal tolerance may actually reflect adaptations to hydrostatic-pressure and/or nutrient duress.

STRESS PARAMETERS

It is tempting to render direct causal links between microbial physiology and the environment from which a given microorganism was collected. This philosophy, while often apt, may not always reflect ecological reality. As mentioned previously, a recent illustration of this potential pitfall is the extreme radiation resistance of *Deinococcus radiodurans*. This bacterium has evolved the capability to repair extensive DNA lesions suffered during desiccation, an adaptation that coincidentally enables cells to repair DNA damage incurred during exposure to high levels of ionizing radiation far beyond those encountered in any natural environment (41). The complexities of the microbial stress response thus require vigilance in their interpretation. Indeed, it may be a cryptic stress response — not necessarily a direct link to the physics and chemistry of a given set of environmental conditions — that accounts for the salt- and metal-tolerance phenotypes of the genus *Halomonas* and other moderately halophilic bacteria.

Geneticists invoke "directed" mutation to explain how, at the level of DNA base changes, microorganisms can adaptively respond to and survive particular environmental challenges. This phenomenon gives the appearance that increased rates of very specific mutations evolve in order to take advantage of precise beneficial conditions (97). The underlying mechanism involves reverting from a hypermutable state while under stress to a normal rate of mutation once an advantageous DNA base change has been acquired (231–233). Stressors which induce a mutator phenotype in *Escherichia coli* and other bacteria include starvation, stationary-phase growth and oxygen stress (63, 187, 317). Sub-optimal growth conditions may expressly induce increased mutation rates as a survival mechanism (or, alternatively, replication errors may accumulate as a direct consequence of stress-induced DNA lesions or during the elevated rates of DNA repair) (63). (I wonder if the observation that marine microorganisms tend to form phylogenetic clusters [197, 198, 215] — here considered as the result of a collective burst of mutations in the 16S rRNA gene — is a reflection of an increased mutation rate throughout their genomes that was induced by environmental stresses.) This notion also highlights the possible role of using stress as a parameter to increase culturability success, as has been observed with nutrient limitation (482) and salt stress (304).

Genome size correlates positively with the number of transcriptional regulatory and signal-transduction genes, and indeed microorganisms that are non-symbiotic and not restricted to stable, extreme habitats have the largest genomes and thus the greatest ability to respond to dynamic environments (103). The chromosome of members of the genus *Halomonas* ranges in size between 1.5 and 2.8 Mb, and species within the genus commonly harbor several plasmids and megaplasmids, up to 600 kb (25, 181, 344, 379, 611). The total genome size alone makes the genus *Halomonas* a candidate for cosmopolitan success in a wide variety of marine and hypersaline habitats (25).

While at first glance it may appear that microorganisms face an overwhelming variety of stresses in the environment, there are only a few generalized cell components that may be damaged: nucleic acids, proteins and phospholipid membranes (245). Proteins may dissociate, denature, lose functionality and aggregate; DNA can be cleaved or chemically modified; and membranes can be disrupted. Stress causes simultaneous down- and up-regulation of genes. Up-regulated genes may produce proteins that (i) induce suites of other repair genes; (ii) directly enable a cell to repair damage by renaturing proteins, mending DNA lesions, or adjusting membrane fluidity; and (iii) proteolyze irreparably damaged polypeptides — and thus survive the stressor (see Table 18 in reference 352).

Hydrostatic pressure

For over a half century microbiologists have studied the influence of hydrostatic pressure on enteric bacteria, foodborne pathogens and microbial isolates and communities collected from shallow and deep oceanic environments. An early review (prior to the discovery of hydrostatic-pressure-adapted bacteria) of the physiological impact of exposing microorganisms to deep-sea hydrostatic pressures notes a multitude of consequences, including effects on protein synthesis and folding, replication, membrane transport, metabolism, enzyme activity and cell morphology (332). The mechanisms by which hydrostatic pressure fundamentally affects cellular processes include the change in volume associated with: (i) a given reaction or macromolecule-macromolecule association; (ii) physicochemical effects on intra- and intermolecular forces and bonds; (iii) alterations in the role of water molecules on macromolecular stability and function; and (iv) modifications of phospholipid membranes to maintain optimal fluidity and transmembrane transporter performance (1, 39, 68, 144, 145).

Hydrostatic pressure does not cause a pressure differential across the membrane but does affect macromolecular packing and interactions, and it promotes protein denaturation as water attempts to penetrate into the core of a folded polypeptide (365). Recent hydrostatic pressure experiments on microorganisms obtained from the deep sea aim to catalogue and characterize hydrostatic-pressure-induced proteins (38, 114, 264, 271, 302, 364, 567, 641, 642), decipher the genetic regulation of the physiological response to hydrostatic pressure (113, 114, 299, 414), and determine how cells maintain the function and fluidity of phospholipid membranes (13, 14).

Psychrophilic (429–431, 653, 657, 661) and hyperthermophilic (468, 489) piezophiles have been cultured from deep-sea sediments, animals, and hydrothermal vents (Table 0.1; 149). Obligate piezophiles from the very deepest reaches of the ocean (up to 11,000-m depth) have been cultured from animal guts (660) and sinking particles (151 and references therein) and have moderate growth rates under cold high-hydrostatic-pressure conditions. Phylogenetic and lipid analyses of abyssal sediment microbial communities reveal the presence of piezophilic bacteria and uncultured taxa with unknown *in situ* activities, along with mesophilic bacteria that presumably are unable to grow at the sample depth and temperature (176, 300). Cultured psychropiezophiles currently belong to five groups within the γ-*Proteobacteria*: members of the genera *Shewanella*, *Photobacterium*, *Moritella* and *Colwellia*, plus a closely related unnamed group (142). This narrow phylogenetic range of cultivated psychropiezophilic microorganisms will undoubtedly expand with time and novel cultivation techniques (149). Deep-sea microbial assemblages from sediments (150, 164, 655), particles (150), and the deep regions of the water column (464) show a piezophilic response when incubated at or near *in situ* conditions.

Deep-sea microorganisms have been observed to contain increased proportions of polyunsaturated fatty acids (PUFA). The kinking produced by multiple double bonds in the acyl hydrocarbon tails of the individual fatty acids is believed to increase membrane fluidity and counteract the “freezing” effects of hydrostatic pressure (quantified as the membrane melting temperature) and/or maintain optimal membrane permeability (39, 144, 145, 658). The presence of PUFA is striking among piezophilic *Shewanella* species obtained from the deep sea when contrasted with their PUFA-free piezosensitive *Shewanella* cousins isolated from shallow marine environments (301). Similarly, the proportion of monounsaturated fatty acids (MUFA) increases with hydrostatic pressure for a given microorganism (39). It has been observed, however, that at least in the psychropiezophile *P. profundum* strain SS9, MUFA, but not PUFA, are required for growth under hydrostatic pressure (14). Many abyssal animals require PUFA but cannot synthesize them, highlighting the importance of indigenous hydrostatic-pressure-adapted microorganisms as part of the foundation of the deep-sea ecosystem (423).

Other experiments have shown that, among *Shewanella*, increased hydrostatic pressure tends to dissociate single-stranded DNA-binding protein from its substrate because the protein-DNA complex occupies more volume than when the macromolecules are separated (115). The dissociation of the protein-DNA complex and concomitant volume change are more pronounced for piezosensitive relative to piezotolerant and piezophilic species (115).

Lastly, it has been observed recently with thermophiles (50) and for several decades with mesophiles, such as *Escherichia coli* and a variety of easily cultivated marine bacteria, that hydrostatic pressure causes cells to elongate, form filaments and contain proportionally less DNA (579, 642, 672–674, 676). These morphologies are signs of incomplete chromosome replication and cell division (642, 672–674, 676).

Osmotic pressure

Microorganisms inhabit environments with salinities ranging from freshwater to saturated salt solutions and typically display at least a small degree of phenotypic plasticity with respect to growth over a salt range (Table 0.1). Different phylogenetic groups occupy different salinity niches, including halophiles adapted to seawater, moderate halophiles adapted to seawater and higher salinities, and obligate halophiles adapted to salinities between 10–15% total salts and saturated conditions (32–37% total salts) (19, 20, 106, 342, 407, 451, 452, 499, 613). Microorganisms able to grow in hypersaline waters are found among most major taxonomic branches, including heterotrophic aerobic *Proteobacteria* and Gram-positive bacteria (613), phototrophic, heterotrophic, methanogenic and sulfate-reducing anaerobes (277, 330, 350, 382, 447, 492, 600), and haloarchaea (440).

When a microorganism is exposed to increased concentrations of external solutes, water leaves the cell causing a temporary decrease in cell volume (652). In natural systems, the only osmotic stress of relevance is that caused by inorganic salts, primarily NaCl. Moderately halophilic bacteria, especially members of the genus *Halomonas*, are osmotic masters, accumulating compatible solutes in response to osmotic duress in order to reconstitute original cell size and water content (613, 650). Compatible solutes are small, neutral (or zwitterionic) organic molecules, such as glycine betaine and ectoine, that are typically derivatives of amino acids, sugars or alcohols (204, 450, 522, 537, 613, 650). Most halotolerant and halophilic bacteria, fungi, and algae utilize the compatible-solute osmoadaptive strategy and can increase the concentration of these molecules to remarkably high levels within the cell (327, 613). The internal concentration of these compatible solutes tracks the external salt concentration as Na^+ and K^+ are actively removed from cells (203).

The osmosensory and osmoregulatory response in bacteria has been studied in great detail. As most scrutinized in *Escherichia coli*, membrane-based turgor sensors, which react to membrane flexure, first trigger the transport of K^+ inside the cells to alleviate the osmotic differential (288, 289). Further investigations with *E. coli*, *Listeria monocytogenes*, *Corynebacterium glutamicum* and *Lactococcus lactis* revealed that a suite of osmoregulatory genes, including glycine betaine and ectoine transporters, are signaled into action directly by similar turgor sensors and by the primary products of those sensors (609, 652). Like *E. coli* and *L. lactis*, the moderate halophile *Halomonas elongata* resiliently accommodates osmotic shifts in the external environment by simultaneously accumulating K^+-glutamate and ectoine during a tightly regulated response (324, 395). After a drop in osmotic pressure, these small organic compounds are instantaneously released into the extracellular milieu or degraded intracellularly (652).

Compatible solutes are hypothesized to function via "preferential exclusion," whereby osmolytes increase the surface tension of a fraction of the intracellular water; consequently, higher-surface-tension water, which contains compatible solutes, is expelled from the protein surface concomitantly as some lower-surface-tension bulk water hydrates proteins (86, 652). The compatible solutes themselves do not interfere with the structural and enzymatic functions of proteins, even at high concentrations (203). In addition, the volume of cytoplasmic water is key to growth and survival, and regulation of the concentrations of K^+ and compatible solutes helps retain sufficient numbers of water molecules inside the cell (105). The intricacies of the thermodynamics of osmoregulation are profound, and it appears that compatible solutes may serve multiple functions, including protein stabilization and of course maintenance of cell volume (also see later section on compatible solutes).

Haloarchaea, unlike bacteria, most often achieve osmotic balance by importing high concentrations of K^+ into their cells with Cl^- as anion (366). Differing from their bacterial counterparts, haloarchaeal proteins have evolved to function optimally under high-potassium conditions and typically denature at seawater salinity (366). Haloarchaeal polypeptides contain high proportions of acidic amino-acid residues, reduced amounts of hydrophobic residues, and increased numbers of salt bridges, ultimately coordinating a protective network of hydrated salt ions around protein surfaces in order to maintain protein integrity and function (152, 334, 366). Though haloarchaea cannot grow in seawater, in some instances they are able to tolerate and perform certain functions at seawater salinity. These abilities may enable them to be transported to new hypersaline habitats via seawater conduits (129, 500). *In vitro* studies reveal that at least some enzymes, such as nucleoside diphosphate kinase, retain activity at low salt concentrations (268), though this haloarchaeal enzyme is exceptional in this regard. The enzymes that are adapted to both low- and high-salinity conditions may enable survival during transport through seawater environments.

There are a few examples of microorganisms that utilize an osmoadaptive strategy entirely inconsistent with fellow members of their prokaryotic domain. For example, the extremely halophilic, obligately anaerobic members of the bacterial family *Halanaerobiaceae* and the obligate aerobe *Salinibacter ruber* accumulate high intracellular concentrations of Na^+, K^+ and Cl^- (but no detectable organic compatible solutes), contain proteins with a high content of acidic amino acids, and require high salinity in order to function (449, 453–455, 486). The intracellular and extracellular concentrations of K^+ and Cl^- are equal, but Na^+ accumulates to levels sufficient to balance any remaining osmotic differential (449). There are also halophilic, methanogenic archaea that predominantly accumulate compatible solutes, or K^+ salts of organic osmolytes (450). Compatible solutes from hyperthermophilic bacteria and archaea are typically negatively charged with K^+ as accompanying cation (di-*myo*-inositol-1,1'-phosphate is the most common), whereas mesophilic bacterial compatible solutes generally have no net charge (523).

Metabolic redox energetics evaluated in conjunction with the cost of salt-resistance strategy — counted in the number of ATP molecules used per compatible-solute molecule produced — appear to dictate the evolved osmoadaptation strategy within a given microbial lineage (450). For example, members of the obligately anaerobic *Halanaerobiaceae* utilize K^+ to counteract high extracellular salt concentrations, the least energetically costly osmoadaptive strategy, which is fitting with its relatively energy-poor anaerobic lifestyle (450). The cell yield of a growing culture is expected to be reduced at high salt concentrations due to the increased amount of ATP molecules consumed during generation of compatible solutes (see Fig. 4 in reference 450; 451).

Haloarchaea show abnormal morphology at low salinities and supra-optimal growth temperatures (212). *Halomonas salina* forms elongate cells and filaments at very high salt concentrations (20%) and in stationary phase (606), presumably a sign of incomplete chromosome replication. Hydrostatic-pressure-stressed cells of piezotolerant bacteria also commonly show incomplete division (579, 642, 672–674, 676), indicating a possible link of the microbial response to salt, hydrostatic-pressure and stationary-phase stresses (see later section on multiple resistances).

Since the 1950s, there have been numerous studies investigating the impact of salinity on phospholipid membranes. Consistent trends with increasing external salt concentration, as monitored in *E. coli*, the euryhaline moderate halophiles *Flavobacterium halmophilum* (reclassified as *H. halmophila* [157]), "*Pseudomonas halosaccharolytica*," *Planococcus halophilus* (reclassified as *Marinococcus halophilus* [236]), *H. salina*, *H. halophila* and *H. elongata*, and halophilic bacteria and archaea in general, include some or all of the following: (i) increased anionic lipids (perhaps to increase cation permeability); (ii) decreased neutral lipids; (iii) decreased MUFA; (iv) increased cyclopropane fatty acids (CFA); and (v) increased saturated fatty acids (SFA) (243, 334, 396, 397, 441, 511, 605, 627). The proportions of MUFA, CFA and SFA determine, in part, the degree of membrane flexibility, with the properties of CFA being intermediate between the respective MUFA and SFA from which they derive (243). Accordingly, increased salinity causes decreased membrane flexibility, or "tightening of the membrane" (605, 626, 627). In "*P. halosaccharolytica*," CFA synthetase was inhibited by NaCl and KCl but strongly stimulated by glycine betaine, thus allowing membranes to decrease in fluidity with increasing external salt concentrations due to a direct interaction of CFA synthetase with the intracellular compatible solute (400).

There is speculation that microorganisms and DNA can be trapped and preserved inside halite crystals for millions, if not hundreds of millions, of years (631). A novel *Bacillus* species (632), haloarchaeal isolates (478), and haloarchaeal 16S rRNA fragments (184) have been cultured or amplified from Permian and Triassic halite crystals, though not without dispute (226, 425). There is no controversy, however, over the finding that haloarchaea can be preserved in natural brine samples taken from the Dead Sea and stored at room temperature for over half a century (23).

Heavy metals

Microorganisms and metals profoundly influence each other in marine settings. Numerous transition metals are required as micronutrients but are frequently in limited supply in aquatic environments; these metals comprise the key reactive centers of enzymes that carry out a broad range of biochemical electron-transferring reactions. The genetics of metal uptake and homeostasis have been explored in great detail for Cu, Zn, Co, Mo and Mn in cyanobacteria (104), Cu in *Enterococcus hirae* (557) and *Escherichia coli* (191, 487), and Fe (17), Ni (410), Mn (308), Zn (64) in bacteria in general (for a quick review, see Finney and O'Halloran [183]). Nutrient metals are enriched to high concentrations (~10 μM to 0.1 mM) within cells (183, 457) and can be sensed at extremely low concentrations, far less than one atom per cell (108, 183). The integral role of metals in routine cellular function and their limited environmental availability have resulted in the evolution of highly efficient, and highly competitive, metal-uptake systems. These uptake systems, when combined with efficient extracellular metal-binding ligands, impose profound controls on biogeochemical cycling of certain elements (223, 402, 421, 436).

Metals can also be utilized for metabolism, either as a source of or sink for electrons. Aerobic chemoautotrophs and mixotrophs that oxidize Fe^{2+}, Mn^{2+} and H_3AsO_3 (arsenite; As^{3+}) are widely known, especially from hydrothermal-vent and acid-mine-drainage habitats (30, 210, 516, 519), as are anaerobic heterotrophs that use Fe^{3+}, Mn^{4+}, $H_2AsO_4^-$ (arsenate; As^{5+}) and SeO_4^{2-} (selenate; Se^{6+}) as terminal electron acceptors during dissimilatory metal reduction (169, 253, 320, 349, 520, 533). Numerous other metals, including Hg^{2+}, CrO_4^{2-} (chromate; Cr^{6+}), Co^{3+}, Pd^{2+}, Au^{3+}, Ag^+, Mo^{6+}, V^{5+} and even the radionuclides UO_2^{2+} (uranyl ion; U^{6+}), Pu^{4+} (yes, plutonium!), Np^{5+} and TcO_4^- (pertechnetate; Tc^{7+}), can be reduced — without necessarily generating metabolic energy — either due to non-specific metal reduction and/or metal-specific detoxification mechanisms (32, 80, 316, 346, 347, 373, 439, 526). Frequently, trace-metal reduction-oxidation chemistry involves a change in metal solubility, further highlighting the impact of microbial metal metabolism and detoxification on biogeochemical cycles — not to mention the enormous potential for bioremediation.

Metals that are utilized as micronutrients or during metabolism — including highly sought Fe (17, 73, 596) — are toxic at high concentrations. In addition, certain metals, like Hg, have no known biological function and are considered toxic at any concentration. Heavy metals can directly cause DNA lesions or catalyze the formation of radical oxygen species with the same effect; perhaps most importantly, they also bind irreversibly to thiol groups in polypeptides, rendering them inoperable (426, 544). The degree of metal toxicity is typically highly correlated with the binding affinity of the metal for H_2S, for which the cellular proxy is the thiol group of proteins (see Fig. 1 in reference 426). Intrinsic metal-resistance mechanisms include modifications of the cell wall, production and release of exopolysaccharides, and an inability to transport a given dissolved metal inside the cell (201, 509). Extrinsic factors include (i) highly specialized, membrane-bound, energy-dependent metal-ion efflux systems; (ii) production of cytoplasmic, periplasmic and extracellular ligands and metal-transport proteins; (iii) alteration of membrane-transport activities; and (iv) enzymatic reductive or oxidative transformation of metals to less toxic species (90, 509, 545). Minor (and easily mutable?) changes in the metal coordination sites of certain metal-sensing regulatory proteins reveal a high level of precision in some metal-stress systems; alteration of the metal recognition site results in either a change in the metal that is sensed or a change in the subsequent regulatory cascade (90).

Completed genome sequences reveal the presence of numerous homologous metal-resistance proteins among widely divergent bacteria and archaea, such as *Bacillus subtilis* (558) and *Methanococcus jannaschii* (87). Resistance genes are commonly, but not always, located on plasmids and may be transferred relatively easily between unrelated taxa. Comparative genomics of dozens of bacteria spanning a range of metal sensitivity and resistance, including the model metal-resistant bacterium *Ralstonia metallidurans* with its 2.9-Mb plasmid (386), has shed further light on the complexities of multiple and extreme metal tolerance. Intriguingly, it appears that the degree of metal resistance is correlated with the number and specificity of partially overlapping metal-resistance systems contained within a given genome (426, 544). Some systems are common among several taxa and confer a general, low-level resistance, while other systems are highly specialized and rare, enabling cells to tolerate exceptionally high concentrations of toxic metals (426).

Despite the remarkable detail with which the genetics of metal regulation and resistance have been elucidated, there remain several significant mysteries. First, why are some microorganisms so strongly resistant to metals? Secondly, why can these organisms sometimes be found distant from any conceivable metal-contaminated area? Moderately halophilic bacteria, including *Halomonas* spp. isolated from seawater, deep-sea sediments and coastal hypersaline habitats, typically tolerate mM concentrations of Cu^{2+}, Zn^{2+}, Cd^{2+}, SeO_4^{2-}, Ni^{2+} and Co^{2+} (135, 428), but metal concentrations are at pM to nM levels in pelagic environments and sediments (217, 436, 651). In experimentally metal-amended and otherwise polluted soils, the growth of certain portions of the microbial community may be inhibited (171, 196, 319, 517, 518) while the activity and concentration of other sub-populations are enhanced (153, 196, 480, 481), indicating both the initial presence of metal-resistant microorganisms in uncontaminated soils and the ability of certain taxa to respond positively to metal stress. *R. metallidurans* tolerates >10 mM Co^{2+}, Mn^{2+} and Zn^{2+}, 5–10 mM Ni^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+}, and 0.01–0.1 mM Ag^{+} and Hg^{2+} and is commonly cultured from polluted areas (386, 426). Where did an organism like *R. metallidurans* originate? Due to its ability to oxidize hydrogen and because of its extreme resistance to metals, researchers often invoke metal-enriched hydrothermal-vent environments as its natural niche (386). Indeed, hydrothermal vents are a source of bacteria and archaea resistant to 0.1–10 mM concentrations of numerous metals (279, 280, 345). Lateral transfer of plasmids encoding metal-resistance genes may account for widespread metal resistance among hydrothermal-vent microorganisms, as has been demonstrated in soils (374, 420, 549) and hypothesized in aquatic systems (89). But these degrees of metal resistance protect far beyond any concentration that would be encountered in hydrothermal fluids of any temperature. End-member hydrothermal fluids at >250°C contain 0.1–1000 mM Fe and Mn, 1–1000 μM Zn, 1–20 μM Cu, 0.01–10 μM As, 0.1–10 μM Pb, 0.1–1 μM Cd and 0.01–1 μM Co (619), but these concentrations are greatly reduced by dilution and precipitation when hydrothermal fluids mix sufficiently with alkaline, oxygenated seawater to temperatures that would permit mesophiles or even (cultured) hyperthermophiles to grow.

There are mechanisms to accumulate metals at growth-permissive temperatures in hydrothermal-vent environments, however. Animal tissues, animal mucous secretions and microbial biofilms concentrate many metals (110, 126, 202, 290, 291, 328, 501, 502, 584, 607, 638, 667) well above the levels found in seawater (436) and low-temperature hydrothermal fluids (619). Bioaccumulation of heavy metals, such as Hg in animal tissues (237), is a widely documented phenomenon that affects marine food chains in polluted environments. For example in hydrothermal-vent habitats, the clams *Vesicomya gigas* from Guaymas Basin and *Calyptogena magnifica* from the East Pacific Rise contained remarkably high concentrations of Cd, Fe, Hg, Mn, Zn, Cu and Pb in various tissues (501, 502, 510). Fe, S, Zn, Cu, Cd, Hg, U and As, among other heavy metals, were measured in high concentrations in association with intracellular sulfur granules, symbiosis organs, organic tubes, soft tissues and mucous secretions of alvinellid polychaetes, vestimentiferans and mussels (110, 126, 202, 290, 291, 667). Microbial biofilms, exopolymeric substances and external cell components also accumulate heavy metals from solution (29, 47, 72, 88, 112, 190, 208, 326, 328, 505, 536, 584, 589, 607, 617, 664). It is this property that explains, in part, the functionality of heavy-metal bioremediation bioreactors (52, 543, 582, 608). It would be ironic if the stress-mitigating exopolymeric architecture of microbial biofilm communities, which protects against high temperature, low nutrient concentrations and other stresses, resulted in the requirement for a metal-resistance phenotype.

There remain significant open questions involving the existence of stress-response systems that confer such high degrees of metal resistance to microorganisms. The specificity of metal resistance and detoxification systems and the high correlation between the degree of metal resistance and the number of overlapping metal-resistance mechanisms strongly suggest a direct causal relationship between high heavy-metal concentrations and metal-resistance systems. However, given the inconsistency of observations of microbial dispersal and a possible preference thereof to spore-forming microorganisms, metal-polluted and metal-enriched sites do not appear to be sufficiently widespread to explain the possible ubiquity of highly metal-resistant bacteria from marine habitats (see later section on multiple resistances).

COMBINATIONS OF STRESSES

Hydrostatic pressure and temperature

One of the first hydrostatic-pressure experiments in marine microbiology documented that increased hydrostatic pressure enhanced growth at elevated temperature and inhibited growth at low temperature among a variety of mesophilic marine bacteria isolated from shallow and deep environments (675). This early work established an important link between two fundamental aspects of living and surviving in the sea. Recent studies of batch-culture growth characteristics are also often coordinated with simultaneous temperature experiments and continue to show an inverse relationship between the effect that hydrostatic pressure and temperature have on cellular growth rates and, more recently, membrane fluidity (144). Hydrostatic pressures equivalent to mid-ocean-ridge or abyssal depths have been shown to increase the cardinal growth temperatures of hyperthermophiles isolated from hydrothermal-vent habitats (100, 247, 468), to increase the maximum growth temperature of a mesophilic *Pseudomonas* strain isolated from the deep sea (293), and to increase the ability of psychropiezophiles isolated from the cold deep sea to tolerate slightly warmer temperatures (151, 657, 659). Stated another way, the higher the hydrostatic pressure, the higher the temperature allowed (for T_{max}) or required (for T_{min}) for growth.

Many hydrostatic-pressure-temperature experiments with batch cultures of hyperthermophilic microorganisms query the nature of the upper temperature limit of life because of the dynamic temperature regime and proximity to intensely hot fluids of up to 400°C in deep-sea hydrothermal-vent and associated subseafloor environments. Hydrostatic pressure was shown to increase growth rates and methanogenesis in *Methanococcus jannaschii* at temperatures up to 90°C and to extend methanogenesis up to 98°C (390). Hydrostatic pressure also raised T_{min} and T_{max} for growth of "*Pyrococcus abyssi*" by 4°C and raised the minimum lethal temperature by an additional 6°C (363). It stimulated growth in *M. thermolithotrophicus* (50), *Pyrococcus* spp. and other hyperthermophiles (276). In addition, hydrostatic pressure increased T_{opt}, T_{max} and the growth rate at temperatures above T_{opt} for certain *Thermococcus* isolates (468). It also increased survival and the induction temperature of a heat-shock response at super-optimal temperatures in *Pyrococcus* strain ES4 (255, 256). Lastly, hydrostatic pressure enhanced survival of hyperthermophiles at temperatures below T_{min} (100).

In vitro studies show that hydrostatic pressure also increases the thermostability of thermophilic and hyperthermophilic proteins, such as certain DNA polymerases (571) and hydrogenases (247). In one experiment, hydrostatic pressure was found to permit protease activity to 130°C (388). (Amylopullulanase activity has been detected at 143°C without hydrostatic pressure but with the addition of 5 mM Ca^{2+} [534].) The hydrostatic-pressure-enhanced thermostability of hyperthermophilic proteins is primarily attributed to hydrophobic interactions (388, 491). Elevation in cardinal growth temperatures of hyperthermophiles (50, 276) and *Escherichia coli* (673) and enzyme thermostabilization (for example, of adenylate kinase) (321, 491) are not always observed, however.

There are fewer experiments with mesophiles. Most mesophilic proteins dissociate or denature under hydrostatic pressure regardless of temperature (39). Hydrostatic pressure increased the survival of *E. coli* and *Streptococcus faecalis* (reclassified as *Enterococcus faecalis* [527]) at temperatures below T_{min} (34). *Staphylococcus aureus* showed a 100-fold increased survival rate under high hydrostatic pressure (200 MPa for 1 h) when pre-incubated at temperatures between –20 and 0°C for 15 min as compared with cultures previously held at 5°C or 30°C (432). (See later section on multiple resistances.)

The combination of hydrostatic pressure and temperature, especially in the context of the cold deep sea, synergistically imposes "freezing" of phospholipid membranes. Both low temperatures and high hydrostatic pressures act to reduce the melting temperature of membranes, rendering them more rigid under deep-sea conditions. To counteract this double effect, membranes tend to have increased proportions of unsaturated fatty acids (39, 658). (See previous section on hydrostatic pressure.)

Osmotic pressure and temperature

The synergistic dependence of microbial growth and nutrient uptake on temperature and salinity has been noted for several decades (270, 404, 562, 563 and references therein). It has even been proposed that more halophilic microorganisms are less able to grow at low temperatures, based on contrasting the minimum growth temperatures of *Halomonas* spp. and haloarchaea isolated from the same Antarctic hypersaline lakes (376). For a given organism, cardinal growth temperatures may vary with salinity (and vice versa), and lag times and maximum cell yield may also depend on the temperature-salinity regime. Growth curves revealed that the optimum and maximum salt concentrations of the moderate halophiles *H. halophila* and *Vibrio anguillarum* (reclassified as *Listonella anguillarum* [354]) increased with increasing temperature (228, 475). Likewise, a greater percentage of strains of *H. halodurans* and *H. elongata* were able to grow at higher salinity only in conjunction with higher temperature (244, 629). "*Pseudomonas halosaccharolytica*" grew faster with elevated salinity at elevated temperature (441). Growth curves of a facultatively halophilic coccoid bacterium isolated from salted mackerel (ultimately classified as *Marinococcus halophilus* [236, 435]) showed that growth at temperatures less than T_{opt} only occurred with decreased NaCl (434). Similarly with haloarchaea, lower temperatures allowed growth at lower salinities and higher temperatures permitted growth at higher salt concentrations (212, 266). With respect to just the maximum salt concentration that permitted growth, experiments with *Moritella marina* (formerly *V. marinus* [604]) (404, 563), certain coliform bacteria (343), several foodborne pathogens (586), "*Pediococcus homari,*" and a *Lactobacillus* sp. (219) showed that T_{max} increased by a few degrees with increasing salinity. By and large, elevated salinity raises both T_{opt} and T_{max}, while decreased salinity allows growth at lower temperatures.

These results are not universal, however. Elevated salinity raised T_{max} for the sea-ice bacterium *Shewanella gelidimarina*, but no salinity effect was observed at temperatures near or below T_{opt} (424). Elevated salinity enhanced growth at T_{opt} for "*P. homari*" and a *Lactobacillus* strain, but $NaCl_{max}$ was still found at T_{opt} (219). $NaCl_{opt}$ and $NaCl_{max}$ did not vary whatsoever with temperature for *H. elongata* (630). While decreased temperature allowed the mesophile *Micrococcus luteus* to grow at lower salt concentrations and higher temperatures permitted growth at higher salinity, no temperature-salinity effects were seen in parallel experiments with "*Mic. morrhuae*" (107). Certain microorganisms (identified as *Brevibacterium* and *Flavobacterium* isolated from solar salterns) grew at lower salinities at lower temperatures, but the dependence of the salinity optimum on temperature was inconsistent (269). *Mor. marina* MP-1, a psychrophile, grew with elevated salinity (8.0%) only at low temperatures between 0 and 10°C; at 20°C, it grew only with 2.5% salt (35). In this case, lower temperatures enabled growth with elevated salinity. Finally, the salt range that permitted growth shrank while the salinity optimum increased when *H. salina* was grown at 42°C versus 32 or 22°C; growth at low salinity only occurred at lower temperatures, but the maximum salt concentration that permitted growth was near the T_{opt} of 32°C, while the smallest salt range was found when cells were grown at 42°C (606).

Elevated-salinity and low-temperature conditions appear to enhance survival of non-growing or stressed cells. While low temperature (4°C) and high salinity (9% NaCl), alone and when combined, decreased the growth rate and cell yield of *L. sakei* below spectrophotometric detection limits, the combination dramatically increased survival times by a factor of seven to sixty-five relative to warmer (30°C) and/or less saline (0% NaCl) conditions, respectively (357). Elevated salinity may also enhance the survival of *Escherichia coli* at low temperature (266). The heat-shock response of *H. halophila* is prolonged at elevated salinity (294); in this case, the physiological response of cells to salt stress is tantamount to a thermoprotective adaptation. In this experiment, it was unclear whether the factors regulating the production of heat-shock proteins were produced in higher quantity at elevated salinity or merely stabilized by the osmoregulatory response and consequent production of compatible solutes (294).

Common ground for the response to sub- or supra-optimal temperatures and salinities may revolve around compatible solutes (521). In *Listeria monocytogenes*, compatible solutes, such as betaine, carnitine and proline, accumulate both at sub-optimal temperatures (–0.1°C) and in response to salt stress (10% NaCl) (547). Glycine betaine in particular may prevent protein aggregation and help maintain membrane fluidity at low temperature (111, 284, 383, 547). Thermophiles, hyperthermophiles, and all thermal classes of methanogens also contain high concentrations (>1 M) of K^+ salts of unusual compatible solutes, such as di-*myo*-inositol-phosphate (DIP; the most common among hyperthermophiles), di-mannosyl-di-*myo*-inositol-phosphate, di-glycerol-phosphate, mannosylglycerate, mannosylglyceramide, and cyclic 2,3-diphosphoglycerate, many of which specifically accumulate in response to increased temperatures and/or salinity (116, 249, 368, 522, 523, 531). For example in *Pyrococcus furiosus*, DIP increased by a factor of twenty at the supra-optimal temperature of 101°C (523) and mannosylglycerate and DIP increased in concentration in response to increases in both temperature and salinity (370). DIP also accumulated because of elevations in both salinity and temperature in *Methanococcus igneus* (reclassified as *Methanotorris igneus* [647]) (116). Likewise, di-glycerol-phosphate accumulated in response to both temperature and salt stresses in *Archaeoglobus fulgidus* (369). *Thermococcus* spp. and other hyperthermophilic archaea have variable responses, with a variety of compatible solutes accumulating specifically in response to temperature or salinity (331, 369). *In vitro* studies reveal that potassium salts of the previously mentioned compatible solutes (523), and even simple salts like KCl and $MgCl_2$ by themselves (358), thermostabilize DNA. Similarly, salt thermostabilizes tRNA in solution and also protects malate dehydrogenase collected from *Haloarcula marismortui* against thermal stress when desiccated and trapped inside salt crystals (583). The trend appears to be that certain salts and compatible solutes function as aids to both temperature and salinity stresses.

Addition of the compatible solute glycine betaine to growth media enabled recombinant protein expressed in *E. coli* to fold properly during osmotic stress. The responsible mechanisms may include induction of heat-shock proteins following salt stress, salt-stabilized periplasmic microenvironments, increased cell survival and function with elevated salinity due to the presence of compatible solutes, and/or, of course, a direct interaction of glycine betaine with the recombinant protein once the compatible solute was transported inside the cells (37). An *in vitro* study concluded that β-hydroxyectoine and betaine not only achieved osmotic balance across the membrane, but also stabilized bovine ribonuclease A against denaturing stressors, perhaps by enabling preferential hydration of the protein (318). Similarly, the compatible solutes glycine betaine and hydroxyectoine protected lactate dehydrogenase against damage by heat, urea and freeze-thaw cycles (220). Ectoine, hydroxyectoine, sucrose, trehalose and maltose all conferred significant protective effects upon lactic dehydrogenase and phosphofructokinase against heat, freezing and desiccation (341).

Hydrostatic and osmotic pressures

There have been fewer studies that examine the combined effects of salinity and hydrostatic pressure on microbial growth. The most obvious ecologically relevant sites at which to study the combined effect of these two parameters on microbial activity are deep brine pools found in the eastern Mediterranean Sea, Red Sea and Gulf of Mexico, but this has rarely been attempted. The Red Sea deeps were initially declared sterile because early culturing efforts at the environmentally appropriate temperatures of 22–56°C, both with and without oxygen, largely failed to obtain any evidence of microbial activity (599, 639). All nitrate-reducer media failed, and high-organic sulfate-reducer media only provided two isolates from Discovery Deep sediment samples collected within the brine-seawater interface and the brine itself (639). The combination (but not any given variable alone) of high hydrostatic pressure, high salinity, warm temperatures, anoxia and high concentrations of heavy metals was thought to totally prevent life (639). A return to the Red Sea brines and brine-seawater interface with updated molecular methods, novel cultural approaches and analysis of potential biomarker molecules has begun to address the sterility hypothesis (21, 165, 166, 182, 387).

The first isolate described from the Red Sea brines was *Flexistipes sinusarabici*, an anaerobic bacterium with a T_{opt} of 45–50°C obtained from multiple locations at 2000-m depth in the Atlantis II Deep (end-member brine characteristics: 25% salt, 64°C, pH 5.7; Table 0.2) (182). The isolate grew over a 3–18% NaCl range (the optimum was not determined) and at pH 6.0, but not pH 5.5 (182). The upper levels of the brine are less salty and at or above pH 6.0 (239), and presumably this organism would be able to grow in these environments but, given that its T_{min} was 30°C, not in the overlying seawater at 22°C (182). Oxygen and hydrostatic-pressure sensitivities were not reported, however.

Most recently, culturing work from the Kebrit Deep brine-seawater interface in the Red Sea (end-member brine characteristics: 26% salt, 23°C, pH 5.5; Table 0.2) led to the isolation of moderately to extremely halophilic, mesophilic, heterotrophic anaerobes of the bacterial genus *Halanaerobium* from 1468-m seawater depth (165). Their fatty acids contained high proportions of monounsaturated fatty acids like other members of *Halanaerobium* but with unusual double bond positions (165), perhaps a reflection of a hydrostatic-pressure adaptation. The novel *Halanaerobium* isolates grew anaerobically with 5–34% NaCl (optimum of 10–20% NaCl) and between 18 and 48°C (optimum of 30–45°C) at pH 6.5, fitting physiologically well with the conditions of the brine-seawater interface (165). A 16S rRNA gene clone library of the Kebrit Deep brine-seawater interface revealed sequences primarily very similar to the characterized *Halanaerobium* isolates and also to other cultivated bacteria such as *Clostridium* and *Propionibacterium* (165). There is also a recent report of the isolation of a novel facultatively aerobic genus within the γ-*Proteobacteria*, *Salinisphaera shabanensis*, obtained from the brine-seawater interface at 1325-m depth in the Red Sea (21). It was found to be piezotolerant and grew with 5–20% NaCl at up to 15 MPa (but not at 20 MPa) (21). At 0.1 MPa, it grew over a 1–28% NaCl range (optimum of 10% NaCl) and accumulated up to 4 M total of ectoine, betaine and small amounts of glycerol when grown in a medium with 25% NaCl (21). (This is the first report of glycerol accumulation among bacteria, though its concentration did not track the extracellular salt concentration [21]). Optimal growth conditions matched the approximate environment of the brine-seawater interface, though the organism would also be able grow in other seawater habitats (21). No hydrostatic-pressure-salinity growth curves have been reported yet for any of these brine-derived bacteria.

16S rRNA gene clone libraries of the sediment community in the anoxic Kebrit Deep at 1515-m depth included sequences of common uncultivated marine Group II and III euryarchaeota, novel archaea, and novel bacteria that branch deeply between the thermophilic *Thermotogales* and *Aquificales* (166). In addition, isoprenoid analysis of sediment samples from the Kebrit and Shaban Deeps indicate a significant contribution to the organic-carbon pool from anaerobic methanogens and acidothermophilic archaea, though, interestingly, the archaeal isoprenoid signatures were not found in asphalt-impregnated sulfide structures, and no haloarchaeal isoprenoids were detected in any sample (387).

The combination of hydrostatic and osmotic pressures affects microbial growth in various ways. Piezosensitivity was shown to depend on salt concentration for *Moritella marina* MP-1, whereby a decreased salinity of 3.5 versus 0.8% decreased P_{max} from 42 to 9 MPa at 9°C and from 33 to 6 MPa at 4°C in defined basal media (461). Low concentrations of chloride salts of Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} have likewise been shown to decrease the piezosensitivity of *Escherichia coli* exposed to the extremely high hydrostatic pressure of 270 MPa; in addition, increasing the Ca^{2+} concentration quantitatively further mitigated piezosensitivity (242). Addition of Mg^{2+} or Ca^{2+} salts enhanced the piezotolerance of *Streptococcus faecalis* (reclassified as *Enterococcus faecalis* [527]) — but not of *E. coli* or *Saccharomyces cerevisiae* — increasing the growth rate at 41 MPa and boosting P_{max} from 55 to 70 MPa (361). Elevated salinity enhanced the piezoresistance of a halophilic, euryhaline strain of *Micrococcus roseus* (reclassified as *Kocuria rosea* [560]) isolated from open seawater, whereby growth with 15% NaCl (versus 3% NaCl) enabled 97% cell survival at 138 MPa (580). Lastly, ethanol, considered an agent of osmotic pressure at low concentrations, combined with hydrostatic pressure alleviates inhibition of cell growth in *E. coli* caused by either stressor alone; above 20 MPa, the combination reverted cell morphology from an elongate shape to the canonical cell size (579).

The response to hydrostatic and osmotic pressures may be linked via compatible-solute production. Hydrostatic pressure and salinity non-linearly augment intracellular solute accumulation in the psychropiezophile *Photobacterium profundum* strain SS9, whereby solutes such as glutamate and betaine can be considered both osmolytes and piezolytes (see Fig. 5 in reference 365). The glutamine synthetase gene *glnA* is up-regulated in response to hydrostatic pressure in the piezophile *Shewanella violacea* (264); glutamine, glutamate, or some derivative thereof, may serve as a piezolyte in this deep-sea bacterial species. In the animal realm, the concentration of unusual intracellular solutes, including trimethylamine *N*-oxide (TMAO), in muscle tissue is correlated with the depth of capture among certain fish, skate, crab and shrimp specimens (213, 315). It has been shown that TMAO stabilizes proteins against hydrostatic pressure, increased salt concentration and urea *in vitro* by helping refold misfolded polypeptides (656).

Additional *in vitro* studies show similar counterbalances between hydrostatic and osmotic pressures. The dissociation of Arc repressor protein from DNA (its substrate) decreased linearly with increasing glycerol concentration (446). Hydrostatic pressure was also shown to reverse the effects of osmotic pressure (created with organic compounds) on DNA site selection by the restriction endonucleases *Eco*RI, *Bam*HI, *Pvu* II and *Eco*RV, highlighting the opposing interplay that these two factors have on hydration of proteins and protein-substrate complexes (495, 496).

Mechanistically, hydrostatic and osmotic pressures exert opposing influences on protein hydration, and therefore they tend to cancel the deleterious effects of the other (365, 656). Hydrostatic pressure favors hydrated protein surfaces due to volume considerations, but osmotic pressure, due to solutes like TMAO, glutamate and betaine, favors folded proteins via preferential exclusion and the minimization of bound water (365, 497, 656).

Heavy metals and antibiotics

Initial metal-resistance studies revealed that cells “infected with R factors,” later called plasmids, contained not only genes for resistance to antibiotics and various other stresses, but also genes encoding highly specific metal resistance (554). Metal-resistant microorganisms are also typically antibiotic-resistant, though the genes for each type of resistance may be located separately on the chromosome and on a plasmid (448). For example, high correlations between Pb and general antibiotic resistance, Cu and penicillin resistance, and Ni and ampicillin resistance were found among eighty-one aerobic heterotrophic marine bacteria isolated near a polluted site (513). Similarly, Hg and ampicillin resistance were co-selected in polluted coastal sediment microbial communities (591). There are few explanatory hypotheses for the apparent link between antibiotic and metal resistance, in spite of how precisely tuned the metal- and antibiotic-detoxification systems appear to be.

I suggest that one connection, an "environmental" hypothesis, is that both heavy metals and naturally produced antibiotics (released by fungi, actinomycetes, etc.) are accumulated in exopolymeric substances produced by animals and in biofilms. If so, it would be instructive to contrast metal-antibiotic resistance patterns from biofilm, free-living and animal-associated populations of microorganisms. An alternative "physiological" hypothesis is that there is some underlying link via a stress-response system (as proposed in the following section for hydrostatic-pressure and salinity stresses). Neither notion, however, explains the remarkable degree of resistance to metals and antibiotics given the reduction in evolutionary fitness caused by maintaining these genes in the genome nor why a particular antibiotic resistance is linked with a particular metal resistance.

Multiple resistances, sigma factors, and the general stress response

Stressors typically induce both specific and general stress responses, such that exposure to an environmental stress may engender increased resistance to it and to seemingly dissimilar stressors (Table 0.3; 246, 466). The literature on food spoilage microbiology and foodborne pathogens is replete with examples of how attempts to kill bacteria usually result in increasing their resistance to other stressors, which is costly and frustrating to the food industry (466). From the medical field, general stress proteins are hypothesized to enable the opportunistic pathogen *Mycobacterium tuberculosis* to exist in a latent state in billions of humans until the host is weakened by some other cause (438). It is difficult to actually kill bacteria that have been pre-conditioned by exposure to multiple stresses. For example, only the most severe combination of conditions — a very high hydrostatic pressure of 345 MPa, a temperature of 50°C, and a pH of 4.5 — destroyed cells of *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* spp. (15). Less extreme conditions of hydrostatic pressure and temperature, especially those relevant to the environment, are frequently insufficient to kill cells and often induce a stress response equipping them to defend against subsequent stress.

There are numerous examples of multiple resistances among bacteria, which are commonly induced during stationary-phase, starvation and osmotic stresses. In *Enterococcus faecalis*, starvation stimulated resistance to heat, ethanol, acid, osmotic, oxidative, bleach and bile-salt stresses (211). Similarly, salt stress induced increased tolerance to ethanol, detergents, hydrogen peroxide and previously lethal temperature in *E. faecalis*, as well as production of an array of stress proteins (185). Mg^{2+} and Ca^{2+} mitigated the deleterious effects of acidic pH on *E. faecalis* at high hydrostatic pressure (361). Stationary-phase *E. coli* cells had enhanced resistance to heat, oxidative stress and antibiotics relative to log-phase cultures; roughly one quarter of proteins induced during salt shock were also produced during starvation (281). A proteomics/mass-spectrometry investigation of *L. monocytogenes*, a common foodborne pathogen, concluded that elevated salinity induced several general stress proteins in addition to osmoregulatory genes, including those found in *Bacillus subtilis* under nutrient starvation and ethanol, heat and salt stresses (159). As another example of a Gram-positive bacterium, when *B. subtilis* was exposed to elevated salt or temperature, it tolerated previously lethal levels of the respective stressor; in addition, heat shock created tolerance to previously lethal levels of salt, though the converse was not quite as robust (618). By and large, these examples do not immediately resonate with combinations of stressors that would ever be encountered in the environment. Their utility, however, is in highlighting the integrated stress response as a means to cope with cellular damage regardless of cause.

Bacterial mutational studies paint the same theme of various multiple resistances that are of questionable environmental relevance at first glance (Table 0.4). Mutants of *E.*

coli that better survived exposure to 220–800 MPa of hydrostatic-pressure treatment were also more heat resistant to 58–60°C at 0.1 MPa (241). Hydrostatic-pressure-resistant mutants of *E. coli* were also found to have enhanced resistance to heat, acid, oxidative and osmotic stresses and produced increased amounts of heat-shock proteins (6). Transient heat-shock and salt stress in these mutants conferred increased piezoresistance, suggesting that managing hydrostatic pressure is likely integrated with the stress-response system (6). *L. monocytogenes* mutants with increased sensitivity to high salinity were also more sensitive to alkaline pH (207). Heat-resistant strains of *E. coli* O157 tend to be more resistant to hydrostatic pressure and, in some instances, also better able to withstand acid, oxidative and salt stresses (48). Again, the lesson from these studies is that injury to proteins, membranes and DNA — regardless of stressor — induces a stress response, which in turn activates cellular damage-control and repair systems.

The cold-shock response provides a more ecologically relevant example of how one stressor (low temperature) enhances the ability of cells to withstand other stressors that would be expected to accompany cold environments. Generally speaking, cold-shock proteins, detected after exposure to low temperature, can also be expressed constitutively during optimal growth or induced by starvation and other stress responses; they are often DNA-binding proteins and may serve as mRNA chaperones (174). *L. monocytogenes* produces cold-shock proteins in response to both cold temperatures (10°C at 0.1 MPa) and high hydrostatic pressure (50–200 MPa at 30°C); cells had a 100-fold increased survival rate under high hydrostatic pressure if preceded by exposure to sub-optimal growth temperatures (643). Similarly, hydrostatic-pressure-induced proteins produced by *E. coli* included cold-shock and heat-shock proteins, with the magnitude of the response positively correlated with the amount of hydrostatic pressure applied (40, 642). (Another experiment showed that the gene *recD*, which encodes for a DNA recombination and repair protein, must be expressed in the psychropiezophile *Photobacterium profundum* strain SS9 for growth under deep-sea hydrostatic pressures [54].) As discussed earlier, low temperatures and high hydrostatic pressures affect proteins and membranes in similar ways, such that this connection between these two linked parameters is sensible. However, cold shock is not synonymous with cold adaptation (not to mention that *E. coli* and *L. monocytogenes* are not marine bacteria), so more research would shed light on how these deep-sea stressors interact in the context of a stress response. A relevant experiment using a psychrophilic marine *Vibrio* sp. found that starvation conditions significantly enhanced subsequent exposure to hydrostatic pressure, which clearly would increase cell survival after transport to the deep sea (433). Other experiments could include contrasting the cold-shock and cold-adaptive responses, with and without hydrostatic pressure, of psychrophilic microorganisms isolated from cold high-hydrostatic-pressure (deep-sea) and low-hydrostatic-pressure (sea-ice) environments.

Sigma factors are small proteins that bind with RNA polymerase to regulate the expression of specific suites of genes. There is evidence from mutational and genome-enabled studies that sigma factors are induced by various environmental stresses and therefore serve as the underpinning, overarching activators of certain generalized, cross-regulated, stress-response systems (Table 0.5). The most studied sigma factors are σ^S in Gram-negative bacteria and σ^B in Gram-positive bacteria (246). In *E. coli*, σ^S is encoded by the *rpoS* gene and itself catalyzes the regulation of 50–60 other genes, including certain catalases, proteases, ATPases, endonucleases, DNA-binding proteins, trehalose-synthesis enzymes, osmoprotectant transporters, and proteins of unknown function (see Fig. 2 in reference 245; 466). The factors σ^S and σ^B respond both to starvation experienced during stationary-phase growth and to elevated salt concentrations in *E. coli* and *B. subtilis*, respectively (246; see Fig. 2 in reference 466). The piezophile *Shewanella violacea* strain DSS12 contains a hydrostatic-pressure-responsive sigma factor that is homologous to σ^{70} in other Gram-negative bacteria (199, 413).

There are numerous examples of the ways in which sigma factors are stimulated into action. When *E. coli* is exposed to high concentrations of NaCl, forty-five genes are up-regulated, including not only classic osmoregulatory genes, such as betaine and proline transporters, but also a suite of genes normally active during stationary-phase growth that are by and large regulated by σ^S (640). *E. coli rpoS* mutants are also more sensitive to the very high hydrostatic pressure of 500 MPa (493). The factor σ^B in *L. monocytogenes* is required for tolerance of low pH (648) and is induced by high salinity and low temperature (46). Production of σ^B in "*Brevibacterium flavum*" is stimulated by acid, salt, ethanol, heat and cold stresses (230), whereas, in *Streptomyces coelicolor*, the relationship between stressor and induction of sigma factor is more so at a one-to-one ratio (616). Two new sigma factors discovered in *Deinococcus radiodurans* are induced by heat and ethanol stress (528). The sigma factor σ^M is induced in *Bacillus subtilis* in response to antibiotics that inhibit cell-wall synthesis, ethanol, heat shock and acid pH (but not hydrogen peroxide, detergents or alkaline pH) and is required for growth during salt stress (587). Among *Vibrio* spp., σ^S plays a role in diverse stress and virulence functions as well (263). Sigma factors are the premier regulators of the stress response; it would be incredibly informative to investigate the degree to which they are active in natural populations of microorganisms.

Compatible solutes represent one possible mechanism that links sigma-factor-mediated multiple resistances. As discussed earlier, compatible solutes protect cell components against numerous stressors, including desiccation, freezing, dehydration, elevated temperature and oxygen radicals; they can thus be considered general "stress protectants" (523). The factor σ^B in *L. monocytogenes* responds to both high salt concentrations and low temperatures, enabling the uptake of compatible solutes which appear to confer protection against both osmotic and low-temperature stresses (45, 46). But there appears to be a different order of events in *E. coli*: the factor σ^S is stimulated normally during stationary phase and in log phase during osmotic stress, heat shock or acid shock, but, after osmotic stress, K^+-glutamate accumulates and appears to stimulate

σ^S production via *rpoS* transcription (248). Another compatible solute, trehalose, protects membrane structure (243) in addition to proteins (203). Further studies are needed to decipher possible reciprocity between the sigma-factor and compatible-solute systems.

Another compound that achieves protection against a variety of stresses is glutathione (γ-GluCysGly), a tripeptide rich in thiol groups that is common among Gram-negative bacteria (175). In *E. coli*, glutathione protects against toxic electrophiles (180), elevated salinity (551) and oxidative stress (101, 552). Though rare among Gram-positive bacteria (175), glutathione was also documented to provide protection against oxidative stress in *Lactococcus lactis* (339). It seems likely as well that glutathione could aid in heavy-metal detoxification via metal-cation sequestration with the thiol groups. Metallothionein, a thiol-rich polypeptide comprised of ~55 amino-acid residues found in *Synechococcus* spp. (602), yeast (595), plants and animals, protects against heavy-metal stress by sequestering metals with sulfhydryl functional groups (229, 545, 603). Though metallothionein has yet to be found in other bacteria (largely for a lack of looking), it is possible that this compound also protects cells against multiple stresses, at least in *Synechococcus*.

The synergistic behavior of sigma factors and compatible solutes has been documented in additional studies. Low-temperature, heat-shock, salt, ethanol, acid, nutrient and oxygen stresses each induced a σ^B-mediated general stress response involving over 150 general stress genes in *B. subtilis*, "preemptively" preparing cells for other stresses (79, 245, 246). Addition of glycine betaine to the growth medium significantly improved growth in wild-type cells and rescued the wild-type phenotype in SigB-deficient mutants, indicating that the σ^B response and accumulation of compatible solutes are independent mechanisms for coping with low-temperature stress (79). Also indicative of this overlap, glycine betaine was found to reduce osmotic-stress up-regulation of σ^S

(and to increase temperature sensitivity) in *Salmonella typhimurium* and to inhibit osmotically induced stress genes and multiple resistances (466).

SUMMARY

There are a variety of spatially discontinuous cold, cool, warm and hot metal-enriched deep-sea brine environments along mid-ocean ridges and in selected pockets in the Gulf of Mexico, the eastern Mediterranean Sea, and the Red Sea, and more undoubtedly will be discovered with time. Detailed hydrologic and chemical models and analyses constrain which microbial physiologies could exist in these environments, but little is known about which microbes actually inhabit these niches (or even if the very hot brines can be cooled while retaining their salt and become habitable). The cold, cool and warm brine habitats are especially confounding given the following reasons. First, many deeps are relatively young, and I wonder how quickly anaerobic extreme halophiles can find their way there. In contrast to anaerobic hyperthermophiles that may colonize new hydrothermal-vent habitats via subseafloor routes or during transit through seawater, anaerobic extreme halophiles may not be as durable when exposed to seawater salinity. Some simple survival experiments would clarify the possibilities. Alternatively, analogous to the possible hydrothermal-vent connectivity via the mid-ocean-ridge subseafloor (570), brine deeps may be connected by subseafloor hypersaline pathways in these regions. Secondly, microbial growth may be constrained by both the thermodynamics of anaerobic metabolism and the energy expenditure required to cope with elevated salinity, opening the question of which lifestyles are possible in these environments. Lastly, how relevant are these isolated environments in the broader marine perspective of the nearly ubiquitous genus *Halomonas* and other moderately halophilic bacteria?

While there is a solid framework outlining how marine microorganisms cope with stressors relevant to the ocean and hydrothermal-vent ecosystems (for example, temperature, hydrostatic pressure, salt and heavy metals), much of the modeling of the microbial stress response is based on research with *Escherichia coli* and by the food industry with non-marine foodborne pathogens. Some of the experiments with non-marine microorganisms have direct applicability to the organism in question, such as the starvation and desiccation response of the soil organism, *Bacillus subtilis*, or the survival along the pathway that ingested pathogens like *Listeria* follow through an acid stomach and a high-osmolarity, low-oxygen small intestine (548). But overall, salted mackerel and rotting meat serve as oddball venues from which to extrapolate the nature of microbial multiple resistances in marine environments. Potential mechanistic links between a generalized stress response and heavy-metal resistance remain elusive, in part because very few studies incorporate exposure to toxic metals along with other stress conditions. Regardless, some key links have been forged between the microbial response to temperature, hydrostatic pressure and salinity: (i) sigma factors that are induced by a multitude of stressors prepare cells to cope with a multitude of other stressors and catalyze the repair of damaged cell components; and (ii) compatible solutes appear to act synergistically with sigma factors to accomplish the same protective goals. These building blocks christen construction on a model that chips away at the rationalization that the presence of *Halomonas* in the ocean may reflect proximity to a briny habitat (304).

The specific motivating questions in this thesis include the following. Which salient features of euryhaline and moderately halophilic bacteria contribute to their apparent success in the deep sea? To what extent and in what manner do *Halomonas* spp. tolerate an array of heavy metals? How does hydrostatic pressure constrain where in the ocean *Halomonas* spp. can grow? Are *Halomonas* spp. restricted to hydrothermal-vent ecosystems or can they proliferate in all deep environments? Do *Halomonas* isolates from hydrothermal-vent environments represent distinct populations associated with vent and deep-sea ecosystems? What does the euryhaline phenotype of *Halomonas* truly mean for the ecology of this and related genera?

The chapters that follow describe the ecology of the moderately halophilic genus *Halomonas* in deep-sea and hydrothermal-vent habitats. Characterization of novel isolates obtained from a variety of vent environments highlight the physiological versatility of this group with particular regard to the organic compounds they consume, Cd^{2+} resistance, and the temperature and salinity ranges (at 0.1 MPa) over which they grow. Phenotypic characteristics are consistent with inhabiting a cool to warm shallow subseafloor environment associated with hydrothermal vents. Molecular-phylogenetic analyses using novel primers designed to detect *Halomonas* and other moderately halophilic bacteria were performed on low-temperature hydrothermal fluids and deep seawater, and the results suggest a distinct biogeography for members of this group (including some of the novel species) and indicate the existence of resident subseafloor and deep-sea populations. The *in situ* activity of these populations initially remained an open question, however, given the low minimum temperature for growth (–1 to 2°C) at 0.1 MPa of the novel hydrothermal-vent species and closely related taxa in conjunction with hydrostatic-pressure-temperature synergism on cellular functions. It was thus critical to perform hydrostatic-pressure-temperature experiments in order to constrain where in the deep sea the *Halomonas* isolates can grow. These experiments also provide an opportunity to examine the combined interaction of hydrostatic pressure and temperature with salinity to investigate possible hydrostatic-pressure-salinity and temperature-salinity synergies as a means to understand why euryhaline bacteria might be found in deep-sea environments distant from known hypersaline habitats. Growth curves and cytosolic and membrane protein patterns indicate hydrostatic-pressure-salinity effects, particularly at low temperature and/or high hydrostatic pressure, that may be connected via a microbial stress response. A linked stress response between Cd^{2+} and Zn^{2+} resistance and nutrient limitation and/or reduced oxygen levels was also suggested by the efficiency with which cells precipitated Cd^{2+} and Zn^{2+} (possibly as

CdS or $CdHPO_4$; unknown for Zn) but only under static growth conditions with high cell densities during late exponential-phase and stationary-phase growth. The ability of *Halomonas* spp. to tolerate an array of heavy metals (Co^{2+} and Cu^{2+} as well) and to impact hydrothermal-vent metal biogeochemistry may be especially relevant in biofilms and in animal mucous secretions where heavy metals accumulate to levels far above the surrounding fluids.

Table 0.1 Terminology (including example organisms and habitats) defining the relationship of microorganisms to temperature, hydrostatic pressure, salinity and heavy metals.

Parameter	Term	Definition	Example organism	Example habitats
Temperature	Obligately psychrophilic	No growth above 15°C	*Colwellia psychrerythraea*	Polar sediments, sea ice
	Psychrophilic	Fastest growth* <20°C	*Moritella marina*	Sediment, sea ice
	Psychrotolerant	Growth down to <0°C; fastest growth 20–40°C	*Halomonas neptunia*	Deep and surface seawater
	Mesophilic	Fastest growth 20–45°C	*Pseudomonas putida*	Surface seawater
	Thermophilic	Fastest growth 45–80°C	*Clostridium caminithermale*	Hydrothermal vents
	Hyperthermophilic	Fastest growth >80°C	*Pyrococcus furiosus*	Hydrothermal vents
Hydrostatic pressure	Piezotolerant	Fastest growth at 0.1 MPa; decreasing growth rate under hydrostatic pressure	*H. pacifica*	Water column
	Piezophilic	Fastest growth >0.1 MPa; growth at 0.1 MPa	*Photobacterium profundum*	Sediment
	Obligately piezophilic	Fastest growth >0.1 MPa; no growth at 0.1 MPa	*Colwellia hadaliensis*	Deep-sea trench
Salinity	Nonhalophilic	Fastest growth 0% salt; growth rate decreases with increasing salinity	*Escherichia coli*	Humans
	Slightly halophilic	Fastest growth 0–2% salt	*Vibrio cholerae*	Estuary
	Halophilic	Fastest growth at seawater salinity	*V. splendidus*	Seawater
	Moderately halophilic	Fastest growth 5–10% salt	*H. elongata*	Hypersaline lagoon
	Extremely halophilic	Fastest growth >10–15% salt	*Halobacterium salinarum*	Dead Sea
	Euryhaline	Growth over a wide salt range	*H. elongata*	Solar saltern
Metals	Metal-sensitive	Sensitive to µM metal concentrations	*E. coli*	Humans
	Metal-resistant	Growth with metals at concentrations above which *E. coli* tolerates	*Ralstonia metallidurans*	Polluted sediment

* The term "fastest growth" is used here where traditionally "optimal growth" would be used.

Table 0.2 Location, depth, size and chemistry of cold, cool and warm brine deeps.

Region	Basin	Depth* (m)	Width (km)	Thickness (m)	T (°C)	S (%)	Selected chemical characteristics	References
Eastern Mediterranean Sea	Tyro	3473	3	70	13	26	Elevated CO_2, H_2S, NH_3, Co, Cu and Zn	133, 285, 610
	Bannock†	3150	7	100–150	ND‡	ND	Elevated H_2S, Co, Cu and Zn; gypsum precipitation	535, 610
	Urania	3462	6	200	17–28	12		377, 634
	Atalante	3344	ND	ND	ND	18§	Elevated K^+	377, 634
	Discovery	3580	ND	ND	ND	>31§	Elevated Mg^{2+}; slightly depleted Na^+ relative to seawater ratio; 40% above NaCl saturation in sediment pore fluids	377, 634
Gulf of Mexico	Orca	2400	23	200	5.6	26	Elevated K^+, Ca^{2+}, PO_4^{3-}, NH_3, Si, Mn^{2+} and Fe^{2+}; depleted Mg^{2+} and SO_4^{2-}; no NO_3^-; salinity at NaCl saturation	541, 597, 540
Red Sea	Atlantis II	1910	ND	160	56–67	16	pH 5.2; Elevated CH_4, ethane, Na^+, K^+, Ca^{2+}, Cl^-, Fe, Mn, Zn, Pb and Si; depleted Mg^{2+}, SO_4^{2-}, NO_3^- and O_2; no H_2S	76, 77, 84, 238, 239, 389, 574, 530
	Discovery	2040	ND	>160	45	16	pH 6.4	76, 238, 239
	Chain	2010	ND	94	56	32		76
	Shaban	1325	ND	215	25	15	pH 5.7–6.2	76, 238, 239
	Kebrit	1466	ND	83	24	15	pH 5.5	238, 239

* Depth of brine-seawater interface

† Depth and thickness inferred from measurements of sound velocity because the CTD cable was not long enough.

‡ ND, not determined.

§ Pore salinity of surface sediment.

Table 0.3 Stress-induced multiple resistances.

Organism	Stress imposed	Resulting in enhanced resistance to:									Reference
		High T	High P	High S	Low pH	Anti-biotics	Deter-gents	Oxidants	EtOH	Bleach	
Bacillus subtilis	High S	+		+							618
B. subtilis	High T	+		+[*]							618
Enterococcus faecalis[†]	High Mg^{2+} or Ca^{2+}				+[‡]						361
E. faecalis	High S	+[*]					+	+	+		185
E. faecalis	Starvation	+		+	+		+	+	+	+	211
Escherichia coli	Stationary phase	+				+		+			281
Listeria monocytogenes	Cold shock		+								643
Salmonella spp.	High T			+							287
Vibrio sp.[§]	Starvation	+									472

[*] The stress imposed enabled cells to survive a previously lethal exposure to the given subsequent stress.

[†] Formerly *Streptococcus faecalis* (527).

[‡] Low pH concomitant with hydrostatic pressure.

[§] A psychrophilic *Vibrio* sp. exposed to a super-optimal temperature of 17°C, 4°C above its T_{max}, after starvation.

Table 0.4 Multiple resistances in *Escherichia coli* mutants.

Primary resistance factor	Resulting enhanced resistance to:					References
	High T	High P	High S	Low pH	Oxidants	
High P	+*		+	+	+	6, 241
Heat shock		+				6
High S		+				6
High T		+	+	+	+	48

* The stress imposed enabled cells to survive a previously lethal exposure to the given subsequent stress.

Table 0.5 Response of sigma factors to various stresses.

Organism	Sigma factor (σ)	Stress that induces σ										References
		High T	Low T	High P	High S	Low pH	Starvation	Antibiotics	EtOH	Low O_2	Stationary phase	
Bacillus subtilis[*]	σ^M	+			+	+		+	+			587
B. subtilis	σ^B	+	+		+		+		+	+		79, 245, 466
B. subtilis	σ^W				+							565
"*Brevibacterium flavum*"	σ^B	+	+		+	+			+			230
Escherichia coli	σ^S	+		+	+	+	+				+	6, 248, 466
Listeria monocytogenes[†]	σ^B	+	+		+	+					+	45, 46, 307, 648
Vibrio vulnificus[‡]	σ^S				+	+						263

[*] The sigma factor σ^M of *B. subtilis* was not induced by high pH or hydrogen peroxide; other stresses were not tested.

[†] Virulence genes of *L. monocytogenes* were also induced by σ^B.

[‡] In *V. vulnificus*, σ^S was not induced by hydrogen peroxide, though it may play a role in virulence.

CHAPTER ONE: Characterization of novel *Halomonas* species from hydrothermal-vent environments

Halophilic and halotolerant microorganisms are typically isolated from hypersaline environments such as lagoons, saline and haloalkaline lakes (the Dead Sea, Great Salt Lake, thalassohaline Antarctic lakes, African rift soda lakes), artificial salterns, cured and salty foods, briny petroleum reservoirs, hypersaline desert soils, and sea ice (5, 69, 70, 160, 161, 192, 273, 367, 405, 477, 485, 525, 606, 613, 614, 629, 666). They have also recently been found in the very dry environment of the walls and murals of a Medieval chapel (251). This skew in sampling has obscured the observation that halophilic and halotolerant bacteria are easily isolated from marine sediments, the open ocean, and low-temperature, seawater-dominated hydrothermal-vent environments (42, 43, 170, 186, 304, 456, 524, 578, 585, 615, 665), where the salinity does not change significantly on the scale of microbial biochemistry. It remains an open question why these halophilic and remarkably halotolerant bacteria inhabit surface seawater and the deep sea distant from coastal hypersaline habitats.

Moderately halophilic bacteria are surprisingly abundant in surface seawater, at mid-ocean depths, in deep water, and in low-temperature hydrothermal fluids and plumes, sometimes comprising upwards of 10% of the total marine microbial assemblage (304). Strains of *Halomonas* and *Marinobacter* as identified by 16S rRNA gene sequence analysis were consistently found in these water-column and hydrothermal-vent samples (304).

Twenty-four species of *Halomonas* have previously been described (22, 24, 251, 485, 504, 666). The strains described here are the first to be characterized from the deep sea and from hydrothermal-vent habitats, and three isolates are also the first isolated using oligotrophic quantities of organic carbon to be characterized. In addition, our results show that four isolates represent new species of the genus *Halomonas*. The proposed names are *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* sp. nov.

METHODS

Sample collection

In 1991, hydrothermal-plume temperature and particle anomalies above the Main Endeavour Field (MEF, 47°57'N 129°06'W) on the Endeavour Segment of the Juan de Fuca Ridge were detected using a conductivity-temperature-depth-transmissometry package. Plume water was procured with Niskin bottles from ~2000-m depth. In 1995 and 1998, sulfide rock and low-temperature hydrothermal-fluid seafloor emissions were collected with manipulator arms, Niskin bottles or titanium syringe samplers (624) mounted onto the Deep-Submergence Vehicle *Alvin* or the Remote-Operated Vehicle *ROPOS*. These samples were obtained from Axial Seamount (45°56'N 129°59'W) located on the Juan de Fuca Ridge during *ROPOS* dive 462 to Cloud vent (27°C), and from the Southern East Pacific Rise (SEPR, 17°25'S 113°12'W) during *Alvin* dive 3300 at a 9°C vent site. The MEF, Axial Seamount and SEPR sites are located at seawater depths of 2200 m, 1530 m and 2580 m, respectively.

Enrichment and isolation

For strains Eplume1^T, Eplume2 and Esulfide1^T, the enrichment medium contained synthetic seawater (SSB), 10 ml (per liter of SSB) of Trace Elements F solution, and additional nutrients. SSB has (per liter deionized water) 19.6 g NaCl, 3.3 g Na_2SO_4, 0.5 g KCl, 0.05 g KBr, 0.02 g H_3BO_3 and 8.8 g $MgCl_2 \cdot 6H_2O$ (33). Trace Elements F solution consists of (per liter of deionized water) 0.05 g $Al_2(SO_4)_3$, 0.1 g H_3BO_3, 0.05 g LiCl, 0.1 g $Na_2MoO_4 \cdot 2H_2O$, 0.05 g KBr, 0.05 g KI, 0.05 g NaF, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.005 g $BaCl_2$, 0.005 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.2 g $MnCl_2 \cdot 4H_2O$, 0.01 g $NiCl_2 \cdot 6H_2O$, 0.005 g Na_2SeO_4, 0.005 g $SrCl_2 \cdot 6H_2O$, 0.005 g H_2WO_4 and 0.005 g

$VOSO_4 \cdot H_2O$ (33, 467). For strains Eplume1^T and Eplume2 the trace elements solution also contained 0.015 g $NiCl_2 \cdot 6H_2O$ but no NaF. The additional components of the enrichment medium are (per liter of SSB) 1.605 g $NaNO_3$, 5.0 g $Na_2S_2O_3 \cdot 5H_2O$, 0.02 g yeast extract, 1.0 g PIPES buffer, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.15 g $MnSO_4 \cdot H_2O$, 0.1 g $CaCl_2$, 0.430 g $(NH_4)_2SO_4$, 0.036 g KH_2PO_4 and 13 g purified agar. The Trace Elements F solution, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $CaCl_2$, $(NH_4)_2SO_4$, 0.605 g of the $NaNO_3$ and KH_2PO_4 were added to the medium after autoclaving via filter sterilization. The pH was adjusted to 7.0.

Strains Eplume1^T and Eplume2 were obtained from plume water that was passed through 0.2-µm polycarbonate filters, placed onto agar slants in stoppered serum bottles, and incubated at 2°C with an air headspace into which CH_4 was injected. Strain Esulfide1^T was isolated from a sulfide rock sample that was smeared onto an agar surface and incubated under an air headspace at 20°C.

Strains Althf1^T, Slthf1 and Slthf2^T were enriched with the medium described above except that it contained 160 g l^{-1} NaCl and 1.0 g l^{-1} trisodium citrate but no agar. Broth tubes were incubated with an air headspace at 20°C onboard ship. These tubes were part of quantitative enrichments using the Most Probable Number (MPN) technique (227).

Growth experiments

The growth medium used to determine the temperature and pH ranges was a broth containing SSB (3.2% total salts; 0.45 M Na^+), 10 ml of Trace Elements F solution, the additional components listed above (except for PIPES), and 1.0 g l^{-1} trisodium citrate. The pH range was performed at 20°C. For the temperature and salt growth curves, Tris buffer (2.4 g l^{-1}) was added and the pH was adjusted to 8.0. Cells were tested for growth at –5°C in this medium with 160 g l^{-1} NaCl (18% total salts; 2.9 M Na^+). For the salt growth curves (performed at 30°C), the amount of NaCl varied from 0 to 250 g

l^{-1}, whereby the total amount of salt ranged from 21 to 270 g l^{-1} (0.12–4.4 M Na^+). Growth was also tested with 0.5% total salts and without any salt. Cd^{2+} tolerance (0.05, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM) was assayed (20°C, SSB salt concentration, pH 7.0) by adding a filter-sterilized solution of $CdCl_2 \cdot 2.5H_2O$ dissolved in SSB, and the lowest concentration that completely inhibited growth is reported. Anaerobic growth and anaerobic nitrate reduction were tested (20°C, SSB salt concentration, pH 7.0) by dispensing the growth medium into Balch tubes, purging and filling the tubes with Ar four times while vortexing (31), reducing with $Na_2S \cdot 9H_2O$ (0.05% final concentration), monitoring oxygen removal with resazurin (0.0002% final concentration), and colorimetrically assaying nitrite production (550). Anaerobic growth was also checked at 20°C with stabs of modified Hugh and Leifson medium with SSB and glucose as the only carbon source (550). Under certain growth conditions when cell yield was low, growth was noted as positive if the number of cells crested $>10^7$ ml^{-1} from an initial concentration of $<10^5$ ml^{-1} as monitored by phase-contrast microscopy.

For growth rate calculations, 40 ml of media were dispensed into autoclaved 125-ml flasks with foam stoppers or aluminum foil covers and inoculated. Growth rates were determined by monitoring optical density at 600 nm using a Lambda2S UV/VIS Spectrophotometer (Perkin Elmer). The slope of the log of the exponential-growth portion of the growth curve was averaged from triplicate runs. In order to convert optical density to cell number, growth rates were simultaneously determined in triplicate by monitoring the increase in cell concentration obtained by direct counts with DAPI (4',6-diamidino-2-phenylindole) (471) at pH 7.0, 20°C, and SSB salt concentration, and the appropriate ratios were calculated.

Phenotypic characteristics

Tests for 121 characteristics, including morphological, cultural, physiological, biochemical and nutritional features, were performed (Table 1.1). Test procedures have been described previously (206, 477, 614).

Transmission electron microscopy

Log-phase cells were harvested by centrifuging at 2,000–3,000 × *g* for 20 min at 4°C. The pellet was resuspended in the growth medium and fixed with electron microscopy-grade glutaraldehyde (3% final concentration) for 2 h at 2°C. For thin sections, cells were pelleted, resuspended in 1% OsO_4, dehydrated, embedded in Spurr's low-viscosity medium, thin sectioned, and stained with 6% uranyl acetate and lead citrate for 10 min (Fig 1.1). For determination of flagellar arrangement, log-phase cells were stained with phosphotungstic acid or ammonium molybdate and directly spotted onto copper grids. Samples were photographed using a JEM 1200 EXII transmission electron microscope (JEOL).

DNA extraction and purification

Cells from the strains included in Table 1.2 were harvested, washed, suspended in 0.15 M NaCl-0.1 M EDTA buffer at pH 8.0 (5 g wet weight in 50 ml of buffer), and lysed with ~10 mg lysozyme at 37°C and with sodium dodecyl sulfate (2% final concentration) at 60°C. The DNA was extracted and purified by standard procedure (359). The purity was assessed from the A_{260}/A_{280} and A_{230}/A_{260} extinction ratios (283).

DNA base composition

The mol% G+C content of the DNA was determined from the midpoint value of the thermal denaturation profile (360) obtained with a model UV/VIS Lambda 20 Spectrophotometer (Perkin Elmer) at 260 nm; this instrument was programmed for a temperature increase of 1.0°C min^{-1}. The G+C content was calculated from the thermal denaturation temperature equation (458). The G+C content of reference DNA from *Escherichia coli* NCTC 9001 was taken to be 51 mol% (459).

Preparation of ^{3}H-labeled DNA and DNA-DNA hybridization experiments

DNA was radioactively labeled by the multiprime system with a commercial kit (RPN 1601Y; Amersham), using (1',2',5-^{3}H) dCTP (Amersham). The average specific activity obtained with this procedure was 8.8×10^6 cpm DNA μg^{-1}. The labeled DNA was denatured before hybridization by being heated at 100°C for 5 min and then placed on ice. DNA-DNA hybridization was studied by the competition procedure (283). Competitor DNA was sonicated (Braun Melsungen) at 50 W for two 15-s intervals. Membrane filters (HAHY; Millipore) containing reference DNA (~25 $\mu g\ cm^{-2}$) were placed in 5-ml screw cap vials that contained the labeled, sheared, denatured DNA and the denatured, sheared competitor DNA. The ratio of the concentration of competitor DNA to the concentration of labeled DNA was at least 150:1. The final reaction concentrations were 2× saline-citrate solution (0.15 M NaCl-0.015 M trisodium citrate, pH 7 ± 0.2) and 30% formamide, and the final volume was 140 µl. The hybridization experiments were carried out under optimal conditions with temperatures ranging between 55.0 and 55.5°C, which is within the limits of validity for the filter method (134). The vials were shaken slightly for 18 h in a water bath (Grant Instruments); these procedures were done in triplicate. After hybridization the filters were measured with a liquid scintillation counter (Beckman Instruments), and the percentage of hybridization was calculated (283). At least two independent determinations were carried out for each experiment; the mean values are reported here. The overall standard deviation was ~6%, and in the case of high DNA similarity values (≥70%), it was ~2%.

Comparative analysis of 16S rRNA gene sequences

Pure cultures of the novel isolates shown in Fig. 1.3 were grown in 500-ml flasks and pelleted by centrifuging at 10,000 × *g* for 20–40 min at 4°C. Genomic DNA was extracted using the IsoQuick kit (Orca Research). Almost complete 16S rRNA gene sequences were obtained by using the polymerase chain reaction with the bacterial primers 8F (5'-AGA GTT TGA TCC TGG CTC AG), 519R (5'-GWA TTA CCG CGG

CKG CTG), 515F (5'-GTG CCA AGC MGC CGC GGT AA), 907R (5'-CCG TCA ATT CMT TTR AGT TT), 926F (5'-AAA CTY AAA KGA ATT GAC GG) and 1492R (5'-GGT TAC CTT GTT ACG ACT T) (333). Gene fragments were sequenced with an Applied Biosystems sequencer (ABI) Model 373A or a MegaBACE 1000 (Molecular Dynamics) at the University of Washington Marine Molecular Biotechnology Laboratory or an ABI100 at the Molecular Genetics Instrumentation Facility at the University of Georgia, Athens. Sequences of approximately 1300 base-pairs length (bases 95–1406, *E. coli* numbering) were aligned with other sequences acquired from the GenBank database after excising variable stem-loops (bases 201–216 and 1135–1139, *E. coli* numbering). A maximum-likelihood phylogenetic tree including other members of the family *Halomonadaceae* was constructed with TREE-PUZZLE version 5.0 (529, 568) and visualized with TreeView version 1.5 (460). GenBank accession numbers are strain Eplume1^T AF212202; strain Eplume2 AF212201; strain Esulfide1^T AF212204; strain Althf1^T AF212206; strain Slthf1 AF212217; and strain Slthf2^T AF212218.

RESULTS

Enrichment and isolation

All six strains derived from various hydrothermal-vent environments in the North or South Pacific Ocean (Table 1.1). Strains Eplume1^T, Eplume2 and Esulfide1^T were isolated from primary enrichment colonies, whereas strains Althf1^T, Slthf1 and Slthf2^T derived from 100-µl aliquots from three different most dilute MPN enrichment tubes (1:50 to 1:500 dilutions) spread onto agar surfaces.

Phenotypic characterization

All strains were motile rods with peritrichous flagella, 2–3 µm in length and 1 µm in width (1.5-µm width for strain Slthf2^T). Transmission electron micrographs are provided in Fig. 1.1. The cells maintained their shape under all growth conditions at different temperatures and salinities, except for strains Eplume2 and Slthf1 which formed chains of incompletely divided cells at ≤2°C. Colonies were round, smooth and cream-colored. Endospores were not observed for any strain under any condition. All strains were psychrotolerant and either slightly or moderately halophilic (Fig. 1.2). Temperature, salt and pH ranges and optima and other phenotypic data are summarized in Table 1.1.

G+C content

The G+C content of the DNA of the six strains ranged between 56.0 and 57.6 mol% (Table 1.2). These values are similar to those described for members of the genus *Halomonas* (24, 158).

DNA-DNA hybridization

The percentage of DNA hybridization obtained between strains Eplume1^T, Esulfide1^T, Althf1^T and Slthf2^T and with previously characterized *Halomonas* spp. was very low (1–53%). Strains Eplume2 and Slthf1 had >70% DNA hybridization with each other and with *H. meridiana* DSM 5425^T, indicating that they are members of this species. Hybridization data are summarized in Table 1.2.

Phylogenetic analysis

The almost complete 16S rRNA genes of the six isolates were sequenced and a phylogenetic tree with other members of the family *Halomonadaceae* was constructed (Fig. 1.3). All six strains were phylogenetically closely related to species in Group 2 (24) of the genus *Halomonas*. DNA-DNA hybridization data were consistent with the 16S rRNA gene phylogeny.

DISCUSSION

Members of the genus *Halomonas* are found in a wide variety of habitats that encompass a broad range in salinity, temperature, hydrostatic pressure, concentration of organic carbon and pH (69, 170, 251, 304, 405, 443, 456, 485, 524, 577, 578, 585, 613, 665, 666). The numerical significance of members of this genus in hydrothermal-vent and pelagic environments (304) underscores why it is important to understand their physiology, growth rates and metabolic capabilities. Their success in diverse marine microbial ecosystems may be attributed to their metabolic and physiological versatility. The *Halomonas* isolates described here and previously can oxidize an extensive variety of organic compounds (42, 43, 304, 372, 613), which may directly contribute to their survival in the marine environment by enabling them to take advantage of many forms of transiently available nutrients. In addition, three of the novel strains were isolated with oligotrophic quantities of organic carbon. This study augments previous reports (384) of members of the genus *Halomonas* able to grow on the low levels of (albeit high-quality) carbon relevant to most of the marine ecosystem.

The phenotypic data presented in Table 1.1 reveal the enzymological and metabolic variability among the members of *Halomonas* 16S rRNA Group 2 (24). The percentage of characteristics shared between the six novel isolates and their closest phylogenetic relatives varied between 51 and 70% for strains Eplume2, Slthf1 and Althf1^T with *H. meridiana*, *H. aquamarina* and *H. magadiensis*, 51 and 78% for strains Eplume1^T and Esulfide1^T with *H. variabilis* and *H. glaciei*, and 69% for strain Slthf2^T with *H. venusta*. Strains Eplume2 and Slthf1 each only shared 51% of traits in common with the type species of *H. meridiana*, consistent with the phenotypic variability observed in this species (273). One caveat of phenotypic comparisons, however, is that the conditions and media used to determine these traits vary between studies, which may partially explain why the phenotypic data poorly mirror the phylogeny. Yet when contrasting the phylogeny based on 16S or 23S rRNA gene sequences (24) with phenotypic dendrograms (372) for which data were generated under identical conditions among the species examined, the incongruity between hypothesized evolutionary history and present-day phenotype is immediately apparent.

It is interesting to note that often the novel strains were more similar to one another than to previously described species. For example, strains Eplume2, Slthf1 and Althf1^T shared 66–83% of traits in common, and strains Eplume1^T and Esulfide1^T gave 70% of the same responses to physiological tests. Of particular note is that the novel strains tolerated 0.05–4.0 mM Cd^{2+} (and were inhibited by 0.5–5.0 mM Cd^{2+}), reduced nitrate to nitrite (strains Slthf1 and Slthf2^T also reduced nitrite), and grew weakly without oxygen. Four strains also reduced nitrate anaerobically. These abilities are hypothesized to be key adaptations for living in warm, heavy-metal-enriched subseafloor habitats associated with hydrothermal vents, where oxygen may become limiting at temperatures as low as ~10°C and nitrate and nitrite may become limiting at ~30°C (92, 261, 274, 378). The higher phenotypic similarity values among the hydrothermal-vent isolates may indeed reflect their common (yet geographically disparate) habitat of origin.

Another consistent difference with previously characterized *Halomonas* spp. was that the cardinal temperatures for growth were more similar to Antarctic and sea-ice *Halomonas* isolates (70, 192, 485), shifted cooler by 5–10°C, than to *Halomonas* strains isolated at higher incubation temperatures and/or from higher temperature and surface-pressure environments (613). This is not surprising given that cold temperatures can be analogous to high hydrostatic pressures with respect to their effects on proteins and phospholipids (Introduction; 39). Strains Eplume1^T, Eplume2, Esulfide1^T and Slthf2^T were obtained from hydrothermal-vent samples at ≤10°C; the downshift in temperature growth characteristics is presumed to reflect an adaptation to cold, pressurized deep-sea habitats. In addition, the isolation of cold-shifted strain Althf1^T from a warm (27°C) deep-sea hydrothermal vent indicates that the cold-shifted phenotype observed at 0.1 MPa may in fact reflect an overarching hydrostatic-pressure adaptation.

The cold-shifted temperature growth characteristics were also reflected in the phylogeny. Isolates Eplume2, Slthf1 and Althf1^T showed a close phylogenetic relationship with *H. meridiana*, which was isolated from a seasonally sub-zero Antarctic hypersaline lake (273). *H. glaciei*, isolated from Antarctic fast ice, was a close relative of strains Eplume1^T and Esulfide1^T as well. (The depth and temperature of the environment from which *H. aquamarina* was isolated was not reported [677]). The phylogenetic link between Antarctic and deep-sea hydrothermal-vent bacteria has been observed previously among numerous *Halomonas* isolates (443). However, given that strain Slthf2^T was closely related to *H. venusta* (isolated from tropical surface seawater [42]) and given that *H. variabilis* (isolated from the Great Salt Lake at 22°C [178]) was as close a cousin as *H. glaciei* to Eplume1^T and Esulfide1^T, the phylogenetic clustering of cold-shifted *Halomonas* was not as robust as the psychropiezophilic monophyly observed among other *γ-Proteobacteria* such as *Colwellia* and *Shewanella* (142, 301).

It is interesting to find bacteria in the deep sea with optimal growth at a salt concentration greater than seawater salinity and with the ability to tolerate up to 22–27% total salts. Low-temperature hydrothermal-vent fluids (<100°C) are a mixture of seawater and hot hydrothermal or crustal fluid (92), the salinity of which can vary between one-tenth to twice that of seawater (619). Typically seawater is the dominant component of low-temperature hydrothermal fluids and its proportion varies inversely with temperature (93, 123, 322). The fluids from which strains Althf1^T, Slthf1 and Slthf2^T were obtained were comprised of >90% seawater and had salt contents (measured as chlorinity) slightly less than the ambient deep-sea concentrations (91, 620). Hydrothermal plumes, from which strains Eplume1^T and Eplume2 were isolated, are even more dilute in hydrothermal component than low-temperature fluids and are

>99.9% seawater (340). The pore-water salinity within sulfide structures, such as where strain Esulfide1^T was isolated, is poorly constrained, however. Takai *et al.* (577) found evidence of obligately halophilic archaea within a sulfide structure based on an environmental 16S rRNA gene sequence phylogeny, suggestive of a stable brine environment. But without direct measurements, the salinity of the interstitial fluids in this dynamic habitat remains unknown. Hot subseafloor brines have also been proposed as a proximal hypersaline habitat (304). It is difficult to envision, however, that conductive cooling of extremely hot hypersaline fluids deep within the oceanic crust would be sufficient to create an accessible, extensive hypersaline habitat at a temperature that would permit the growth of *Halomonas* spp.

Habitats such as sea ice, Antarctic hypersaline lakes, and saline alkaline waters confront microorganisms with multiple physiological stresses. These environments also contain *Halomonas* spp. Therefore it was not surprising to culture novel *Halomonas* isolates from deep-sea hydrothermal-vent habitats, where microorganisms are typically responding to dynamic nutrient regimes and fluctuating temperatures and heavy-metal concentrations, all while under moderate hydrostatic pressures (295). The novel microorganisms described here comprise the first complete characterizations of *Halomonas* strains isolated from deep-sea and hydrothermal-vent habitats, some of which are also the first to be enriched with oligotrophic concentrations of organic carbon. Based on the phylogeny, levels of DNA-DNA hybridization, and phenotypic differences among the novel and previously described taxa, we conclude that four of the strains represent novel species within the genus *Halomonas*.

NOVEL STRAIN AND SPECIES DESCRIPTIONS

The following novel *Halomonas* species names are used through the remainder of this thesis.

Description of *Halomonas neptunia* sp. nov.

Halomonas neptunia (nep.tu'ni.a. L. fem. adj. *neptunia*, Neptunian, pertaining to Neptunus, Roman god of the sea).

Cells are rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 µm in length and 1 µm in width under all growth conditions. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic and anaerobic conditions. Nitrite is not reduced under aerobic conditions. Positive activity for cytochrome oxidase and catalase. Negative activity for phenylalanine deaminase, lysine and ornithine decarboxylase, and DNAse. No growth on Simmon's citrate agar. Indole and H_2S are not produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between –1 and 35°C, with an optimum of 30°C. No growth at –5°C (with 18% total salts) or 40°C. Growth from pH 5.0 to 12.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 13.0. Halophilic, growing with 0.5–27% total salts and optimally at 2–3% total salts. No growth without salt. Growth with up to 2.0 mM Cd^{2+}.

Tween-80, starch, casein and gelatin are not hydrolyzed. Acid is produced from D-galactose and D-glucose, but not from lactose, maltose, D-mannose, D-trehalose, D-xylose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melibiose, L-raffinose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, erythritol, ethanol, glycerol, D-mannitol, D-sorbitol, acetate, butyrate, citrate, fumarate, D,L-glycerate, L-glutamate, D,L-malate, malonate, D-saccharate, succinate, tartrate, L-alanine, L-arginine, L-asparagine, betaine, L-glutamine, L-lysine and L-proline. No growth on inulin, propanol, aconitate, α-ketoglutarate, propionate, creatine, glycine, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 57.3 mol% (T_m method).

Type strain: Eplume1^T, isolated from a hydrothermal plume at 2000-m depth above the Main Endeavour Field of the Endeavour Segment of the Juan de Fuca Ridge, northeast Pacific Ocean, has been deposited as ATCC BAA-805^T = CECT 5815^T = DSM 15720^T = CCM 7107^T.

Description of *Halomonas sulfidaeris* sp. nov.

Halomonas sulfidaeris (sul.fid.ae'ris. N.L. neut. n. *sulfidum*, sulfide; L. neut. gen. n. *aeris*, of ore; N.L. neut. gen. n. *sulfidaeris*, from sulfide ore).

Cells are rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 µm in length and 1 µm in width under all growth conditions. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic and anaerobic conditions. Nitrite is not reduced under aerobic conditions. Positive activity for cytochrome oxidase and catalase. Negative activity for phenylalanine deaminase, lysine and ornithine decarboxylase, and DNAse. No growth on Simmon's citrate agar. Indole and H_2S are not produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between –1 and 35°C, with a broad optimum between 20–35°C. No growth at –5°C (with 18% total salts) or 40°C. Growth from pH 5.0 to 10.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 11.0. Halophilic, growing with 0.5–24% total salts and optimally at 2–3% total salts. No growth without salt. Growth with up to 0.05 mM Cd^{2+}.

Casein, but not Tween-80, starch or gelatin, is hydrolyzed. Acid is produced from D-galactose, D-glucose, lactose, maltose, D-mannose and D-xylose, but not from D-trehalose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-fructose, sucrose, D-trehalose, D-xylose, ethanol, D-mannitol, propanol, butyrate, fumarate, D,L-glycerate, L-glutamate, D,L-malate, malonate, D-saccharate, tartrate, L-arginine, L-asparagine, betaine and L-lysine. No growth on D-cellobiose, D-galactose, D-glucose, inulin, lactose, maltose, D-mannose, D-melibiose, L-raffinose, L-rhamnose, D-ribose, erythritol, glycerol, D-sorbitol, acetate, aconitate, α-ketoglutarate, citrate, propionate, succinate, L-alanine, creatine, glycine, L-glutamine, L-proline, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 56.0 mol% (T_m method).

Type strain: Esulfide1^T, isolated from a metal-sulfide rock at 2200-m depth from the Main Endeavour Field of the Endeavour Segment of the Juan de Fuca Ridge, northeast Pacific Ocean, has been deposited as ATCC BAA-803^T = CECT 5817^T = DSM 15722^T = CCM 7108^T.

Description of *Halomonas axialensis* sp. nov.

Halomonas axialensis (a.xi.a.len'sis. N.L. masc./fem. adj. *axialensis*, pertaining to Axial Seamount, Juan de Fuca Ridge, northeast Pacific Ocean).

Cells are rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 µm in length and 1 µm in width under all growth conditions. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic conditions and slightly under anaerobic conditions. Nitrite is not reduced under aerobic conditions. Positive activity for cytochrome oxidase and catalase. Negative activity for phenylalanine deaminase, lysine and ornithine decarboxylase, and DNAse. No growth on Simmon's citrate agar. Indole and H_2S are not produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between –1 and 35°C, with an optimum of 30°C. No growth at –5°C (with 18% total salts) or 40°C. Growth from pH 5.0 to 12.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 13.0. Moderately halophilic, growing with 0.5–24% total salts and optimally at 4% total salts. No growth without salt. Growth with up to 0.05 mM Cd^{2+}.

Tween-80, starch, casein and gelatin are not hydrolyzed. Acid is produced from D-glucose and D-trehalose, but not from D-galactose, lactose, maltose, D-mannose, D-xylose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-fructose, D-glucose, maltose, sucrose, ethanol, butyrate, fumarate, L-glutamate, D,L-malate and tartrate. No growth on D-cellobiose, D-galactose, inulin, lactose, D-mannose, D-melibiose, L-raffinose, L-rhamnose, D-ribose, D-trehalose, D-xylose, erythritol, glycerol, D-mannitol, propanol, D-sorbitol, acetate, aconitate, α-ketoglutarate, citrate, D,L-glycerate, malonate, propionate, D-saccharate, succinate, L-alanine, L-arginine, L-asparagine, betaine, creatine, glycine, L-glutamine, L-lysine, L-proline, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 57.6 mol% (T_m method).

Type strain: Althf1^T, isolated from low-temperature hydrothermal fluid (Cloud Vent) at 1530-m depth on Axial Seamount on the Juan de Fuca Ridge, northeast Pacific Ocean, has been deposited as ATCC BAA-802^T = CECT 5812^T = DSM 15723^T = CCM 7103^T.

Description of *Halomonas hydrothermalis* sp. nov.

Halomonas hydrothermalis (hy.dro.ther.ma'lis. N.L. masc./fem. adj. *thermalis* pertaining to hot springs; N.L. masc./fem. adj. *hydrothermalis*, pertaining to hydrothermal vents).

Cells are squat rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 μm in length and 1.5 μm in width under all growth conditions. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic, but not anaerobic, conditions. Nitrite is reduced under aerobic conditions. Positive activity for cytochrome oxidase, catalase and DNAse. Negative activity for phenylalanine deaminase and lysine and ornithine decarboxylase. No growth on Simmon's citrate agar. Indole and H_2S are not produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between 2 and 40°C, with an optimum of 30°C. No growth at –1 or 50°C. Growth from pH 5.0 to 12.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 13.0. Moderately halophilic, growing with 0.5–22% total salts and optimally at 4–7% total salts. No growth without salt. Growth with up to 0.05 mM Cd^{2+}.

Tween-80, starch, casein and gelatin are not hydrolyzed. Acid is produced from D-glucose, but not from D-galactose, lactose, maltose, D-mannose, D-trehalose, D-xylose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-fructose, D-galactose, D-glucose, inulin, maltose, D-mannose, sucrose, D-trehalose, glycerol, D-mannitol, acetate, butyrate, α-ketoglutarate, citrate, fumarate, D,L-glycerate, L-glutamate, D,L-malate, malonate, propionate, L-arginine, L-asparagine, L-glutamine, L-lysine and L-proline. No growth on D-cellobiose, lactose, D-melibiose, L-raffinose, L-rhamnose, D-ribose, D-xylose, erythritol, ethanol, propanol, D-sorbitol, aconitate, D-saccharate, succinate, tartrate, L-alanine, betaine, creatine, glycine, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 56.3 mol% (T_m method).

Type strain: Slthf2^T, isolated from low-temperature hydrothermal fluid at 2580-m depth at 17.5°S on the SEPR, South Pacific Ocean near Easter Island, has been deposited as ATCC BAA-800^T = CECT 5814^T = DSM 15725^T = CCM 7104^T.

Description of *Halomonas meridiana* strain Eplume2

Cells are rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 μm in length and 1 μm in width under all growth conditions. Chains of incompletely divided cells are common when grown at 2°C. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic conditions and slightly under anaerobic conditions. Nitrite is not reduced under aerobic conditions. Positive activity for cytochrome oxidase and catalase. Negative activity for phenylalanine deaminase, lysine and ornithine decarboxylase, and DNAse. No growth on Simmon's citrate agar. Indole is not produced. H_2S is produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between 2 and 35°C, with an optimum of 30°C. No growth at –5°C (with 18% total salts) or 40°C. Growth from pH 5.0 to 11.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 12.0. Slightly halophilic to halophilic, growing with 0.5–24% total salts and optimally at 2–3% total salts. No growth without salt. Growth with up to 3.0 mM Cd^{2+}.

Tween-80, starch, casein and gelatin are not hydrolyzed. Acid is produced from D-galactose, D-glucose and D-trehalose, but not from lactose, maltose, D-mannose, D-xylose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melibiose, L-raffinose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, erythritol, glycerol, D-mannitol, propanol, D-sorbitol, acetate, butyrate, citrate, fumarate, D,L-glycerate, L-glutamate, D,L-malate, malonate, D-saccharate, succinate, tartrate, L-arginine, L-asparagine, betaine, L-glutamine, L-lysine and L-proline. No growth on inulin, ethanol, aconitate, α-ketoglutarate, propionate, L-alanine, creatine, glycine, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 56.7 mol % (T_m method).

New strain of *H. meridiana*: strain Eplume2, isolated from a hydrothermal plume at 2000-m depth above the Main Endeavour Field of the Endeavour Segment of the Juan de Fuca Ridge, northeast Pacific Ocean, has been deposited as ATCC BAA-804^T = CECT 5816^T = DSM 15721^T = CCM 7106^T.

Description of *Halomonas meridiana* strain Slthf1

Cells are rods with rounded ends, stain Gram negative, are motile with peritrichous flagella and are found primarily as single cells and doublets. Individual cells measure 2–3 μm in length and 1 μm in width under all growth conditions. Chains of incompletely divided cells are common when grown at 2°C. No spores are observed under any condition. Colonies are round, smooth and cream colored. Facultative aerobic growth. Nitrate is reduced under aerobic, but not anaerobic, conditions. Nitrite is reduced under aerobic conditions. Positive activity for cytochrome oxidase and catalase. Negative activity for phenylalanine deaminase, lysine and ornithine decarboxylase, and DNAse. No growth on Simmon's citrate agar. Indole and H_2S are not produced. Negative reaction to methyl red and Voges-Proskauer test.

Psychrotolerant growth between –1 and 40°C, with a broad optimum of 20–35°C. No growth at –5°C (with 18% total salts) or 50°C. Growth from pH 5.0 to 12.0 with an optimum of pH 7.0–8.0. No growth at pH 4.0 or 13.0. Halophilic to moderately halophilic, growing with 0.5–22% total salts and optimally at 2–7% total salts. Growth with up to 2.0 mM Cd^{2+}.

Tween-80, starch, casein and gelatin are not hydrolyzed. Acid is produced from D-galactose and D-glucose, but not from lactose, maltose, D-mannose, D-trehalose, D-xylose, L-arabinose, D-fructose, glycerol or sucrose. Growth on L-arabinose, D-fructose, D-glucose, inulin, maltose, D-ribose, sucrose, D-trehalose, ethanol, glycerol, acetate, butyrate, citrate, fumarate, D,L-glycerate, L-glutamate, D,L-malate, malonate, propionate, L-alanine, L-arginine, L-asparagine, L-glutamine and L-proline. No growth on D-cellobiose, D-galactose, lactose, D-mannose, D-melibiose, L-raffinose, L-rhamnose, D-xylose, erythritol, D-mannitol, propanol, D-sorbitol, aconitate, α-ketoglutarate, D-saccharate, succinate, tartrate, betaine, creatine, glycine, L-lysine, D-fucose, galactosamine, D-gluconolactone, D-glucosamine, adonitol, dulcitol, α-aminobutyrate, α-aminovalerate, caprylate, lactate, oxalate, L-histidine, L-methionine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine or L-valine.

DNA G+C content is 56.5 mol % (T_m method).

New strain of *H. meridiana*: strain Slthf1, isolated from a low-temperature hydrothermal vent at 2580-m depth at 17.5°S on the SEPR, South Pacific Ocean near Easter Island, has been deposited as ATCC BAA-801^T = CECT 5813^T = DSM 15724^T = CCM 7105^T.

Table 1.1 Differences among novel isolates and previously characterized *Halomonas* 16S rRNA Group 2 species (24), including sample source, enrichment conditions and phenotypic characteristics.

Species included: 1, strain Eplume1^T; 2, strain Eplume2; 3, strain Esulfide1^T; 4, strain Althfl^T; 5, strain Slthfl; 6, strain Slthf2^T; 7, *H. aquamarina* DSM 30161^T; 8, *H. meridiana* DSM 5425^T; 9, *H. variabilis* DSM 3051^T; 10, *H. magadiensis* NCIMB 13595^T; 11, *H. venusta* DSM 4743^T; 12, *H. halodurans* DSM 5160^T; 13, *H. subglaciescola* DSM 4683^T; and 14, *H. glaciei* JCM 11692^T. Data for sample characteristics, enrichment conditions, temperature and salt ranges, temperature optima, and cadmium tolerance from ZoBell and Upham (677), Baumann *et al.* (44), Baumann *et al.* (42), Baumann *et al.* (43), Rosenberg (508), Franzmann *et al.* (192, 193), Fendrich (178), Fendrich and Schink (179), McMeekin and Franzmann (375), Nieto *et al.* (428), James *et al.* (273), Duckworth *et al.* (161), Duckworth *et al.* (160), Kaye and Baross (304), Arahal *et al.* (24), Reddy *et al.* (485) and this study. Other characteristics for taxa 1–6 from Kaye and Baross (304) and this study; for taxa 7–13 from Mata *et al.* (372); and for taxon 14 from Reddy *et al.* (485). Traits are scored as: +, positive; –, negative; ND, not determined.

Characteristic[*]	1	2	3	4	5	6	7	8	9	10	11	12	13	14[†]
Sample characteristics:														
Habitat	Hydrothermal plume	Hydrothermal plume	Sulfide rock	Low-T hydrothermal fluid	Low-T hydrothermal fluid	Low-T hydrothermal fluid	Temperate ocean	Cold hypersaline lake	Hypersaline lake	Hypersaline soda lake	Tropical coastal water	Temperate estuary	Cold hypersaline lake	Fast ice
T (°C)	2	2	~10	27	9	9	ND	–4	22	36	ND	ND	–7	<0
S (%)	3	3		3	3	3	3	18	22	Saturated	3	3	17	
Depth (m)	2000	2000	2200	1530	2580	2580	ND	6	0	0	0	0	2	0
Enrichment conditions:														
T (°C)	2	2	20	20	20	20	22	10	30	37	25	20	10	2
Headspace	Air/CH_4	Air/CH_4	Air	Air	Air	Air	Air	Air	Air	Air	Air	Air	Air	Air
Carbon source	0.002% yeast extract	0.002% yeast extract	0.002% yeast extract	1.0% sodium citrate[‡]	1.0% sodium citrate	1.0% sodium citrate	0.5% peptone	0.01% yeast extract	0.5% organic carbon	1.5% organic carbon	0.1% *o*-hydroxybenzoate	0.2% organic carbon	Trace organic acids[§]	0.6% organic carbon
S (%)	4	4	4	18	18	18	3	15	21	6	3	2	17	3
Growth:														
T range (°C)	–1 to 35	2–35	–1 to 35	–1 to 35	–1 to 40	2–40	5–40	–5 to 45	15–37	20–50	4–40	4–35	0–25	4–30
T optimum (°C)	30	30	20–35	30	20–35	30	20–25	28–40	33	37	ND	20	24[‖]	22
S range (%)	0.5–27	0.5–24	0.5–24	0.5–24	0.5–22	0.5–22	0–20	0.01–25	7–29	0–20	0–20[¶]	2–12	1–17.5	0–15
S optimum (%)	2–3	2–3	2–3	4	2–7	4–7	7.5–10	1–3	10	0–7	0.5–7	8	5–10	2
pH range	5–12	5–11	5–10	5–12	5–12	5–12	5–10	5–10	6–9	5–11	5–10	5–10	5–10	6–12
Aerobic NO_3^- reduction	+	+	+	+	+	+	+	–	–	+	+	–	+	–
Aerobic NO_2^- reduction	–	–	–	–	+	+	–	–	–	–	–	–	–	ND
Facultative aerobe	+	+	+	+	+	+	–	–	–	–	–	–	–	ND

Table 1.1 continued.

Characteristic*	1	2	3	4	5	6	7	8	9	10	11	12	13	14†
Anaerobic NO_3^- reduction	+	+	+	+	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Pigmentation	Cream	Cream	Cream	Cream	Cream	Cream	Cream	White	Cream	Cream	Yellow	None	Cream	White
Cd^{2+} inhibition (mM)	5.0	4.0	0.5	4.0	3.0	0.5	0.5	ND	ND	ND	ND	ND	5.0	ND
Hydrolysis of:														
Casein	–	–	+	–	–	–	–	–	–	–	–	+	–	ND
Starch	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Tween-80	–	–	–	–	–	–	+	+	–	+	–	+	–	–
H_2S production	–	+	–	–	–	–	–	+	+	+	+	–	–	–
DNAse	–	–	–	–	–	+	ND	ND	ND	ND	ND	ND	ND	ND
Lysine decarboxylase	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Ornithine decarboxylase	–	–	–	–	–	–	–	+	–	–	–	+	–	ND
Phenylalanine deaminase	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
Acid production from:														
L-Arabinose	–	–	–	–	–	–	+	+	–	–	–	–	–	–
D-Fructose	–	–	–	–	–	–	+	+	–	–	–	+	–	ND
D-Galactose	+	+	+	–	+	–	+	+	–	–	–	+	–	ND
D-Glucose	+	+	+	+	+	+	+	+	–	–	–	+	–	–
Lactose	–	–	+	–	–	–	+	+	–	–	–	–	–	–
Maltose	–	–	+	–	–	–	+	+	–	–	–	–	–	ND
D-Mannose	–	–	+	–	–	–	+	+	–	–	–	–	–	ND
Sucrose	–	–	–	–	–	–	+	+	–	–	–	+	–	ND
D-Trehalose	–	+	–	+	–	–	+	+	–	–	–	–	–	–
D-Xylose	–	–	+	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Growth on:														
L-Arabinose	+	+	+	+	+	+	+	–	+	+	–	+	+	+
D-Cellobiose	+	+	–	–	–	–	–	–	+	–	+	+	–	ND
D-Fructose	+	+	+	+	+	+	–	–	+	+	–	+	+	–
D-Galactose	+	+	–	–	–	+	–	+	+	+	+	+	–	ND
D-Glucose	+	+	–	+	+	+	+	+	+	+	–	+	–	+
Inulin	–	–	–	–	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Lactose	+	+	–	–	–	–	+	–	+	+	–	+	–	–
Maltose	+	+	–	+	+	+	–	+	+	–	–	–	–	–
D-Mannose	+	+	–	–	–	+	–	–	+	+	+	+	–	–
D-Melibiose#	+	+	–	–	–	–	+	+	+	–	–	–	+	–
D-Raffinose	+	+	–	–	–	–	–	–	–	+	–	–	–	ND
L-Rhamnose	+	+	–	–	–	–	–	–	–	+	–	–	–	ND

Table 1.1 continued.

Characteristic[*]	1	2	3	4	5	6	7	8	9	10	11	12	13	14[†]
Growth on:														
Ribose[**]	+	+	–	–	+	–	–	–	+	–	+	+	+	ND
Sucrose[#]	+	+	+	+	+	+	+	+	+	+	+	–	+	–
D-Trehalose	+	+	+	–	+	+	+	+	+	+	+	+	–	+
D-Xylose	+	+	+	–	–	–	–	–	–	+	–	+	–	ND
Adonitol	–	–	–	–	–	–	–	–	–	+	–	–	+	–
Dulcitol	–	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	–
Erythritol[#,††]	+	+	–	–	–	–	–	–	+	–	–	–	–	–
Ethanol	+	–	+	+	+	–	+	+	+	+	+	+	–	ND
Glycerol	+	+	–	–	+	+	+	+	+	+	–	+	–	–
D-Mannitol	+	+	+	–	–	+	–	+	+	+	+	–	–	–
Propanol	–	+	+	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Sorbitol[**]	+	+	–	–	–	–	–	–	–	+	+	+	–	–
Acetate	+	+	–	–	+	+	+	–	+	+	–	+	+	+
Aconitate[#,‡‡]	–	–	–	–	–	–	–	+	+	+	+	+	–	ND
Butyrate	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Citrate	+	+	–	–	+	+	+	–	+	+	+	+	+	–
Fumarate	+	+	+	+	+	+	+	–	+	+	+	+	+	ND
D,L-Glycerate	+	+	+	–	+	+	ND	ND	ND	ND	ND	ND	ND	ND
α-Ketoglutarate[#]	–	–	–	–	–	+	+	+	+	+	+	+	–	ND
Lactate[#,§§]	–	–	–	–	–	–	+	+	+	+	+	+	–	ND
Malonate	+	+	+	–	+	+	+	–	–	–	–	+	+	ND
Propionate	–	–	–	–	+	+	+	–	+	+	–	+	–	ND
D-Saccharate[#]	+	+	+	–	–	–	+	+	+	+	+	–	–	ND
Succinate	+	+	–	–	–	–	+	+	+	+	+	+	+	ND
Tartrate	+	+	+	+	–	–	ND	ND	ND	ND	ND	ND	ND	ND
D-Glucosamine[#]	–	–	–	–	–	–	+	+	+	–	+	–	–	ND
L-Alanine	+	–	–	–	+	–	+	+	+	+	+	+	+	–
L-Arginine	+	+	+	–	+	+	ND	ND	ND	ND	ND	ND	ND	–
L-Asparagine[#]	+	+	+	–	+	+	–	+	+	+	+	–	+	–
Betaine	+	+	+	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND
L-Glutamine	+	+	–	–	+	+	ND	ND	ND	ND	ND	ND	ND	ND
L-Glutamate[#]	+	+	+	+	+	+	–	–	+	+	+	+	+	–
L-Histidine	–	–	–	–	–	–	–	–	–	+	+	+	+	–
L-Lysine	+	+	+	–	–	+	+	+	–	+	+	+	+	ND
D,L-Malate	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND

Table 1.1 continued.

Characteristic*	1	2	3	4	5	6	7	8	9	10	11	12	13	14†
Growth on:														
L-Methionine	–	–	–	–	–	–	–	–	–	+	–	+	–	–
L-Ornithine#	–	–	–	–	–	–	+	+	+	–	–	–	–	ND
L-Proline	+	+	–	–	+	+	–	+	+	+	+	+	–	ND
Putrescine#	–	–	–	–	–	–	+	–	+	–	+	–	–	ND
L-Serine	–	–	–	–	–	–	–	+	+	+	–	+	+	–
L-Threonine#	–	–	–	–	–	–	–	+	–	+	–	–	–	–
L-Valine	–	–	–	–	–	–	–	–	+	+	–	+	+	–

* Characteristics that were all positive: rod morphology, motility, and cytochrome oxidase and catalase activity; all negative: Gram stain, spore production, indole production, methyl red and Voges-Proskauer tests, and hydrolysis of gelatin. Characteristics that were all negative for taxa 1–6 but not determined by Mata *et al.* (372) or Reddy *et al.* (485) for taxa 7–14: growth on Simmon's citrate medium, acid production from glycerol, and growth on D-fucose, α-aminobutyrate, α-aminovalerate, caprylate, oxalate, creatine, galactosamine, D-gluconolactone, glycine and sarcosine. The optimum pH was between 7.0 and 8.0 for taxa 1–6.

† Isomers of organic compounds not indicated in Reddy *et al.* (485).

‡ As trisodium citrate. Medium contained 0.002% yeast extract as well.

§ Isolation medium was filtered Organic Lake water containing trace quantities of acetate, formate and lactate and was sometimes amended with 0.05% L-cystine.

|| Model prediction (375).

¶ Salt range from Mata *et al.* (372); it was not determined during the original or an early characterization of the organism.

\# Organic carbon utilization determined with a BIOLOG plate for taxa 7–13 (372).

** D form of isomer for taxa 1–6.

†† Specified as *i*-erythritol for taxa 7–13.

‡‡ Specified as *cis*-aconitic acid for taxa 7–13.

§§ Specified as D,L-lactic acid for taxa 7–13.

Table 1.2 G+C content of DNA (T_m method) and degrees of DNA-DNA hybridization between the novel strains and related species of the genus *Halomonas*.

Strain	G+C (mol%)	% Hybridization with ^{3}H-labeled DNA from strain:					
		Eplume1^T	Eplume2	Esulfide1^T	Althf1^T	Slthf1	Slthf2^T
Eplume1^T	57.3	100	50	37	39	17	39
Eplume2	56.7	1	100	52	33	71	22
Esulfide1^T	56.0	46	46	100	7	21	28
Althf1^T	57.6	6	45	35	100	41	30
Slthf1	56.5	2	78	21	31	100	6
Slthf2^T	56.3	25	54	17	53	7	100
H. aquamarina DSM 30161^T	58.0	23	38	31	28	21	43
H. meridiana DSM 5425^T	59.5	6	88	33	32	84	29
H. venusta DSM 4773^T	52.3	2	31	1	30	1	22
H. variabilis DSM 3051^T	61.0	28	40	44	38	13	40
H. marina DSM 4741^T	62.8	ND*	41	38	ND	1	13
H. halmophila DSM 5349^T	62.8	ND	20	2	ND	2	27
H. campisalis ATCC 700597^T	66.0	ND	2	2	ND	1	1
H. pacifica DSM 4772^T	68.0	ND	40	11	ND	1	24

* ND, Not determined.

Figure 1.1 Transmission electron micrographs of (A) strain Eplume1^T, (B) strain Esulfide1^T, (C) strain Althf1^T and (D) strain Slthf2^T. Bars, 0.2 μm.

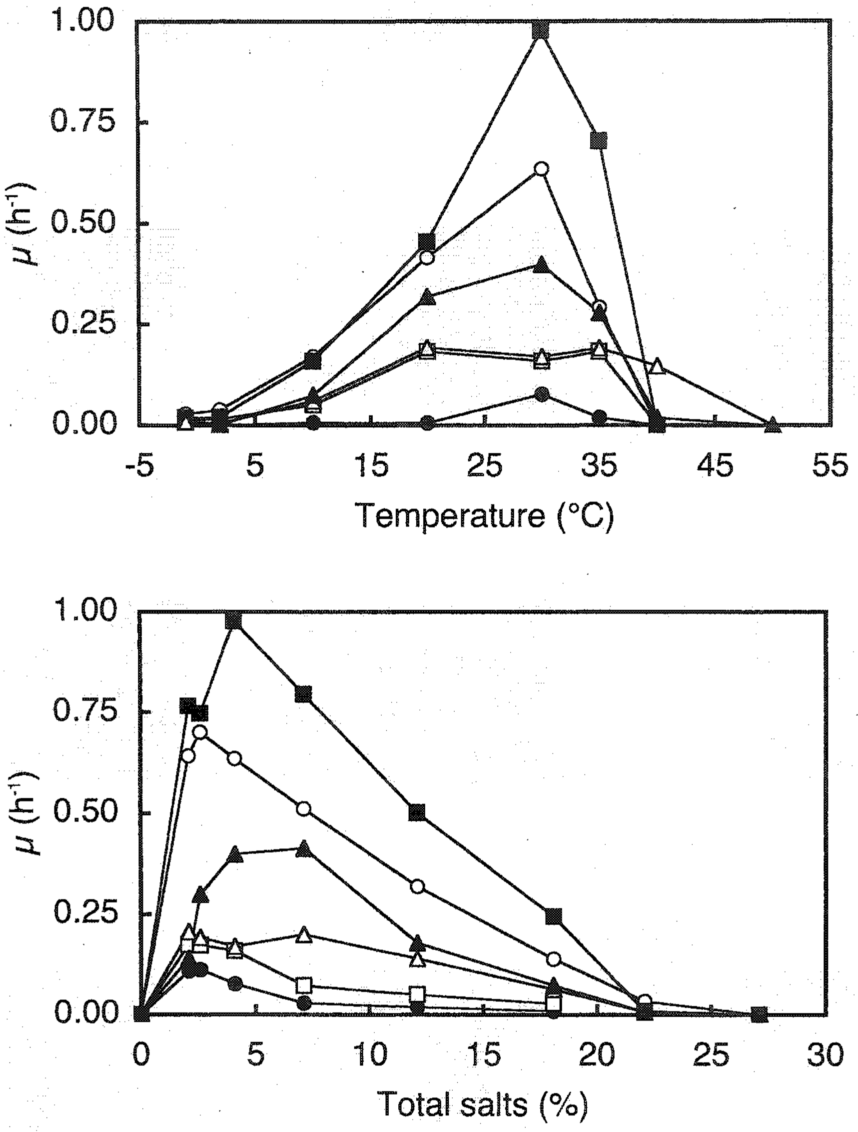

Figure 1.2 Growth rates (μ) at different temperatures and salt concentrations. Symbols: ○, strain Eplume1^{T}; ●, strain Eplume2; □, strain Esulfide1^{T}; ■, strain Althf1^{T}; Δ, strain Slthf1; ▲, strain Slthf2^{T}.

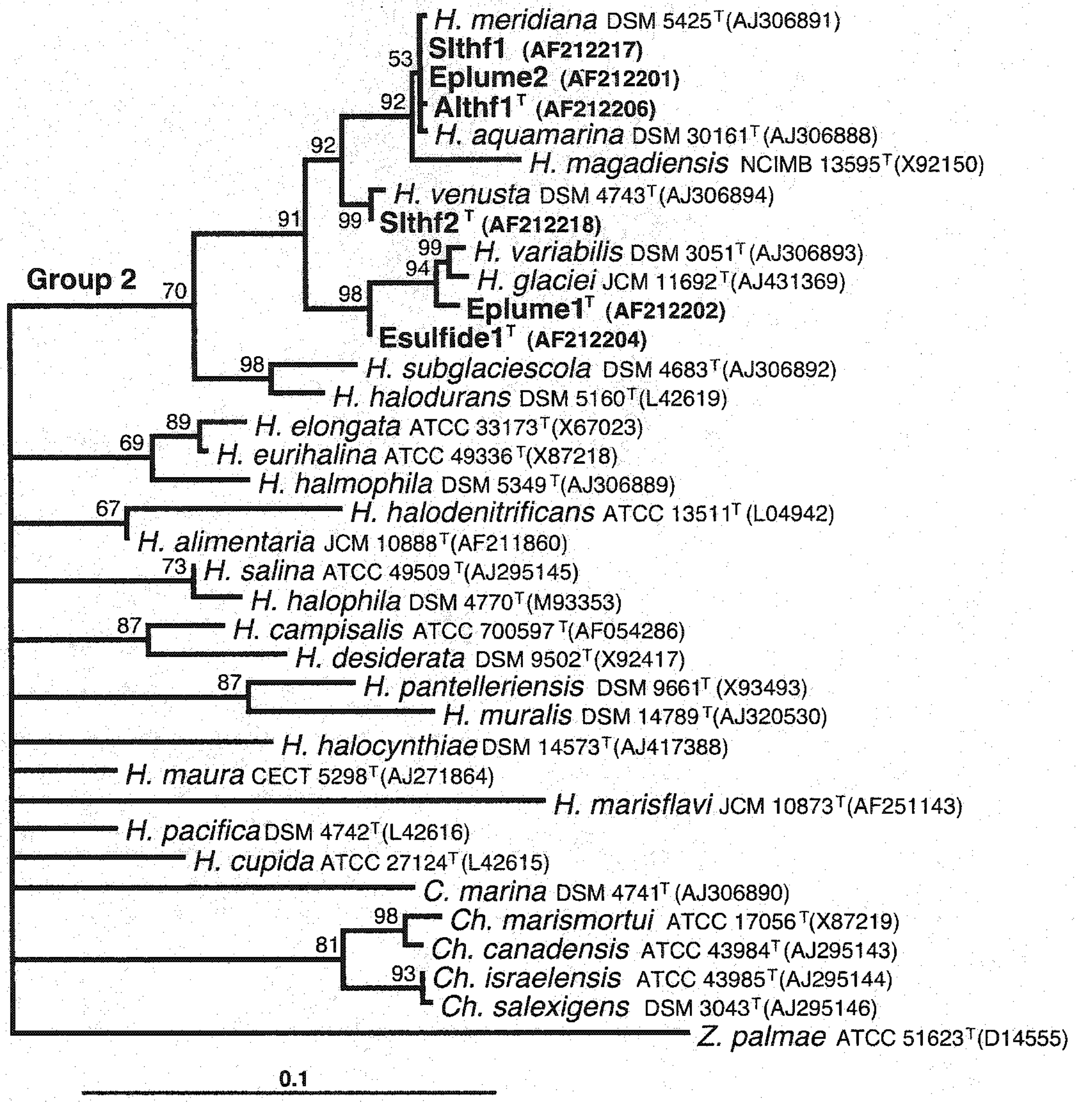

Figure 1.3 Maximum-likelihood 16S rRNA gene phylogeny of novel isolates (Eplume1^{T}, Eplume2, Esulfide1^{T}, Althf1^{T}, Slthf1 and Slthf2^{T}) and previously described *Halomonas* (*H.*), *Chromohalobacter* (*Ch.*), *Cobetia* (*C.*) and *Zymobacter* (*Z.*) species with accession numbers in parentheses. Quartet-puzzling support values are shown at branch points. *Z. palmae* is used as the outgroup. Bar, nucleotide changes per site.

CHAPTER TWO: **Phylogenetic analysis of moderately halophilic bacterial communities from hydrothermal-vent and deep-sea environments**

Easily cultivated microorganisms from marine environments, such as *Pseudomonas* and *Vibrio* of the γ-*Proteobacteria*, are rarely observed in bacterial phylogenies based on the 16S rRNA gene (143, 197, 198, 573). Though an emerging exception to this trend is in polar sediments and sea ice where a high incidence of commonly cultivated bacteria characterizes clone libraries (71, 81, 85), those derived from other marine habitats continue to contain a dearth of gene sequences belonging to fast-growing, historically cultivated bacteria. Less easily cultured microorganisms, as represented by the ubiquitous bacterial SAR clades, have been shown to comprise up to 26% of pelagic 16S rRNA gene sequences in every ocean examined thus far (214, 215, 409, 483). Numerically dominant bacteria have just begun to be enriched and characterized physiologically with novel cultivation techniques (122, 292, 482).

Culture-independent community analyses of various hydrothermal-vent habitats indicate the presence of diverse bacterial phylotypes. ε-*Proteobacteria* are the most frequently identified microorganisms and have been found as a significant or dominant proportion of 16S rRNA gene sequences in clone libraries derived from almost all fluid, sulfide-rock and animal-associated hydrothermal-vent environments (11, 102, 125, 261, 348, 408, 469, 490). Other significant populations of cloned 16S rRNA gene sequences include members of the *Cytophaga-Flavobacterium-Bacteroides* group, *Aquificales*, "*Verrucomicrobia*," *Xanthomonas*, *Desulfurobacterium* and uncharacterized taxa, with considerable variability depending on sample type (11, 348, 408, 490). Bacterial culturing efforts have focused on heterotrophic (including Fe^{3+}-reducing), mixotrophic, and autotrophic thermophiles and hyperthermophiles along with chemoautotrophic mesophiles that oxidize Fe^{2+} or sulfur compounds (12, 18, 82, 83, 99, 163, 168, 172, 173, 275, 295, 297, 298, 362, 393, 394, 412, 419, 514, 515, 542, 575, 576, 644).

Successful cultivation of numerically significant taxa and culture-independent analyses of marine microbial communities augment the importance and utility of each other. The combination of laboratory-determined physiology and a map of the distribution of bacterial clades, such as the SAR groups in pelagic environments and the ε-*Proteobacteria* in hydrothermal-vent habitats, reveals where and how microbial metabolisms may be impacting transformations of organic compounds and inorganic nutrients. There are still caveats, however, including the caution with which one must infer phenotype from 16S rRNA gene sequence as well as the distinction between the genotypic potential of a microorganism and the phenotype actually expressed *in situ*.

In between the rare, easily cultivated heterotrophic weeds and the numerically dominant bacterial taxa in both pelagic and hydrothermal-vent environments lie groups such as *Halomonas* and *Marinobacter* (304). These genera were commonly isolated from enrichments on media with elevated Fe^{2+} and Mn^{2+} at 2 and 20°C and were the sole representatives obtained with quantitative enrichments using the same medium augmented with elevated salt and incubated at 20°C. Moderately halophilic and euryhaline taxa, as represented by *Halomonas* and *Marinobacter* spp., comprised 0.01 to >10% of the total microbial community in North and South Pacific Ocean hydrothermal-vent and water-column samples (304). Characterization of six novel *Halomonas* strains revealed that they were psychrotolerant, heterotrophic, euryhaline, slightly to moderately halophilic, Cd^{2+}-resistant, and facultatively aerobic, consistent with a phenotype suitable for growth in hypothesized low-temperature hydrothermal-vent (diffuse flow) and subseafloor environments (Chapter 1; 92, 261, 378). Partial characterization of novel *Marinobacter* strains from vent habitats highlighted their ability to oxidize Fe^{2+} from sulfide minerals and basalt at low temperature (168). These *Marinobacter* strains were closely related to the numerically significant euryhaline strains isolated at 20°C from seawater and diffuse-flow environments (168, 304).

Our goal in the present study is to understand the biogeography and diversity of moderately halophilic and euryhaline bacterial communities in warm hydrothermal-vent and nearby cold deep-sea environments given their numerical significance and possible contribution to transformations of organic carbon and Fe. Though *Halomonas* and *Marinobacter* spp. were abundantly represented in culturing efforts (304), they are not generally detected using a molecular-phylogenetic approach. We designed specific PCR primers to determine the species diversity of *Halomonas* and *Marinobacter* and to ascertain whether certain sub-populations of these genera live in particular hydrothermal-vent and deep-sea habitats.

METHODS

Sample collection

Low-temperature hydrothermal fluids and deep seawater were collected in the northeast Pacific Ocean (Fig. 2.1; Table 2.1). Diffuse-flow fluids were collected at Axial Seamount (282) and the Main Endeavour Field (MEF) (141) on the Endeavour Segment of the Juan de Fuca Ridge with a suction sampler and either filtered on deck or *in situ* using a hydrothermal fluid and particle sampler mounted on the Remote-Operated Vehicle *ROPOS* or the Deep-Submergence Vehicle *Alvin*. In some instances, these fluids were filtered first through cellulose membranes (3.0-µm pore size; Millipore) and then Sterivex-GP filters (0.2-µm pore size; Millipore) and frozen in liquid nitrogen (260). The former fraction was defined as "particle-attached" and subsequently analyzed. Other diffuse-flow samples were filtered only with Sterivex-GP filters. Deep seawater was collected at Axial Seamount, in the axial valley near the MEF, and near Baby Bare (406) with Niskin bottles jointly mounted with a conductivity-temperature-depth-transmissometry package onto a steel frame that was lowered over the side of the ship. These deep seawater samples were filtered only through Sterivex-GP filters on deck. Lastly, sections of sulfide structures measuring several meters in length were retrieved hot and fresh from the Mothra vent field (311) on the Endeavour Segment during the Edifice Rex sulfide-recovery program (139), subsampled according to mineralogical zonation, and frozen in liquid nitrogen (532).

DNA extraction

Community DNA was extracted from filters (260) and rocks (532) as previously described.

Primer construction

Oligomeric polymerase chain reaction (PCR) primers were designed to amplify selected euryhaline, halophilic and moderately halophilic bacterial taxa from the γ-*Proteobacteria*. Thirty-six 16S rRNA gene sequences from the genera *Halomonas* (29 strains), *Cobetia* (2 strains) and *Chromohalobacter* (5 strains), the three genera that comprise the family *Halomonadaceae*, were retrieved from the GenBank database via the National Center for Biotechnology Information (NCBI) website and aligned with other named and uncharacterized *Halomonas* isolates from hydrothermal vents (Chapter 1; 304). Ten *Marinobacter* 16S rRNA gene sequences from hydrothermal-vent isolates and other ecosystems were included in the alignment. Segments with near consensus among the *Halomonadaceae* were identified by eye and checked against the GenBank database to ascertain specificity. Three DNA sequences were selected that matched many, but not all, euryhaline, halophilic and moderately halophilic bacterial species belonging to the genera after which the primers were designed with zero or one base difference. All three oligomeric sequences also fortuitously matched numerous *Marinobacter* 16S rRNA gene sequences with zero or one base difference despite the phylogenetic distance between this group and the *Halomonadaceae*. (A primer specific to the *Marinobacter* sp. IC022 group from sea ice was recently reported [81].) The oligomeric sequences were additionally checked for self-complementarity and tailored to be approximately 20 bases in length and rich in guanine and cytosine residues at their 3' ends in order to facilitate DNA transcription during PCR. The sequences were named based on the first nucleotide position using *Escherichia coli* numbering: 115F (5'-GAG TAA TGC ATA GGA ATC TGC C); 327F (5'-ACA CGG CCC GAA CTC CTA CGG G); and 1446R (5'-TAG GCT AAC CAC TTC TG).

PCR optimization

Primers 115F (22-mer) and 1446R (17-mer) were chosen for subsequent PCR optimization in order to cover as much of the 16S rRNA gene as possible. With genomic *Halomonas* DNA from three species as template, the annealing temperature for each primer was optimized with temperature-gradient PCR using the following combinations of primers: 115F and the universal bacterial primer 1492R (5'-GGT TAC CTT GTT ACG ACT T) (333); 1446R and the universal bacterial primer 8F (5'-AGA GTT TGA TCC TGG CTC AG); and 115F and 1446R. The PCR cocktail for each 20-μl reaction contained 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 0.25 μM of each primer, 1× PCR buffer (Promega), 1 U *Taq* DNA polymerase (Promega), and 1 μl template. The profile had an initial denaturation at 94°C for 5 min followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 54–61°C for 45 s and extension at 72°C for 2 min, with a final 10-min extension at 72°C. A compatible optimum annealing temperature of 56°C for both primers enabled a subsequent check for primer species specificity by contrasting PCR amplifications of genomic DNA from *Halomonas* (9 strains), *Marinobacter* (7 strains), *Cobetia*, *Marinococcus, E. coli*, *Desulfurobacterium thermolithotrophum*, *Salinisphaera*, *Vibrio* (2 strains) and *Thermococcus* (2 strains) using the primer pairs of 115F-1446R and 8F-1492R. The same cocktail and profile as above were used, with a 56°C annealing temperature for the 115F-1446R primer combination and 55°C annealing temperature for the 8F-1492R pair.

Environmental DNA extracts were amplified using the primers 115F and 1446R again over a temperature gradient to confirm that optimal performance was still at 56°C. Diffuse-flow, sulfide-rock and deep seawater samples were optimized separately for cycle number if a band was obtained at all (Table 2.1). Multiple PCR reactions were run with each sample using a cycle number that indicated product saturation had not yet been reached (470, 572). Each product was reconditioned by running a three-cycle PCR on a 1:10 dilution of the initial product using the same cocktail and profile (590). The reconditioned products were pooled and cleaned with Qiaquick PCR purification columns (Qiagen) and then further purified by running 10 µl on a 1% (w/v) agarose gel and using the Qiaquick Gel Extraction Kit (Qiagen).

Clone libraries and sequencing

The final cleaned PCR products were then cloned into *E. coli* using the TOPO TA kit (Invitrogen) and One Shot TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. White colonies were examined for vector insert by growing transformed *E. coli* in Luria-Bertani broth with ampicillin (100 mg l^{-1}) for 75 min at 37°C on a shaker table at 200 rpm and running a 50-µl PCR (with 1 µl of cell culture) using M13F (5'-GTA AAA CGA CGG CCA G) and M13R (5'-CAG GAA ACA GCT ATG AC) vector primers. The cocktail consisted of 1 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphates, 0.4 µM of each primer, 1× PCR buffer (Promega), and 1.875 U *Taq* DNA polymerase (Promega). The profile began with denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 54°C for 45 s, and 72°C for 2 min, and ended with a 10-min extension at 72°C. PCR products were cleaned with Qiaquick PCR purification columns (Qiagen), and sometimes dried down in a Vacufuge™ (Eppendorf) and resuspended in deionized water. Sequences were obtained using the sequencing reaction mix provided in the DYEnamic ET Dye Terminator kit (Amersham) using the newly designed primers 115F and 1446R in addition to the universal bacterial primers 515F (5'-GTG CCA AGC MGC CGC GGT AA), 519R (5'-GWA TTA CCG CGG CKG CTG), 907R (5'-CCG TCA ATT CMT TTR AGT TT) and 926F (5'-AAA CTY AAA KGA ATT GAC GG) (333) and analyzed on a MegaBACE 1000 (Molecular Dynamics) at the University of Washington Marine Molecular Biotechnology Laboratory.

Phylogenetic analysis

Six unidirectional sequences from the 16S rRNA gene were obtained for each clone and were cleaned and rendered as a contiguous sequence using Sequencher version 4.1.2 (Gene Codes Corp.). With SeqApp version 1.9a169, the novel sequences were then aligned with each other and other *Halomonas*, *Marinobacter*, *Chromohalobacter* and *Cobetia* gene sequences, including the closest BLAST matches (Table 2.2; 16, 355), obtained from GenBank via the NCBI website and from Kaye and Baross (304). Sequences were confirmed as non-chimeric by using the Chimera Check function of the Ribosomal Database Project-II (RDP-II) website (119, 262), consistent with their seamless fit into a master alignment of closely related taxa. Variable stem-loops at bases 201–216 and 1135–1139 (*E. coli* numbering) were excised to facilitate the alignment. Maximum-likelihood phylogenetic trees were constructed using a Hasegawa-Kishino-Yano evolutionary model (240) with 10,000 puzzling steps and with mutation rates estimated from the data sets using TREE-PUZZLE version 5.0 with collapsed branches for quartet-puzzling support values of <50% (529, 568) and graphed using TreeView version 1.5 (460). Trees were developed using bases 196–1361 (library G), bases 197–1385 (library D), bases 179–1384 (library E), bases 179–1369 (library F) and bases 217–1352 (library H), *E. coli* numbering. Sequences of characterized and unnamed *Halomonadaceae* and *Marinobacter* isolates were included on the trees (Table 2.2). A sequence identity matrix was generated with BioEdit version 4.7.8 (234).

16S rRNA gene sequence accession numbers

The 16S rRNA gene sequences from clone libraries D, E, F, G and H have been submitted to GenBank. Accession numbers are forthcoming.

RESULTS

Primer construction and preliminary PCR amplification

Three primers were crafted from regions of the 16S rRNA gene with near consensus for forty-six halophilic to moderately halophilic and euryhaline bacterial taxa including *Halomonas*, *Chromohalobacter*, *Cobetia* and *Marinobacter*. No single region of the portion of the 16S rRNA gene examined was unique to these genera or to the genus *Halomonas* alone. The probe-match function of the RDP-II website (119) revealed that with one base mismatch the primers frequently hit phylogenetically disparate members of the α-, β-, γ-, δ- and ε-*Proteobacteria*, cyanobacteria, Gram-positive bacteria, spirochetes, the *Cytophaga-Flavobacterium-Bacteroides* group and some archaea. Specifically, primer 115F matched identically to 16S rRNA gene sequences of thirty-three members of the *Halomonadaceae* and twenty-one other species (including members of *Beggiatoa*, *Ehrlichia* and *Methylomonas*); with one mismatch it hit the 16S rRNA gene sequences of an additional forty-two intended bacterial strains but also hundreds of others, most notably strains of *Pseudomonas*, *Helicobacter*, *Acinetobacter* and *Moraxella*. Primer 327F uniquely matched the 16S rRNA gene sequences of fifty-one intended members of the *Halomonadaceae* and *Marinobacter* in addition to five other strains; with one mismatch it hit the 16S rRNA gene sequences of six more desired taxa as well as numerous others, including *Aerococcus*, *Blastobacter*, *Caulobacter* and *Lactobacillus*. Lastly, primer 1446R matched identically to the 16S rRNA gene sequences of thirty-eight strains within the *Halomonadaceae* and numerous strains of *Moraxella*; with a single base mismatch this primer paired with the 16S rRNA gene sequences of fifteen additional desired taxa but also with an extensive, broad selection of seemingly random microorganisms. None of the probe-match results reported here includes the hits of hundreds of clones without annotated affiliations in the RDP-II database.

Primers 115F and 1446R were selected for optimization with the notion that using two novel primers would focus amplification on the desired taxa (since fewer undesired taxa matched both primers) and because the combination of these two new primers provided maximal coverage of the 16S rRNA gene. Overlapping named taxa matched by both of these primers with zero mismatches included only certain *Halomonas* spp.; with one base mismatch selected members of the genera *Acinetobacter*, *Flavobacterium*, *Marinobacter*, *Methylomonas*, *Moraxella*, *Pseudomonas*, *Thialkalimicrobium* and *Vibrio*, along with symbionts and additional *Halomonas* spp., also matched. Temperature-gradient PCR using each primer in concert with a universal bacterial primer and with each other (using genomic *Halomonas* and *Marinobacter* DNA as template) revealed that the optimum annealing temperature for each primer was 56–57°C, the lower temperature being compatible with both of the new primers. The primer pair amplified a DNA fragment of approximately 1300 base-pairs length from genomic extracts of numerous strains of *Halomonas*, *Marinobacter* and *Cobetia*, but not of *Marinococcus* (Gram-positive), *Desulfurobacterium*, *Thermococcus* (archaea) or other strains of γ-*Proteobacteria* including *E. coli*, *Salinisphaera* and *Vibrio*.

Amplification of environmental DNA extracts

The primers 115F and 1446R were used to attempt to amplify 16S rRNA genes from DNA extracts from various diffuse-flow, sulfide-rock and deep seawater samples (Table 2.1). Some samples never produced a band while others did so inconsistently. Five samples amplified sufficiently consistently to undergo cycle optimization (31–35 cycles) and cloning, ultimately resulting in 12–44 16S rRNA gene sequences from each clone library of approximately 1200–1250 base-pairs length.

Clone libraries

The 16S rRNA gene sequences identified in all of the clone libraries belonged exclusively to the genera *Halomonas* (97% of clones) and *Marinobacter* (3% of clones). The *Halomonas* 16S rRNA gene sequences fell among only three phylogenetic branches, all within 16S rRNA Group 2 of the genus (24) with high quartet-puzzling support values (Fig. 2.2–2.7; Table 2.3). Within each of the three *Halomonas* (sub-groups 2A, 2B and 2C) and one *Marinobacter* clades, clone sequences shared approximately >99% sequence identity with each other and with the named and unnamed isolates included in the given sub-group (except in cases of outliers like *H. magadiensis*). Identity between a given sub-group and its closest neighboring sub-group was approximately >98%. The closest BLAST matches and all 16S rRNA Group 2 *Halomonas* are included on the phylogenetic trees. The source of isolation of both named and incompletely characterized *Halomonas* and *Marinobacter* taxa included in the phylogenetic analysis is summarized in Table 2.2.

DISCUSSION

The newly designed PCR primers enabled detection of the moderately halophilic and euryhaline genera *Halomonas* and *Marinobacter* in deep seawater and low-temperature hydrothermal-fluid emissions. It is unknown if certain members of these genera were excluded during amplification, nor can it be determined which other bacteria could have been amplified but were not due to their possible absence from the fluids examined. The 16S rRNA gene sequences obtained do indicate, however, clear population delineations among the taxa that the primers could amplify. In addition, the dominance of *Halomonas* phylotypes over *Marinobacter* sequences suggests that *Halomonas* spp. are more abundant than *Marinobacter* in the environments sampled, although PCR is not quantitative and previous enrichments of *Halomonas* spp. and *Marinobacter* spp. in diffuse-flow, hydrothermal-plume and other deep-sea samples were in more equitable proportions (304).

Library H from Easy vent (10°C) on Axial Seamount only contained clones that fell into the *H. meridiana*-*H. axialensis*-*H. aquamarina* branch (sub-group 2A) of the

Halomonas phylogeny. The other diffuse-flow sample, Library G from marker 33 (a 37–55°C hydrothermal vent) on Axial Seamount, also contained a high proportion of clones (68%) from this *Halomonas* clade, as did the nearby background seawater sample, Library F (42% of clones), collected 700 m to the southeast of marker 33. The other two deep background seawater samples from sites 230–260 km northeast along the Juan de Fuca Ridge and onto the eastern ridge flank contained relatively few clones from sub-group 2A. The observations that sub-group 2A were (i) dominant in diffuse-flow fluids sampled in 1998, (ii) well represented in a seawater sample taken in 1999 located <1 km away, and (iii) poorly represented in two other deep seawater libraries collected in 2000 and 2002, strongly suggest that the *H. meridiana-H. axialensis-H. aquamarina* clade represents a resident subseafloor population at Axial Seamount and that low-temperature vents may be seeding the nearby water column with these bacteria.

Members of sub-group 2A were present in all samples analyzed, but their significant enrichment in low-temperature vents indicates their growth in this niche. The maximum temperature for growth within the genus *Halomonas* is 40–50°C (372), though the maximum temperature for growth for most members of 16S rRNA Group 2 within the genus, including *H. meridiana*, *H. axialensis*, *H. aquamarina*, *H. variabilis*, *H. neptunia*, *H. glaciei* and *H. sulfidaeris*, is 30–40°C (Chapter 1; 372). The maximum temperature for growth may increase by several degrees under hydrostatic pressures equivalent to mid-ocean-ridge depths (100, 247, 293, 657, 659). While the marker 33 hydrothermal vent may be too warm (37–55°C) to permit growth of certain *Halomonas* spp., members of sub-groups 2A, 2B and 2C may be growing in cooler nearby habitats and flushed from the seafloor during the vigorous hydrothermal flow associated with that vent (260). Easy vent at 10°C may be a more hospitable environment for growth of *Halomonas*; indeed, 100% of clones from this library belonged to sub-group 2A.

The samples from which the low-temperature hydrothermal-fluid libraries were generated derived from the >3.0-µm, "particle-attached" fraction of the microbial population. Particle-attached bacteria and archaea are believed to represent subseafloor populations because the subseafloor life style may be characterized by biofilm communities attached to solid substrates (260, 261).

The cultured members sub-group 2A, *H. axialensis*, *H. meridiana* and *H. aquamarina*, were isolated from a 27°C diffuse-flow vent on Axial Seamount in 1998, from an Antarctic hypersaline lake, and from an unreported location and depth off the coast of California, respectively (Chapter 1; 273, 677). Characterized *H. meridiana* strains Slthf1 and Eplume2 were cultured from a 9°C diffuse-flow site on the Southern East Pacific Rise and from a 2°C hydrothermal plume above the MEF on the Juan de Fuca Ridge, respectively (Chapter 1). (*H. magadiensis*, isolated from a warm, alkaline east African rift lake [160, 161], is consistently a more distant cousin, phylogenetically and phenotypically.) Hydrostatic-pressure-temperature growth experiments suggest that *H. axialensis* and *H. meridiana* strain Slthf1 can thrive in cool to warm pressurized habitats (13–30°C, >25 MPa) but can grow only very slowly or not at all under cold (2°C) deep-sea conditions (Chapter 3), another indicator of the likely endemism of this sub-group in cool to warm subseafloor habitats. The uncharacterized *Halomonas* isolates belonging to sub-group 2A primarily derived from cold deep seawater and sediments in the Atlantic and Pacific Oceans, where they may or may not be active *in situ*.

Ninety-five percent of the clones from Library D, a deep seawater sample from the eastern flank of the Juan de Fuca Ridge 4.6 km from the basaltic outgroup at Baby Bare, fell into the *H. variabilis*-*H. neptunia*-*H. glaciei* sub-group 2B. This clade was also moderately well represented in the marker 33 library but not detected in any other sample. *H. neptunia* and *H. variabilis* were isolated from a 2°C hydrothermal plume above the MEF and an Antarctic hypersaline lake, respectively. (*H. variabilis*, more distantly related to the other members of this sub-group, was isolated from the Great Salt Lake, USA [178, 179]). The remaining isolates in sub-group 2B were obtained from a variety of cold environments: 2°C deep-sea hydrothermal plumes and basalt, Arctic seawater and various Antarctic habitats. One strain isolated from 194°C hydrothermal fluid from a vent on the TAG hydrothermal mound on the Mid-Atlantic Ridge clearly did not derive from that high-temperature environment (443). The clones in sub-group 2B may represent a cold-adapted, deep-sea *Halomonas* lineage.

Library E, derived from a deep seawater sample procured in 2000 from within the axial valley at the MEF without a detectable hydrothermal-plume signal, was comprised almost exclusively of clones belonging to sub-group 2C with *H. sulfidaeris* as its only characterized member. *H. sulfidaeris* was isolated from a sulfide-rock sample collected from the MEF in 1995 (Chapter 1). The three incompletely characterized isolates in this group were collected from a deep sediment layer, deep Arctic Ocean seawater and a terrestrial Antarctic sample. The low clone diversity again implicates a resident population that is locally enriched, which may be linked directly to the widespread massive metal-sulfide deposits for which the MEF and the encompassing 15-km stretch of the Endeavour Segment is known (141, 311, 494, 593). The presence of sub-group 2C clones in the marker 33 diffuse-flow and deep seawater samples from Axial Seamount indicate that this group is not restricted to the MEF and may be found in proximity to other metal-sulfide hydrothermal-vent structures and metalliferous sediments. The ability of members of sub-group 2C to grow under cold deep-sea conditions is unknown.

As mentioned previously, the deep seawater sample taken near Axial Seamount contained members of sub-groups 2A and 2C, but it also was the only sample that included *Marinobacter* spp. Like *Halomonas*, the genus *Marinobacter* is nearly ubiquitous, contains moderately halophilic and euryhaline species, and includes strains that have been isolated frequently from hydrothermal-vent environments (168, 304). Partial characterization of *Marinobacter* spp. from Endeavour Segment and Middle Valley samples of oxidized sulfide rock and metalliferous sediments revealed that some species are psychrophilic Fe^{2+}-oxidizers, a phenotype consistent with success in cold, oxic hydrothermal-vent environments (168). Moreover, these and other Fe^{2+}-oxidizing bacteria likely play a significant role in seafloor weathering of hydrothermal deposits (167). It is possible that the *Marinobacter* strains found in the library generated from the deep seawater sample near Axial Seamount may also be psychrophilic or psychrotolerant Fe^{2+}-oxidizers, perhaps utilizing Fe^{2+} and iron-sulfide particles in hydrothermal plumes (340) emanating from the caldera.

One must always be cautious when inferring phenotype from phylogenetic relationships, especially for the genus *Halomonas* within which 16S rRNA gene sequences of different species are typically >97% identical (24). Although it is not yet possible to demonstrate the precise phenotype of the bacteria represented in the clone libraries, the results suggest that certain sub-populations of *Halomonas* dominate over other *Halomonas* sub-groups in different hydrothermal-vent, subseafloor and deep-sea habitats. Intra-specific population delineations in marine environments are rarely documented. We assume that the clones represent euryhaline species, but the exact temperature ranges for growth (including possible psychrophily), especially under *in situ* hydrostatic pressures, and possible mixotrophic or autotrophic growth on Fe^{2+}, have yet to be determined. It would be instructive to attempt to isolate psychrophilic *Halomonas* and *Marinobacter* spp. using cold temperatures, elevated salinity and *in situ* hydrostatic pressure during enrichment.

Low-temperature hydrothermal-fluid emissions and eruption-induced releases of subseafloor fluids have yielded microorganisms that grow only at high temperatures and have a minimum growth temperature well above the sample temperature; these isolates are very likely subseafloor residents (257, 569, 570). It is more difficult, however, to constrain which psychrotolerant and psychrophilic microorganisms specifically inhabit shallow subseafloor environments, given that they also may grow at the deep seawater temperature of 2°C and that both seawater and crustal fluids comprise low-temperature hydrothermal-vent emissions (92, 93, 123, 322, 463). The results from this study begin to characterize the biogeography of *Halomonas* and *Marinobacter* spp. and indicate the presence of distinct deep-sea and subseafloor populations of moderately halophilic and euryhaline bacteria.

Table 2.1 Source of DNA extracts amplified with primers designed for moderately halophilic and euryhaline mesophilic bacteria.

Region	Sample	Site	Sample type	T (°C)	Depth (m)	Volume filtered (L)	Size fraction (µm)	Date	Dive or cast	PCR[*]
Axial Seamount	SS001	Marker 33	Diffuse flow	37[†]	~1520	1.00	>3.0	Aug 1998	*ROPOS* 462	G
	SS002	Milky vent	Diffuse flow	5	~1520	1.25	>3.0	Sept 1998	*ROPOS* 463	+
	FS004	Marshmallow vent	Diffuse flow	100	~1520	0.60	>3.0	Sept 1998	*ROPOS* 469	+
	FS009	Easy vent	Diffuse flow	10	~1520	1.00	>3.0	Sept 1998	*ROPOS* 473	H
	FS024	Magnesia/Whiteout vent	Diffuse flow	5.0	~1520	1.02	>3.0	July 1999	*ROPOS* 488	–
	FS045	Cloud pit, marker N6	Diffuse flow	15.9	~1520	1.00	>3.0	July 2000	*ROPOS* 547	–
	FS047	Marker 33	Diffuse flow	31.3	~1520	1.00	>3.0	July 2000	*ROPOS* 551	–
Near Axial Seamount	CTD003	Background seawater	Deep seawater	2	1295	1.00	>0.2	July 1999	CTD[‡]	F
Mothra vent field	G3FeSi	Finn	Iron and zinc sulfide, silica[§]	ND[‖]	2270	NA[¶]	Total cells	June 1998	NA	+
MEF[#]	MEF166	Near Puffer vent	Diffuse flow	39	~2200	3.00	>0.2	Sept 2000	*Alvin* 3618	+
Near MEF	MEF131	On-axis background seawater	Deep seawater[**]	2	2350	3.00	>0.2	Sept 2000	CTD	E
Near Baby Bare	CTD005	Background seawater	Deep seawater	2	2540	3.00	>0.2	Sept 2002	CTD	D

[*] Letters correspond to clone library constructed; +, preliminary or inconsistent amplification; –, no amplification.

[†] 55°C measured on a later dive during the cruise.

[‡] CTD, Conductivity-temperature-depth package that was attached to a rosette of Niskin bottles for water sampling.

[§] Zone FZ2b, 2–7 cm within a sulfide structure (532).

[‖] ND, Not determined.

[¶] NA, Not applicable.

[#] MEF, Main Endeavour Field, Endeavour Segment, Juan de Fuca Ridge.

[**] Hydrothermal plume not detected.

Table 2.2 Sources and accession numbers for isolates included on phylogenetic trees.

Clade	Isolate	Accession No.	Source	Sample depth (m)	T range for growth at 0.1 MPa (°C)*	References
Halomonas aquamarina-*H. meridiana*-*H. axialensis* (sub-group 2A)	*H. aquamarina* DSM 30161^{T}	AJ306888	Temperate ocean (western Pacific)	ND†	2–40‡	304, 677
	H. axialensis ATCC BAA-802^{T}	AF212206	Diffuse flow (Axial Seamount, northeast Pacific)	1530	−1 to 35	Chapter 1
	H. magadiensis NCIMB 13595^{T}	X92150	Hypersaline soda lake (Kenya)	0	20–50	160, 161
	H. meridiana DSM 5425^{T}	AJ306891	Cold hypersaline lake (Antarctica)	2–6	−5 to 37	273
	H. meridiana strain Slthf1 ATCC BAA-801^{T}	AF212217	Diffuse flow (SEPR§, South Pacific)	2580	−1 to 40	Chapter 1
	H. meridiana strain Eplume2 ATCC BAA-804^{T}	AF212201	Hydrothermal plume (MEF$^{\|}$, northeast Pacific)	2000	2–35	Chapter 1
	Halomonas sp. 1818c.Ssw1	AF212212	Deep seawater (South Pacific)	2000	ND	304
	Halomonas sp. 1856c.Ssw2	AF212215	Deep seawater (South Pacific)	1000	ND	304
	Halomonas sp. DII1a	AF254112	Sediment (Atlantic continental slope)	1500	ND	585
	Halomonas sp. HTB069	AB010846	Deep-sea sediment (eastern Pacific)	1050–10,897¶	ND	578
	Halomonas sp. HTB111	AB010870	Deep-sea sediment (eastern Pacific)	1050–10,897¶	ND	578
	Halomonas sp. V4.MS.05	AJ244730	Seawater (western Mediterranean)	ND	ND	195
H. variabilis-*H. neptunia*-*H. glaciei* (sub-group 2B)	*H. glaciei* JCM 11692^{T}	AJ431369	Fast ice (Antarctica)	0	4–30	485
	H. neptunia ATCC BAA-805^{T}	AF212202	Hydrothermal plume (MEF, northeast Pacific)	2000	−1 to 35	Chapter 1
	H. variabilis DSM 3051^{T}	AJ306893	Hypersaline lake (Great Salt Lake, Utah, USA)	0	15–37	178, 179
	Halomonas sp. d3.Eplume3	AF212203	Hydrothermal plume (MEF, northeast Pacific)	2000	ND	304
	Halomonas sp. Ko501	AF550585	Basalt or extinct sulfide structure	ND	ND	566
	Halomonas sp. NT N97	AB085658	Sediment (Nankai Trough, western Pacific)	4000	ND	442, 444
	Halomonas sp. R7074	AJ278781	Antarctic seawater	ND	ND	384
	Halomonas sp. R7102	AJ293827	Arctic seawater	ND	ND	384
	Halomonas sp. SYM P12	AB085660	Hydrothermal fluid (Suiyo Seamount)	1400	ND	442, 444
	Halomonas sp. TAG C2	AB042500	194°C hydrothermal fluid (TAG hydrothermal mound, MAR$^{\#}$)	3650	<4 to >37	443
	Halomonas sp. TNB I1	AB085648	Terrane (near Terra Nova Bay, Antarctica)	0	ND	442, 444
	Halomonas sp. TNB I20	AB085649	Terrane (near Terra Nova Bay, Antarctica)	0	ND	442, 444

Table 2.2 continued.

Clade	Isolate	Accession No.	Source	Sample depth (m)	T range for growth at 0.1 MPa (°C)[*]	References
H. sulfidaeris (sub-group 2C)	*H. sulfidaeris* ATCC BAA-803^T	AF212204	Sulfide rock (MEF, northeast Pacific)	2200	−1 to 35	Chapter 1
	Halomonas sp. ARD M34	AB085656	Deep seawater (Arctic Ocean)	3000	ND	442, 444
	Halomonas sp. DSM.25.14	AB094459	Subseafloor sediment (Sea of Okhotsk)	ND	ND	265
	Halomonas sp. TNB I59	AB085652	Melted terrestrial ice (near Terra Nova Bay, Antarctica)	0	ND	442, 444
Other 16S rRNA Group 2 *Halomonas*	*H. halodurans* DSM 5160^T	L42619	Temperate estuary (New Hampshire, USA)	0	4–43[**]	244, 508
	H. hydrothermalis ATCC BAA-800^T	AF212218	Diffuse flow (SEPR, South Pacific)	2580	2–40	Chapter 1
	H. subglaciescola DSM 4683^T	AJ306892	Cold hypersaline lake (Antarctica)	2–4	0–45[††]	192, 372
	H. venusta DSM 4743^T	AJ306894	Tropical surface seawater (North Pacific)	0	<4 to >40	42, 43
	Halomonas sp. 1840c.Sbplume1	AF212214	Buoyant hydrothermal plume (SEPR, South Pacific)	2644	ND	304
	Halomonas sp. 1864c.Splume4	AF212216	Hydrothermal plume (SEPR, South Pacific)	2566	ND	304
Other *Halomonas*	*H. desiderata* DSM 9502^T	X92417	Sewage (Germany)	0	10–45	49
	H. elongata ATCC 33173^T	X67023	Solar saltern (Holland)	0	4–45	629
	H. eurihalina ATCC 49336^T	X87218	Hypersaline soil and Mediterranean Sea (Spain) and lagoon (Chile)	0	4–45	372, 476
	H. halocynthiae DSM 14573^T	AJ417388	Gill tissue of ascidian (Japan Sea)	3	7–35	504
	H. pacifica DSM 4742^T	L42616	Tropical surface seawater (North Pacific)	0	2–45[‡‡]	Chapter 1; 43
Other *Halomonadaceae*	Strain 3012dK	AY196981	Deep on-axis seawater; possible weak hydrothermal-plume signal (MEF, northeast Pacific)	~2200	ND	305
	Cobetia marina DSM 4741^T	AJ306890	Tropical and temperate surface seawater (North Pacific and North Atlantic)	0	10–42[§§]	22, 42, 118
	Chromohalobacter marismortui ATCC 17056^T	X87219	Hypersaline lake (Dead Sea) and saltern (Mediterranean Sea)	0	5–45	380
	Cobetia sp. 1556b.Msw1	AF212205	Deep seawater (northeast Pacific)	1400	2–37	304, 306
	Zymobacter palmae ATCC 51623^T	D14555	Palm sap	0	21–39	445
Marinobacter	*M. aquaeolei* DSM 11845^T	AJ000726	Offshore petroleum well (Vietnam)	Subsurface	13–50	422
	"*M. arcticus*"	AF148811	Ballast water from *Mobile Arctic* (Alaska, USA)	0	ND	96
	M. hydrocarbonoclasticus 1 ATCC 27132^T	AB021372	Tropical coastal seawater (North Pacific)	0–1100	>4 to >45	42, 559

Table 2.2 continued.

Clade	Isolate	Accession No.	Source	Sample depth (m)	T range for growth at 0.1 MPa (°C)*	References
Marinobacter	*M. hydrocarbonoclasticus* 2 DSM 8798^{T}	X67022	Sediment at mouth of petroleum refinery outlet (western Mediterranean)	0	10–45	209
	Marinobacter sp. 1727a.Aplume1	AF212207	Hydrothermal plume (Axial Seamount, northeast Pacific)	1530	ND	304
	Marinobacter sp. 1741a.Asw1	AF212208	Deep seawater (northeast Pacific)	1100	ND	304
	Marinobacter sp. 1747a.Asw2	AF212209	Surface seawater (northeast Pacific)	10	ND	304
	Marinobacter sp. 1802c.Splume1	AF212210	Hydrothermal plume (SEPR, South Pacific)	2610	ND	304
	Marinobacter sp. 1814c.Splume2	AF212211	Hydrothermal plume (SEPR, South Pacific)	2510	ND	304
	Marinobacter sp. 1825c.Splume3	AF212213	Hydrothermal plume (SEPR, South Pacific)	2400	ND	304
	Marinobacter sp. 3030dQ	AY196982	Diffuse flow (marker DK2, MEF, northeast Pacific)	~2200	ND	305

* For species overall.
† ND, Not determined.
‡ Also reported as 15–37°C (372).
§ SEPR, Southern East Pacific Rise.
|| MEF, Main Endeavour Field, Endeavour Segment, Juan de Fuca Ridge.
¶ Sample depth not specified within range provided.
# MAR, Mid-Atlantic Ridge.
** Original T range reported as 4–35°C (508).
†† Original T range reported as –5 to 37°C (192, 273).
‡‡ Original T range reported as 4–45°C (43).
§§ Original T range reported as <6 to 40°C (118).

Table 2.3 Composition of clone libraries.

Clone library	Sample	Number of clones	Clones (%) related to phylogenetic branch			
			Sub-group 2A: *Halomonas meridiana, H. axialensis, H. aquamarina*	Sub-group 2B: *H. variabilis, H. neptunia, H. glaciei*	Sub-group 2C: *H. sulfidaeris*	*Marinobacter*
G	Marker 33 vent	37	68	16	16	0
H	Easy vent	44	100	0	0	0
F	Near Axial Seamount	12	42	0	25	33
D	Near Baby Bare	19	5	95	0	0
E	Near the Main Endeavour Field	26	8	0	92	0

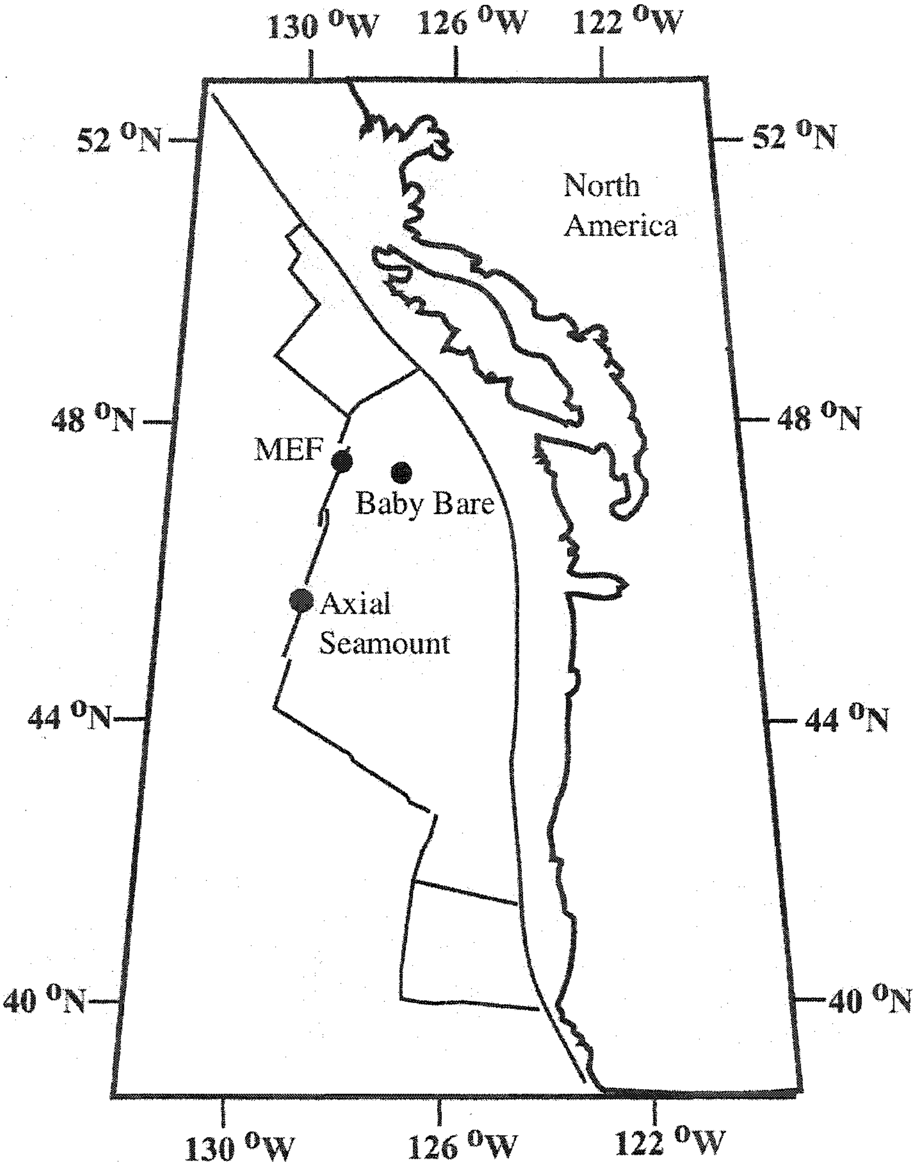

Figure 2.1 Map of the Juan de Fuca Ridge, associated ridge flanks and sampling sites.

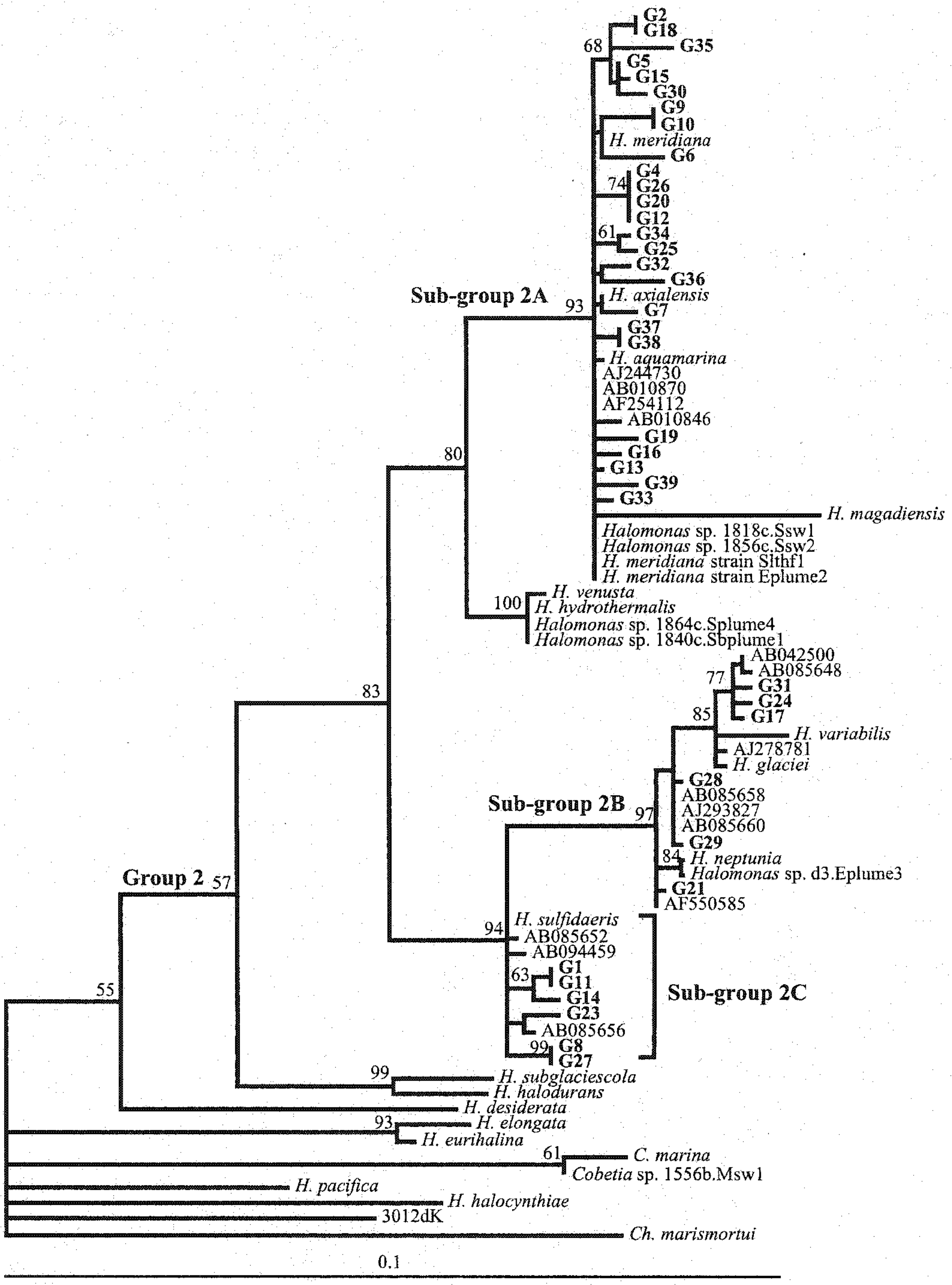

Figure 2.2 *Halomonadaceae* phylogeny from marker 33 vent, Axial Seamount (library G), including *Halomonas* (*H.*), *Cobetia* (*C.*) and *Chromohalobacter* (*Ch.*) species. Quartet-puzzling support values shown only at major tree nodes. *Ch. marismortui* is used as the outgroup. Bar, nucleotide changes per site.

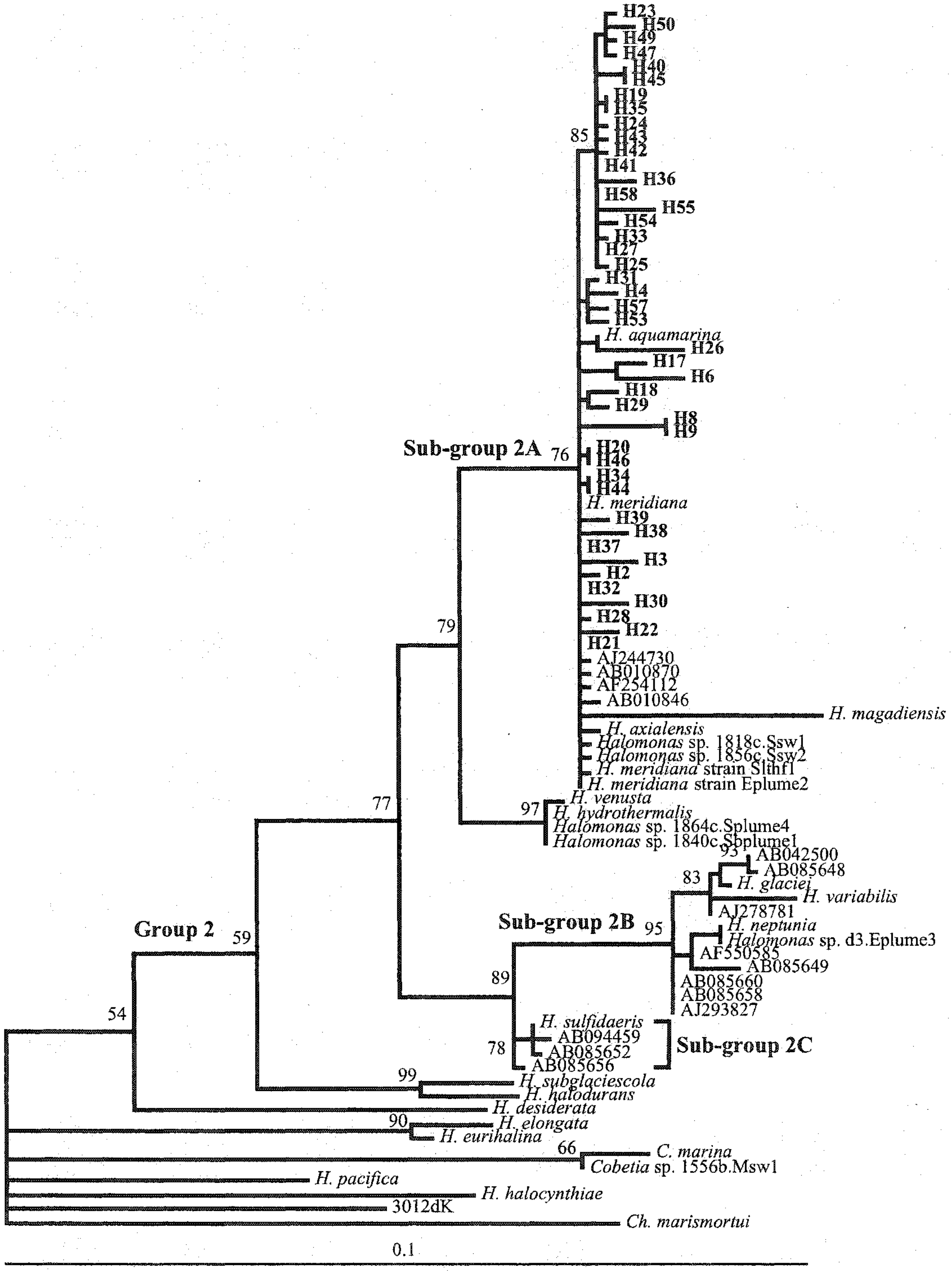

Figure 2.3 *Halomonadaceae* phylogeny from Easy vent, Axial Seamount (library H). Other features as in Fig. 2.2.

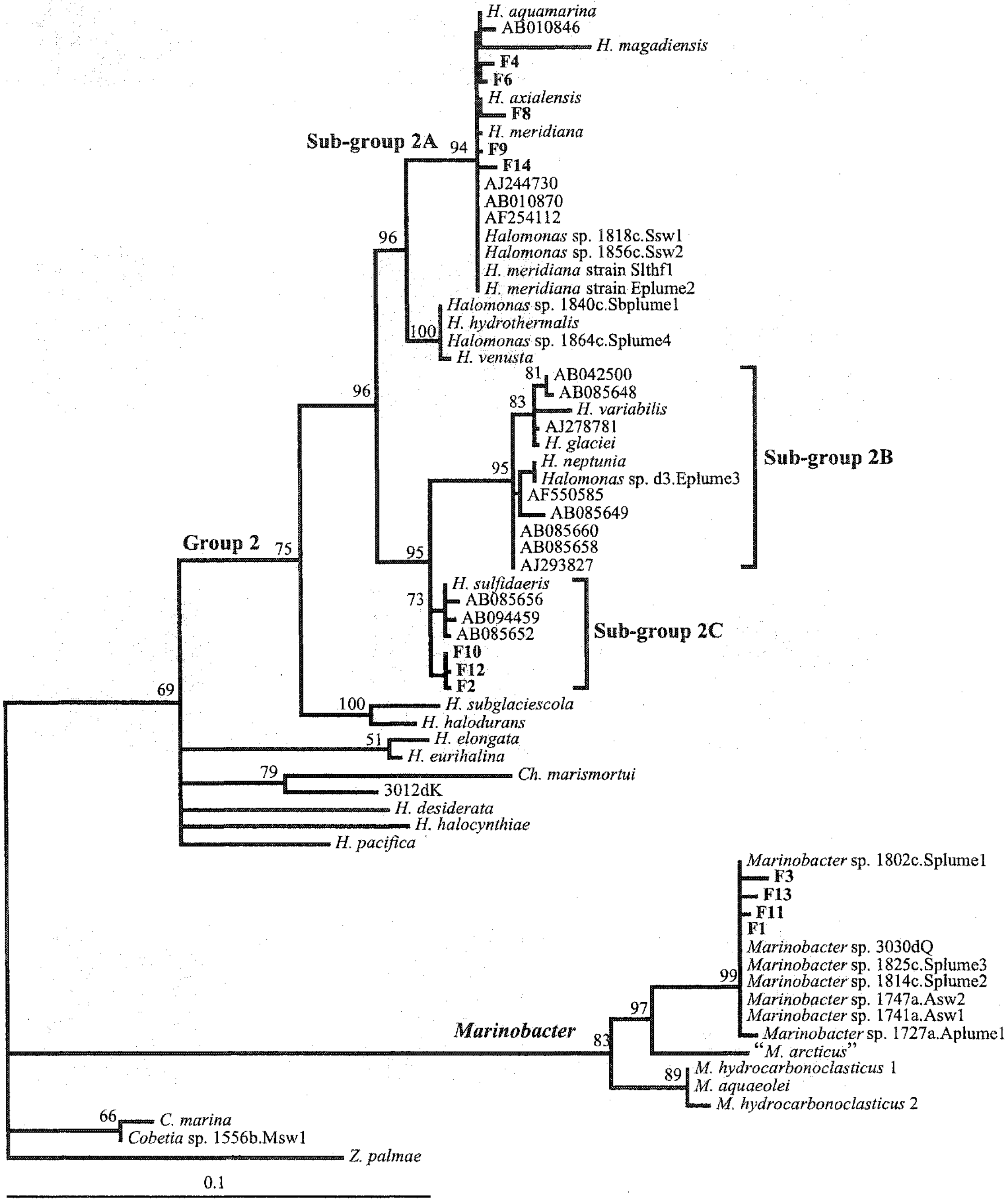

Figure 2.4 *Halomonadaceae* and *Marinobacter* (*M.*) phylogeny from deep background seawater near Axial Seamount (library F). *Zymobacter* (*Z.*) *palmae* is used as the outgroup. Other features as in Fig. 2.2.

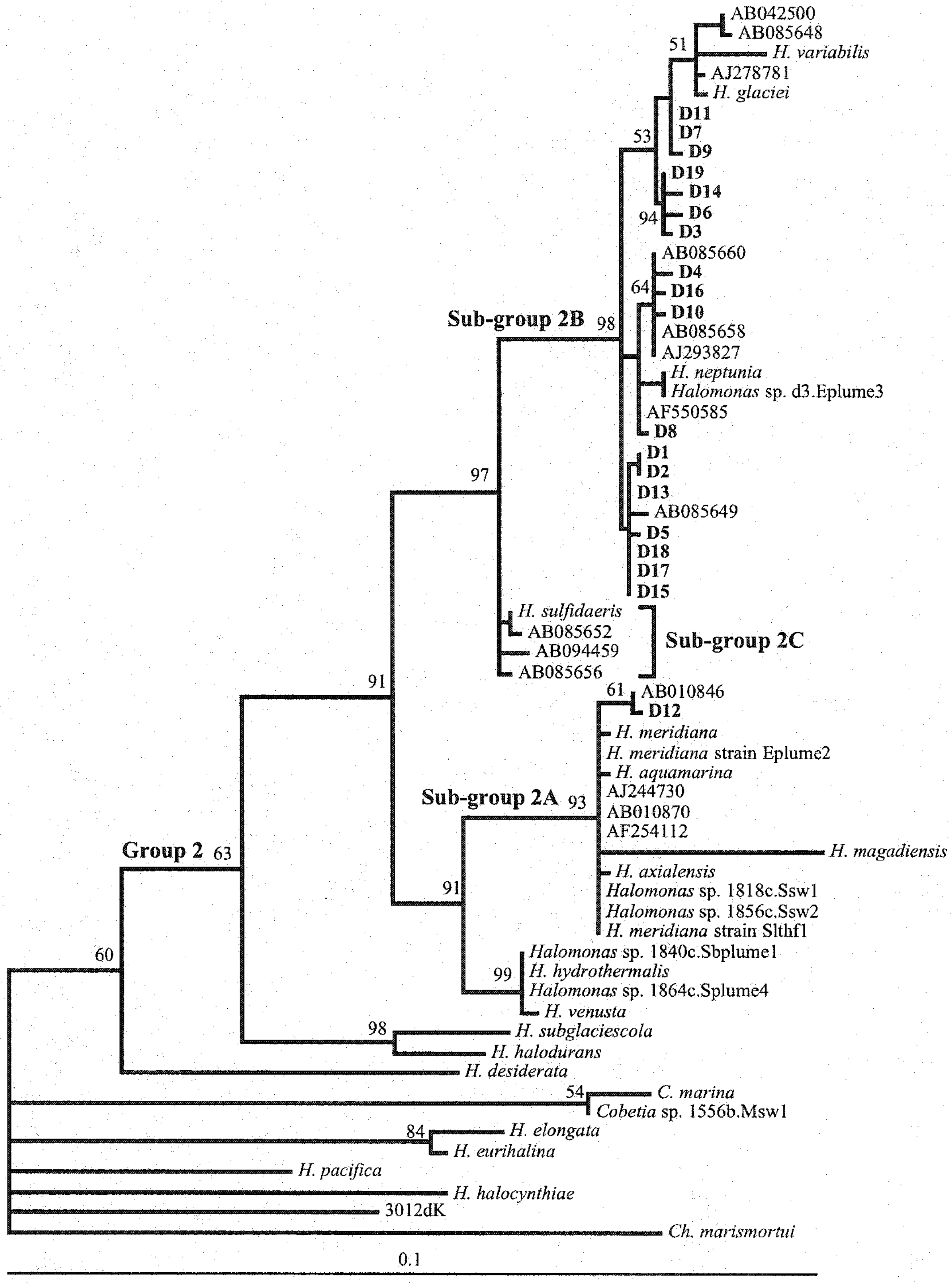

Figure 2.5 *Halomonadaceae* phylogeny from deep background seawater near Baby Bare (library D). Other features as in Fig. 2.2.

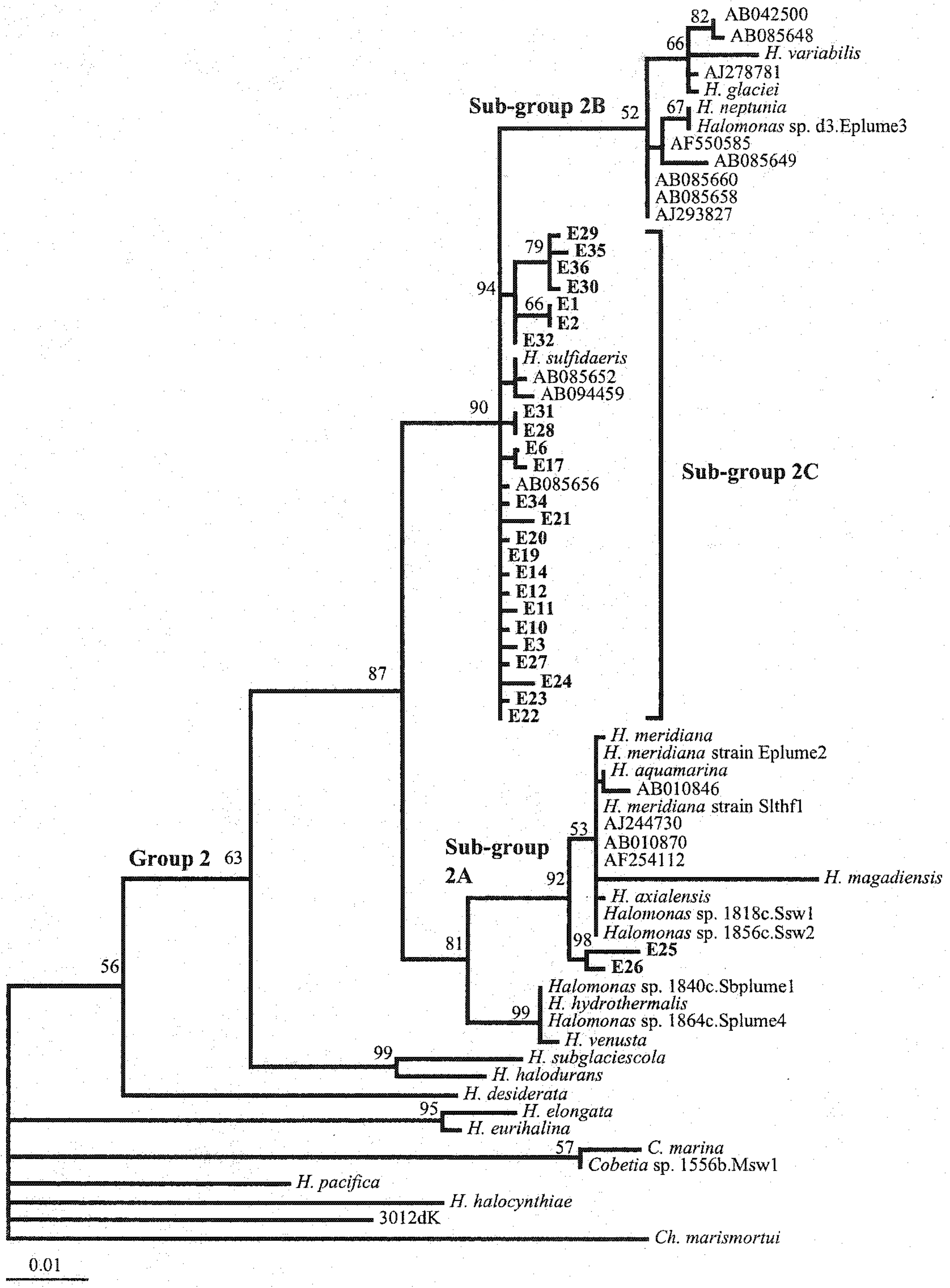

Figure 2.6 *Halomonadaceae* phylogeny from deep background seawater near the Main Endeavour Field (library E). Other features as in Fig. 2.2.

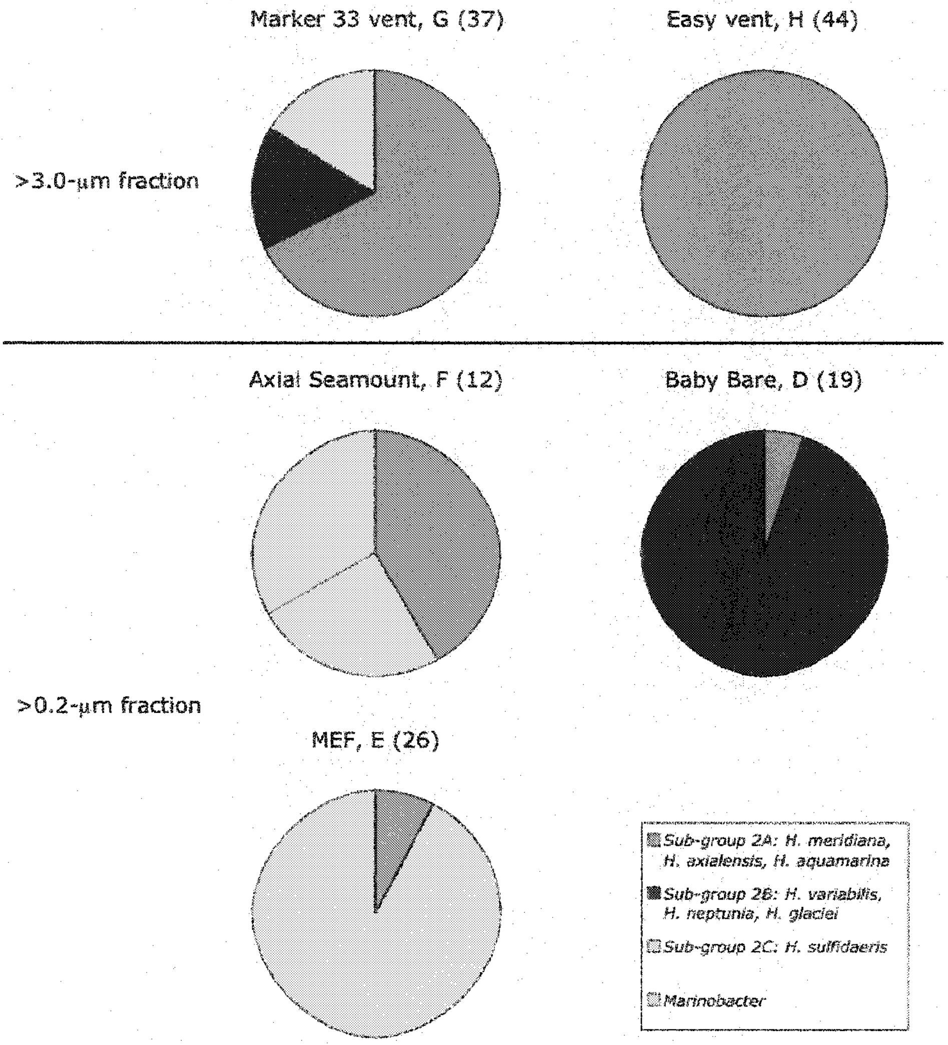

Figure 2.7 Composition of clone libraries based on phylogenetic clades within *Halomonas* (*H.*) and *Marinobacter*. Libraries G and H derived from the >3.0-µm filter fraction of cool to warm diffuse-flow hydrothermal fluids, whereas libraries D, E and F were constructed from cold deep background seawater samples (>0.2-µm filter fraction) located near the sites mentioned. MEF, Main Endeavour Field. Number of clones in parentheses.

CHAPTER THREE: The combined effects of temperature, hydrostatic pressure and salinity on growth, phospholipid profiles and protein patterns of four *Halomonas* species

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 (304, 443, 578, 613). Microorganisms 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 Oceans (304). 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, similar to Antarctic *Halomonas* isolates (Chapter 1; 372, 485, 613). This low minimum temperature for growth closely matches the temperature of the deep sea below 1500 m (and shallower towards the poles) and leaves open the question of whether *Halomonas* spp. 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 >0.1 MPa (146, 658). 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 (39, 144, 145, 659). 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 (100, 247), to increase the maximum growth temperature of a mesophilic *Pseudomonas* strain isolated from the deep sea (293), and to increase the ability of psychropiezophiles isolated from the cold deep sea to tolerate (or prefer [151]) slightly warmer temperatures (657, 659). Stated another way, the higher the hydrostatic pressure, the higher the temperature allowed (for T_{max}) or required (for T_{min}) to permit growth. Given that the minimum temperature for growth typically increases with increasing hydrostatic pressure, we hypothesized that *Halomonas* spp. with a T_{min} of –1 to 2°C 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 yield may also depend on the temperature-salinity regime. Growth curves revealed that the optimum and maximum salt concentrations for growth of the moderate halophiles *Halomonas halophila* and *Vibrio anguillarum* (reclassified as *Listonella anguillarum* [354]) increased with increasing temperature (228, 475). Similarly, "*Pseudomonas halosaccharolytica*" grew faster with elevated salinity at elevated temperature (441). Growth curves of a facultatively halophilic coccoid bacterium isolated from salted mackerel (ultimately classified as *Marinococcus halophilus* [236, 435]) likewise showed that growth at temperatures less than T_{opt} only occurred with decreased NaCl (434). This salinity-temperature relationship, however, is sometimes observed only for a single cardinal growth parameter, or not at all (35, 107, 219, 269, 424, 606, 630).

Moderately halophilic and euryhaline bacteria 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 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 (22–25°C) at 1325-m and 1466-m seawater depth, respectively, in the Red Sea (21, 165). At 30°C, *S. shabanensis* grew with 1–28% NaCl at 0.1 MPa and with 5–20%

NaCl at 15 MPa, but it did not grow at ≥20 MPa (21). At 0.1 MPa, this bacterium accumulated up to 4 M of compatible solutes under salt stress (165), though 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 production of compatible solutes, whereby the combination of the two stresses resulted in a disproportionate increase in accumulation of these compounds (365).

The current set of experiments utilizes four *Halomonas* strains, three isolated from deep-sea hydrothermal vents and one isolated from surface seawater, and explores several facets of the combined effects of temperature, hydrostatic pressure and salinity on microbial growth. The selected parameters are 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* spp.

METHODS

Growth curves

Three deep-sea *Halomonas* strains isolated from hydrothermal vents, *H. axialensis* ATCC BAA-802^T, *H. meridiana* strain Slthf1 ATCC BAA-801^T and *H. hydrothermalis* ATCC BAA-800^T, and *H. pacifica* ATCC 27122 isolated from surface seawater on the Hawaiian coast (Table 3.1; 43) were grown over 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). The growth medium (4% total salts, pH 8.0) described in Kaye and Baross (304) was augmented with NaCl to achieve higher salt concentrations. Cells were inoculated into fresh media and kept at the relevant incubation temperature for 3–12.5 h (for the 30°C experiments), 22.25–27.5 h (for the 13°C experiments) or 2–5 d (for the 2°C experiments) to allow cells to acclimate to the temperature and salinity conditions before pressurization. The inoculated media was then used to completely fill 4.6-ml polyethylene transfer pipets (Samco Scientific) (113) which were sealed by melting the tip in a Bunsen flame and crimping with needle-nose pliers. The pipets were then placed into stainless steel pressure vessels (Tem-Pres Division of LECO Corp.), topped off with distilled water of the appropriate temperature, manually pressurized with an Enerpac 11-400 pump outfitted with a quick-connect coupling device (663) 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. Vessels were sequentially sacrificed at five time intervals and slowly depressurized (30–60 s), and a triplicate set of pipets was harvested for each time point and each organism. Growth was assessed at 0.1 MPa using the same general protocol, with pipets submerged in distilled water.

Growth at 13 and 30°C was monitored by optical density using a Lambda UV/VIS Spectrophotometer at 600 nm and at 2°C by direct counts by filtering and staining cells with DAPI (4',6-diamidino-2-phenylindole) (471). Growth rates were 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 an increase in cell number with time by calculating the ratio of growth rates determined simultaneously by cell counts and optical turbidity 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.

Phospholipid fatty acid (PLFA) analysis

H. axialensis and *H. hydrothermalis* were grown at 30°C and 0.1 or 45 MPa in 23-ml transfer pipets (Samco Scientific) using media with either 4 or 11% total salts. Cultures were grown in pressure vessels as described above and harvested in early- to mid-exponential-phase growth. Cells were centrifuged at 10,000 × *g* at 26–29°C immediately following decompression; pellets were resuspended in a 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 PLFA analysis using a modified Bligh and Dyer method (646). 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, Wisc.) for fractionation into cytosolic and membrane-bound proteins and subsequent 1-D gel electrophoresis. Samples were lysed with an osmotic lysis buffer with nuclease and protease inhibitors and centrifuged for 30 min, after which the supernatant was removed and the entire procedure repeated upon 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 less β-mercaptoethanol was added to the remaining pellet, which was mixed by vortex and held in a boiling water bath for 5 min. The protein concentration for each fraction was then determined with a bicinchoninic-acid assay (556). 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 protein ml^{-1}, and placed in a boiling water bath for 5 min. SDS slab gel electrophoresis (329, 437) was run for each protein fraction in triplicate using a 10% acrylamide slab gel (125 mm long, 150 mm wide and 0.75 mm thick) overlaid with a 25-mm 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, 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 using Nonlinear Dynamics 1D Advanced software (version 5.0). Triplicate runs were used to ensure consistency of protein band patterns and to calculate standard deviations of band intensities. Images provided here were obtained by re-running gels with a single lane devoted to each sample and each protein fraction.

RESULTS

Growth curves

From previous studies, *H. axialensis*, *H. meridiana* strain Slthf1, *H. hydrothermalis* and *H. pacifica* all exhibited psychrotolerant and euryhaline growth at 0.1 MPa (Table 3.1). The three strains isolated from hydrothermal vents were able to grow at temperatures as low as –1 to 2°C and grew significantly better at 2°C than *H. pacifica*. The previously reported T_{min} at 0.1 MPa for *H. pacifica* is 4°C, though slight growth was found at 2°C with the growth medium employed in this study.

Temperature-hydrostatic-pressure-salinity growth curves (Fig. 3.1–3.9) showed that temperature had a dominant effect on the growth rate, whereby fastest growth (0.17–0.74 h^{-1}) occurred at 30°C for each *Halomonas* strain regardless of the tested combination of hydrostatic pressure and salinity. (Note that color coding on the growth plots is reset for each bacterium, such that, for example, red indicates fastest growth for each organism though the corresponding growth-rate value is different for each strain.) In addition, increased hydrostatic pressure generally resulted in a decreased growth rate for each organism at any given salinity and temperature combination, indicating piezotolerant growth for each strain. Growth of each strain was very slow or did not occur above 25 MPa at 2°C at all salinities tested. Similarly, decreased temperature generally resulted in a decreased maximum hydrostatic pressure for growth. For example, at 30°C and 4% total salts, the highest hydrostatic pressure that permitted growth for *H. axialensis* (Fig. 3.1) and *H. meridiana* strain Slthf1 (Fig. 3.4) was 45 and >55 MPa, respectively, but at 2°C these values dropped to 25 and 45 MPa, respectively (Fig. 3.3 and 3.6).

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 at both 30°C (Fig. 3.1) and 2°C (Fig. 3.3) but equally well with 4 and 11% total salts at 13°C (Fig. 3.2). Growth was favored by 11% total salts at 30°C at the higher hydrostatic pressures of 7.5–55 MPa (Fig. 3.1). Salt-enhanced growth was not seen at 13°C (Fig. 3.2) or 2°C (Fig. 3.3). At 13°C, growth rates were roughly equal at 4 and 11% total salts between 7.5 and 55 MPa (Fig. 3.2), whereas at 2°C growth rates declined with increasing hydrostatic pressure and/or salinity (Fig. 3.3).

H. meridiana strain Slthf1 showed a consistently piezotolerant and psychrotolerant response at 30°C (Fig. 3.4) and 13°C (Fig. 3.5), with 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 yielding growth (0.1 to >35 MPa) (Fig. 3.6). 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. 3.9). Growth curves of *H. hydrothermalis* at 13°C (Fig. 3.8) and 30°C (Fig. 3.7) had large 95% confidence intervals, masking any growth trends.

H. pacifica also showed salinity-enhanced growth, but only at 30°C and >45 MPa (Fig. 3.10). Growth was faster with 11 and 17% total salts under these conditions as compared 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 salinity and hydrostatic pressure (Fig. 3.11). No significant growth occurred at 2°C (Fig. 3.12).

Salt-enhanced growth with hydrostatic pressure and at low temperature 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 four conditions tested (30°C; 0.1 and 45 MPa; 4 and 11% total salts), the predominant phospholipids for both strains examined, *H. axialensis* and *H. hydrothermalis*, were 18:1ω7c, 16:0 and 16:1ω7c, comprising 94.2–96.3% of the total (Fig. 3.13; Table 3.2). *H. hydrothermalis* contained slightly more of the minor components 18:1ω9c and 18:1ω7t than *H. axialensis*, lipids that decreased in concentration with increased salinity in *H. hydrothermalis*. *H. axialensis* contained slightly more cy19:0 (0.6–1.0%) than *H. hydrothermalis* (0.0–0.2%). At 0.1 MPa, the degree of lipid saturation in *H. hydrothermalis* increased with increasing salinity from 20.0 to 23.4%. The monounsaturated fatty acids (MUFA) 18:1ω7c and 16:1ω7c comprised 71.4–76.7% and 84.5–85.7% of the total at 0.1 and 45 MPa, respectively, while the saturated fatty acid (SFA) 16:0 decreased in concentration from 17.5–24.1% to 10.3–11.8% simultaneously as hydrostatic pressure and the proportion of MUFA increased. The medium used to grow 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 analyzed for lipids contained 1.2–6.7 mg protein ml^{-1} and 0.94–14.5 mg protein ml^{-1}, respectively. Forty micrograms of protein were run in each gel lane in triplicate with computerized band matching (Fig. 3.16–3.19) and are summarized for comparison (Fig. 3.14 and 3.15).

When grown at 30°C with 4% total salts at both 0.1 and 45 MPa, the overall protein patterns for *H. axialensis* were similar within the respective cytosolic- and membrane-protein fractions (Fig. 3.14 and 3.15), although a suite of proteins was slightly induced or inhibited by hydrostatic pressure (Table 3.3). Two proteins were significantly induced by hydrostatic pressure and are considered hydrostatic-pressure-induced proteins (PIP; cytosolic band *f*; membrane band *c*; Table 3.3).

Overall, the protein profiles within each protein fraction for *H. hydrothermalis* at 30°C were also similar to each other (Fig. 3.14 and 3.15) whether the strain was grown at approximate seawater salinity (4% total salts) or elevated salinity (11% total salts) and at sea-surface (0.1 MPa) or deep-sea (45 MPa) hydrostatic pressures. However, comparison of individual bands did reveal a variety of interactions between salinity and hydrostatic pressure (Table 3.4). 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*; 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 elevated salinity (cytosolic bands *l*, *m*, *n*, *o*, *p*, *v* and *w*). Other cytosolic proteins were inhibited only with the combination of elevated salinity and hydrostatic pressure (cytosolic bands *r*, *s* and *t*). One membrane protein was significantly inhibited by hydrostatic pressure but enhanced with 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 salt concentration or hydrostatic pressure were not induced by the combination of the two (cytosolic band *u*; membrane band *h*).

DISCUSSION

The *Halomonas* strains tested were psychrotolerant and piezotolerant, able to grow only slowly or not detectably under cold deep-sea conditions (2°C and >15 MPa) with the medium employed. *Halomonas* spp. appear to be much more piezotolerant than the moderate halophile *S. shabanensis* isolated from 1325-m depth in the brine-seawater interface above the Shaban Deep in the Red Sea (21). Growth rates for all of the *Halomonas* strains were faster under the cool to warm conditions that characterize low-temperature hydrothermal fluids and associated subseafloor environments (13–30°C, >15 MPa). *Halomonas* spp. are therefore potential 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 at 10–15°C at depth, may also be conducive to the growth of *Halomonas* spp.). 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 strongly in fact indicate than *H. axialensis* is a subseafloor resident atop this volcano (Chapter 2). In addition, these growth-rate data 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 (540, 541), eastern Mediterranean Sea (65, 98, 131, 132, 200, 285, 377, 535) and Red Sea (137, 239). Indeed, *H. aquamarina* was one of only three taxa cultured from the brine-seawater interface above the Urania Basin at ~3500-m depth in the eastern Mediterranean Sea (524). The brine pools themselves are anoxic, however, and anaerobic growth at elevated salinity and hydrostatic pressure was not tested in this study.

Elevated salinity appeared partially to compensate for the depression of the growth rate caused by low temperature. Vent *Halomonas* isolates showed an increasingly halophilic response at low temperature and >7.5 MPa. It is possible that a salinity enhancement of growth at low temperature would be seen in *H. pacifica* if cells were tested at a slightly warmer temperature, a few degrees above its T_{min} of 2–4°C (304, 372). Low temperature is analogous to elevated hydrostatic pressure in its effects on proteins and membranes (39), 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 the other (365, 656). 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 preferential exclusion and minimization of bound water (365, 497, 656). It is important to bear it 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 (203, 613).

Antagonistic interactions between hydrostatic pressure and increased solute concentrations have been observed in a variety of disparate experiments. Elevated salinity was previously shown to increase the maximum hydrostatic pressure for growth in the psychrophile *Moritella marina* (461, 604) and *Streptococcus faecalis* (reclassified as *Enterococcus faecalis* [527]) (361) and to decrease the piezosensitivity of *Escherichia coli* to an extremely high hydrostatic pressure of 270 MPa (242). Likewise, 15% NaCl (as compared with 3% NaCl) dramatically enhanced the survival of a halophilic, euryhaline marine isolate of *Micrococcus roseus* (reclassified as *Kocuria rosea* [560]) under a very high hydrostatic pressure of 138 MPa (580). Osmotic pressure (created with low concentrations of ethanol) also mitigated the hydrostatic-pressure-induced inhibition of cell division in *E. coli*, enabling reversion of cell morphology from an elongate form to its canonical rod shape (579). Additional *in vitro* studies show similar counterbalances between hydrostatic and osmotic pressures. The dissociation of Arc repressor protein from DNA, its substrate, decreased linearly with increased glycerol concentration (446). Hydrostatic pressure was also seen to reverse the effects of osmotic pressure (created with organic compounds) on DNA site selection by the restriction endonucleases *Eco*RI, *Bam*HI, *Pvu* II and *Eco*RV, highlighting the opposing interplay that these two stresses have on hydration of proteins and protein-substrate complexes (495, 496). Salt-induced enhancement of growth with elevated hydrostatic pressure (at cold and warm temperatures) is consistent with the opposing influence 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 increasing hydrostatic pressure (39, 658), presumably as a means to maintain membrane fluidity. Consistent with previous studies of *Halomonas* strains and other moderately halophilic and euryhaline species (396, 397, 399, 441, 605, 627), the proportion of MUFA decreased in concentration (and the proportion of SFA concomitantly increased in concentration) with increasing salinity as was observed with *H. hydrothermalis* at 0.1 MPa and 4 or 11% total salts. 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 (0.0–1.0%), and changes in their proportions were not apparent as previously documented with *Halomonas* and other moderately halophilic and euryhaline bacteria (252, 396, 397, 400, 441, 605). While changes in the proportion of CFA with salinity are not always observed among *Halomonas* spp. (411), the CFA cy17:0 and cy19:0 typically comprise a much greater proportion of the phospholipids present (up to 37.2%) (194, 384, 546). It should be noted as well that lipid profiles vary significantly with growth phase and between the members of the family *Halomonadaceae* (51, 398). In this study, cells were grown to early- to mid-exponential phase, whereas for the other lipid analyses cited, cells were usually harvested in late-exponential or stationary phase. This difference may explain the very low concentration of CFA observed in this experiment.

The cytosolic- and membrane-protein patterns determined at 30°C showed overarching consistency at low and high hydrostatic pressure and salinity, indicating that cellular functions would be generally consistent between warm shallow and deep, marine and hypersaline habitats. At 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 regimes, and while some proteins appeared to be salt- or hydrostatic-pressure-specific and likely serve osmoregulatory or hydrostatic-pressure-adaptation functions, frequently the combination of elevated salinity and hydrostatic pressure illustrated that the two stresses mitigate the effect of the other. Salinity and hydrostatic pressure were thus competitive, not synergistic, for these proteins. These proteins may be involved in adaptation of phospholipid membranes given that salinity and hydrostatic pressure exerted opposing influences on lipid saturation. These proteins may also be expressed during salt- or hydrostatic-pressure-induced stress and function to repair or chaperone damaged macromolecules and polypeptides. A link between a salt- or hydrostatic-pressure-induced general stress response and/or salt- or hydrostatic-pressure-adapted growth is possible given the behavior of the interaction of these stresses. If so, it is possible that the euryhaline phenotype of deep-sea *Halomonas* spp. and other bacteria may 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 expression of certain genes during growth. Both the protein-expression and growth-rate data thus suggest that deep-sea hypersaline habitats may be amenable to the proliferation and success of *Halomonas* spp.

Table 3.1 Source of *Halomonas* strains used in hydrostatic-pressure experiments and basic growth features at sea-surface pressure.

Strain	Source of strain			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)	T (°C)	T_{min}	T_{opt}	T_{max}	S_{min}	S_{opt}	S_{max}
*H. axialensis**	Diffuse flow	1533	27	–1	30	35	0.5	4	24
H. meridiana strain Slthf1*	Diffuse flow	2580	9	–1	20–35	40	0.5	2–7	22
*H. hydrothermalis**	Diffuse flow	2580	9	2	30	40	0.5	4–7	22
H. pacifica†	Tropical seawater	0	ND‡	2–4§	ND	45	0	0.5–3	20

* Chapter 1; Kaye and Baross (304).

† Baumann *et al.* (43); Mata *et al.* (372).

‡ ND, Not determined.

§ Equivocal growth a 2°C. T_{min} reported as 2 or 4°C (Chapter 1; 372).

Table 3.2 Phospholipid fatty acid (PLFA) profiles of *Halomonas axialensis* and *H. hydrothermalis* grown at 30°C under different salinity and hydrostatic-pressure conditions.

PLFA category and identity	Total PLFA (%)					
	H. axialensis		*H. hydrothermalis*			
	4% total salts		4% total salts		11% total salts	
	0.1 MPa	45 MPa	0.1 MPa	45 MPa	0.1 MPa	45 MPa
Terminally branched saturates						
i15:0	0.0	0.4	0.1	0.0	0.0	0.0
a15:0	0.0	0.0	0.0	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
Summary						
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
Branched monoenoics	0.0	0.0	0.0	0.0	0.0	0.0
Mid-chain branched	0.0	0.0	0.0	0.0	0.0	0.0
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

Table 3.3 Impact of increased hydrostatic pressure (45 versus 0.1 MPa) at 30°C on proteins of *Halomonas axialensis*.

Protein fraction	Protein			Effect of P*	Comment
	Band label	Band # in control lane	Approximate weight (kDa)		
Cytosolic (Fig. 3.14)	*a*	4	100	– –	P repression
	b	5	90	– –	P repression
	c	9	60	+	P enhancement
	d	10	50	+	P enhancement
	e	12	43	+	P enhancement
	f	14	42	+ + +	PIP†
	g	15	41	– –	P repression
	h	19	38	–	P repression
	i	21	36	–	P repression
	j	39	20	+	P enhancement
	k	45	15	–	P repression
Membrane (Fig. 3.15)	*a*	2	160	+ +	P enhancement
	b	3	150	– –	P repression
	c	11	60	+ + +	PIP
	d	26	33	+	P enhancement
	e	30	26	–	P repression
	f	31	25	+	P enhancement

* Symbols indicate degree of change of intensity of protein band.

† PIP, Hydrostatic-pressure-induced protein.

Table 3.4 Impact of increased hydrostatic pressure (45 versus 0.1 MPa) and salinity (11 versus 4% total salts) at 30°C on proteins of *Halomonas hydrothermalis*.

Protein fraction	Protein			Effect of[*]			Comment
	Band label	Band # in control lane	Approximate weight (kDa)	P	S	P and S	
Cytosolic (Fig. 3.14)	*l*	3	190	+			S cancels P enhancement
	m	10	100	+			S cancels P enhancement
	n	13	90	+			S cancels P enhancement
	o	17	70	+			S cancels P enhancement
	p	19	65	+			S cancels P enhancement
	q	21	60	+	–		S and P cancel each other
	r	22	50			–	Repression by combination of S and P
	s	24	43			–	Repression by combination of S and P
	t	25	40			–	Repression by combination of S and P
	u	28	35	+	+		S and P combined cancel protein induction by either stressor alone
	v	44	16	+			S cancels P enhancement
	w	46	15	+			S cancels P enhancement

Table 3.4 continued.

Protein fraction	Protein			Effect of[*]			Comment
	Band label	Band # in control lane	Approximate weight (kDa)	P	S	P and S	
Membrane (Fig. 3.15)	*g*	4	200		+ + +	+ + +	SIP[†] only
	h	24	47	+	+ +		S and P combined cancel protein induction by either stress alone
	i	27	40	– –	+	– –	P repression dominates over S enhancement
	j	33	25	–	+		S and P cancel each other
	k	34	23		+	+	SIP only
	l	35	21	–	+		S and P cancel each other
	m	36	19	–	+		S and P cancel each other
	n	37	18	–	+		S and P cancel each other
	o	38	15	–	+		S and P cancel each other

[*] Symbols indicate degree of change of intensity of protein band; a lack of symbol indicates no significant change.

[†] SIP, Salt-induced protein.

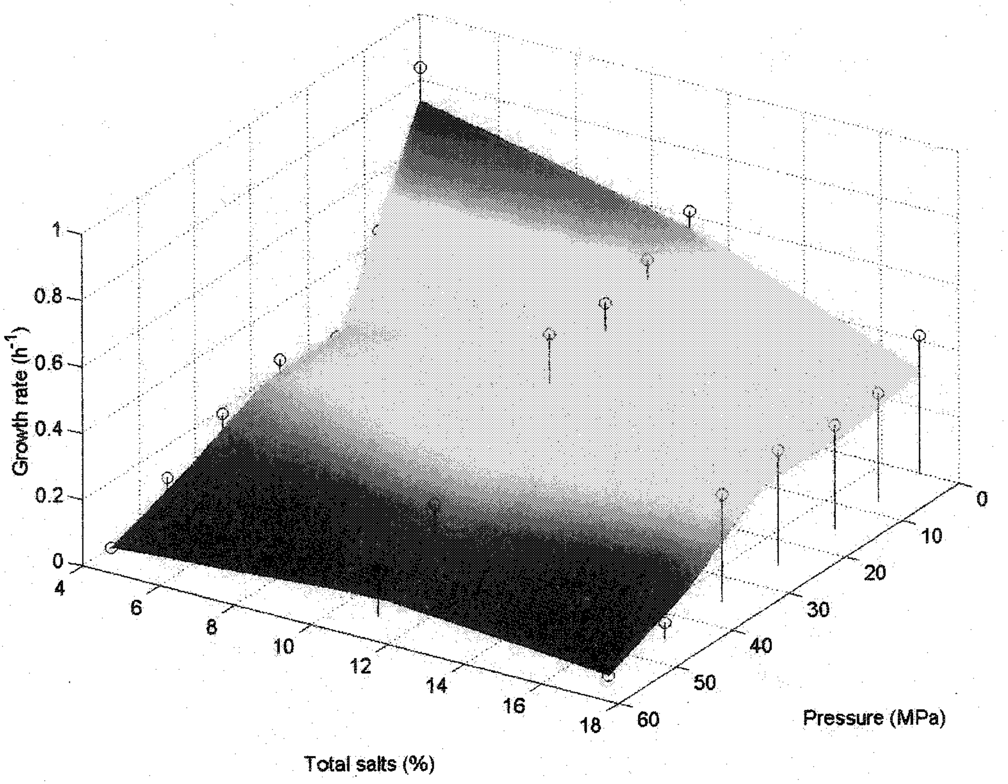

Figure 3.1 Growth of *Halomonas axialensis* at 30°C, 4, 11 and 17% total salts, and under 0.1, 7.5, 15, 25, 35, 45 and 55 MPa of hydrostatic pressure. Warm colors (red) indicate faster growth. Note differences in *z*-axis scale among Fig. 3.1–3.12.

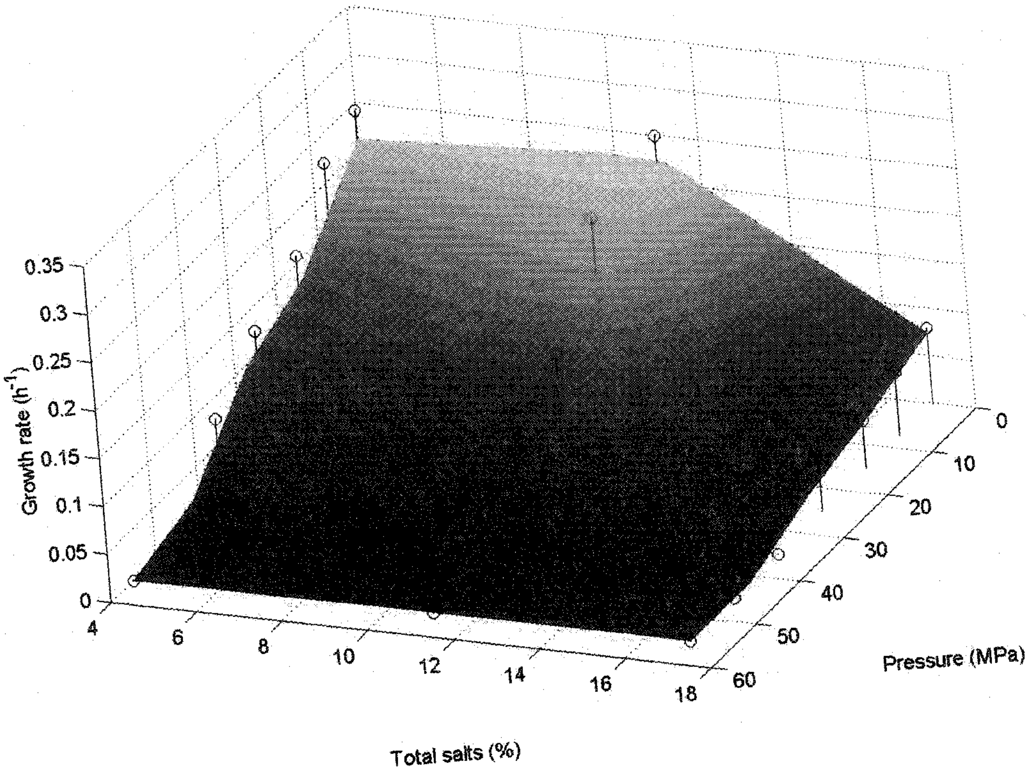

Figure 3.2 Growth of *Halomonas axialensis* at 13°C. Other features as in Fig. 3.1.

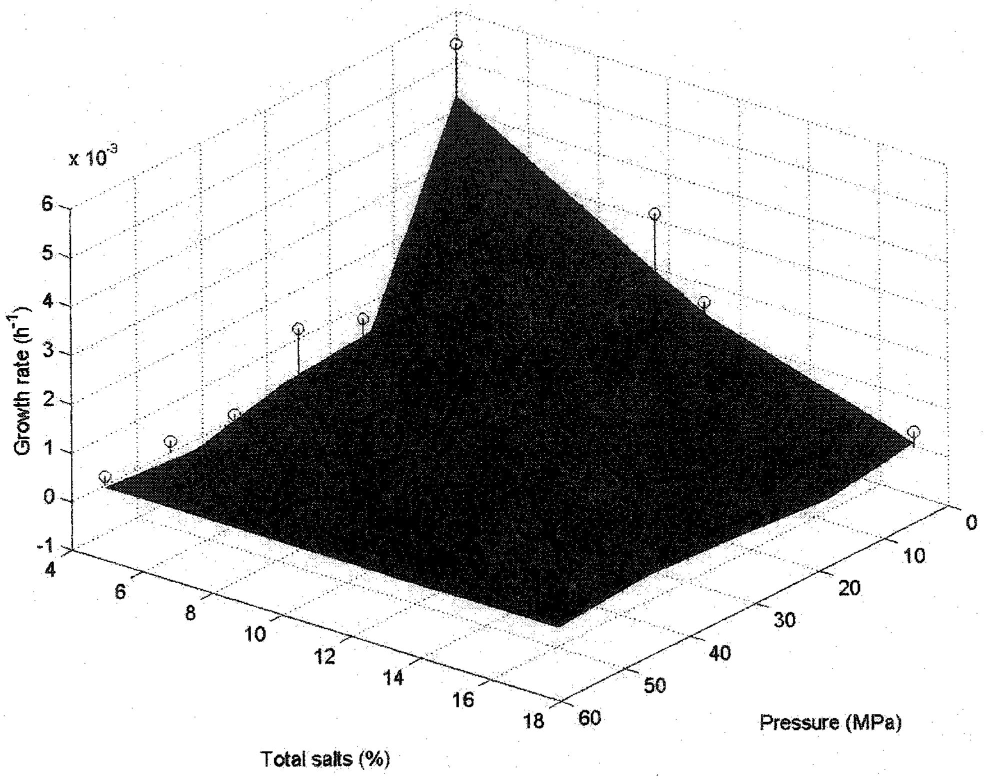

Figure 3.3 Growth of *Halomonas axialensis* at 2°C. Other features as in Fig. 3.1.

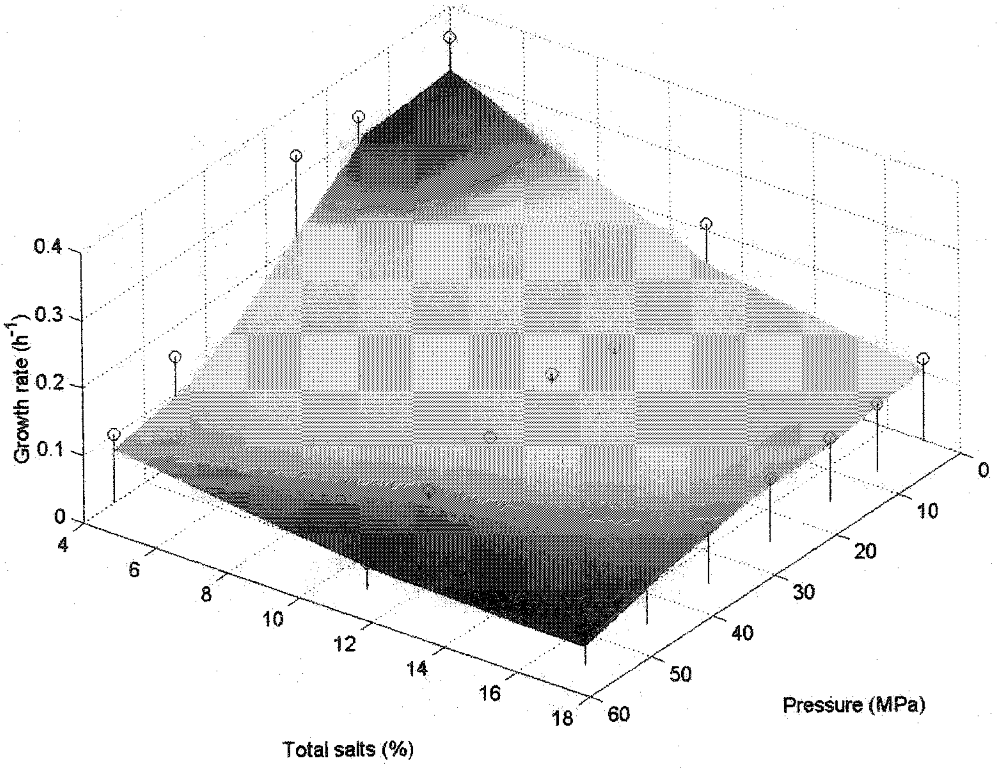

Figure 3.4 Growth of *Halomonas meridiana* strain Slthf1 at 30°C. Other features as in Fig. 3.1.

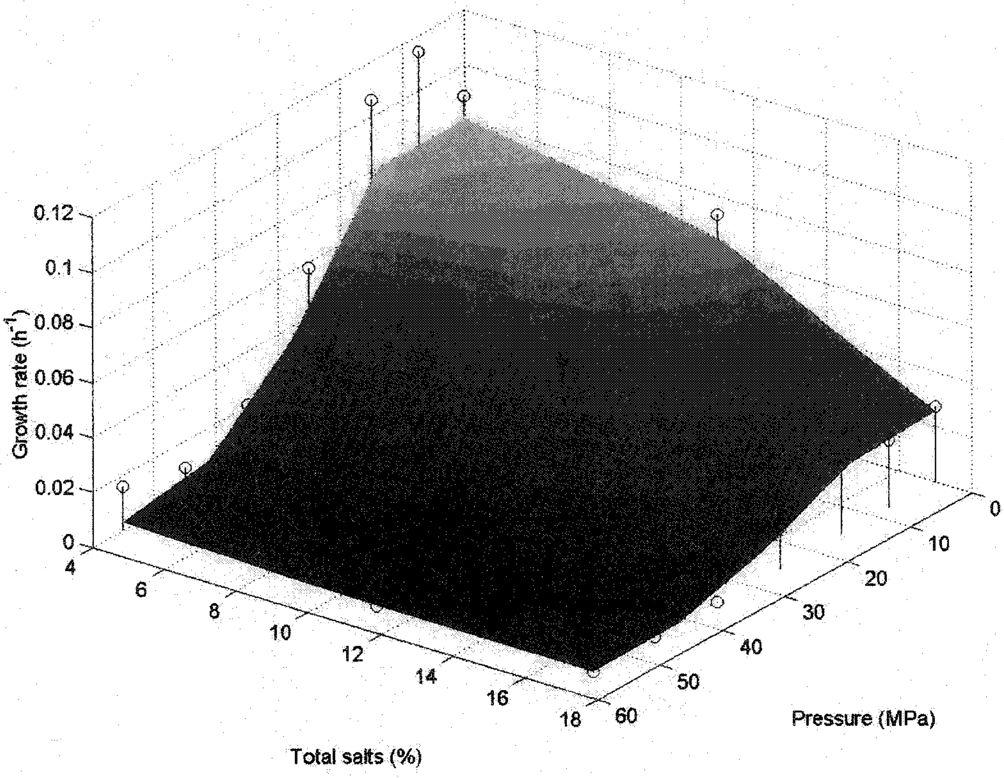

Figure 3.5 Growth of *Halomonas meridiana* strain Slthf1 at 13°C. Other features as in Fig. 3.1.

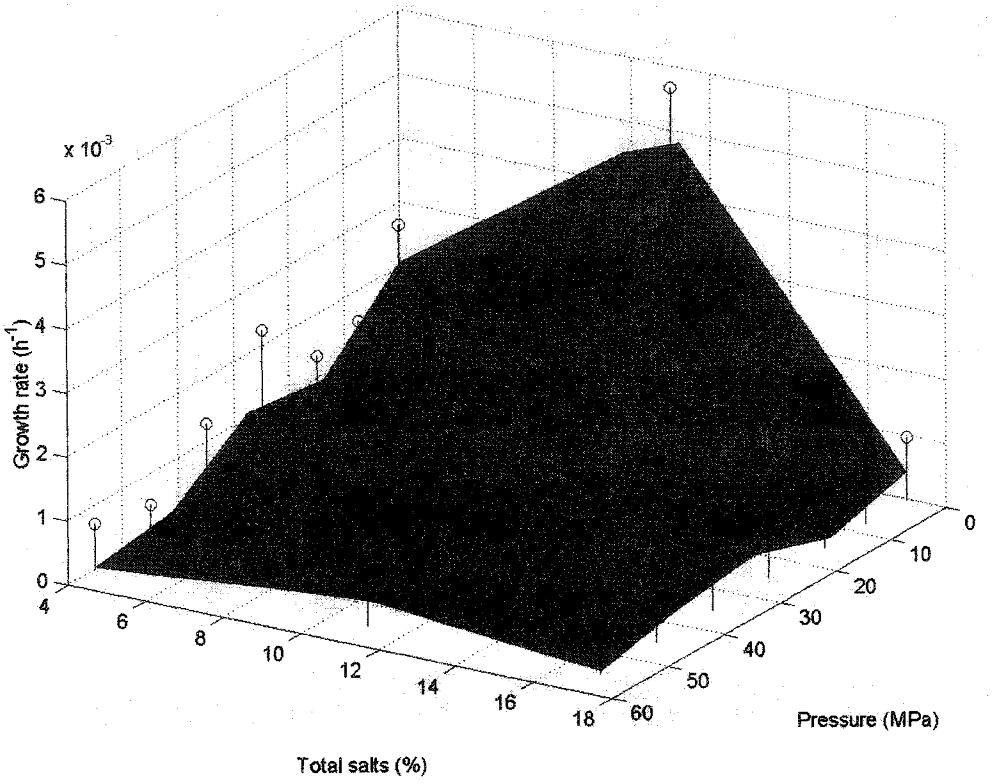

Figure 3.6 Growth of *Halomonas meridiana* strain Slthf1 at 2°C. Other features as in Fig. 3.1.

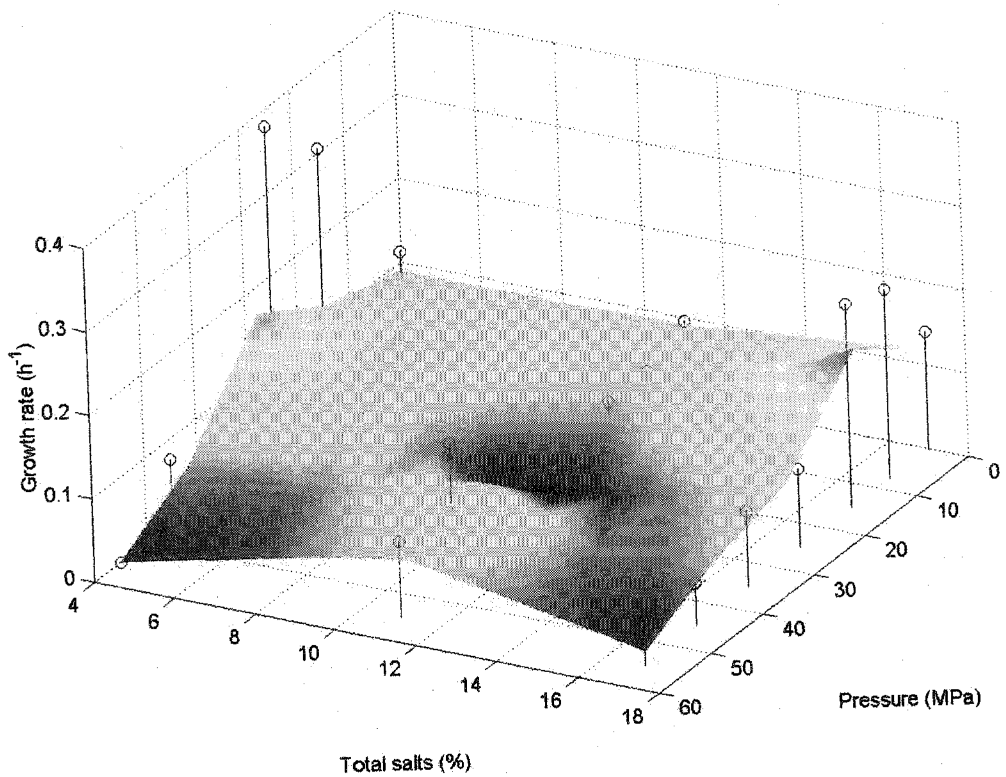

Figure 3.7 Growth of *Halomonas hydrothermalis* at 30°C. Other features as in Fig. 3.1.

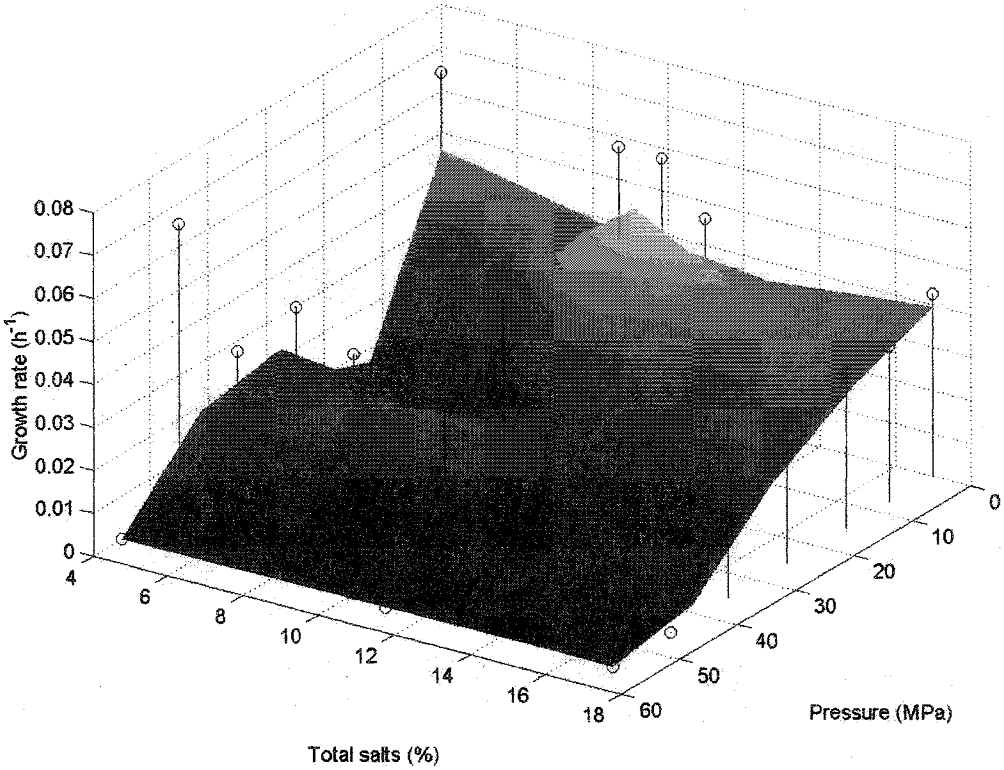

Figure 3.8 Growth of *Halomonas hydrothermalis* at 13°C. Other features as in Fig. 3.1.

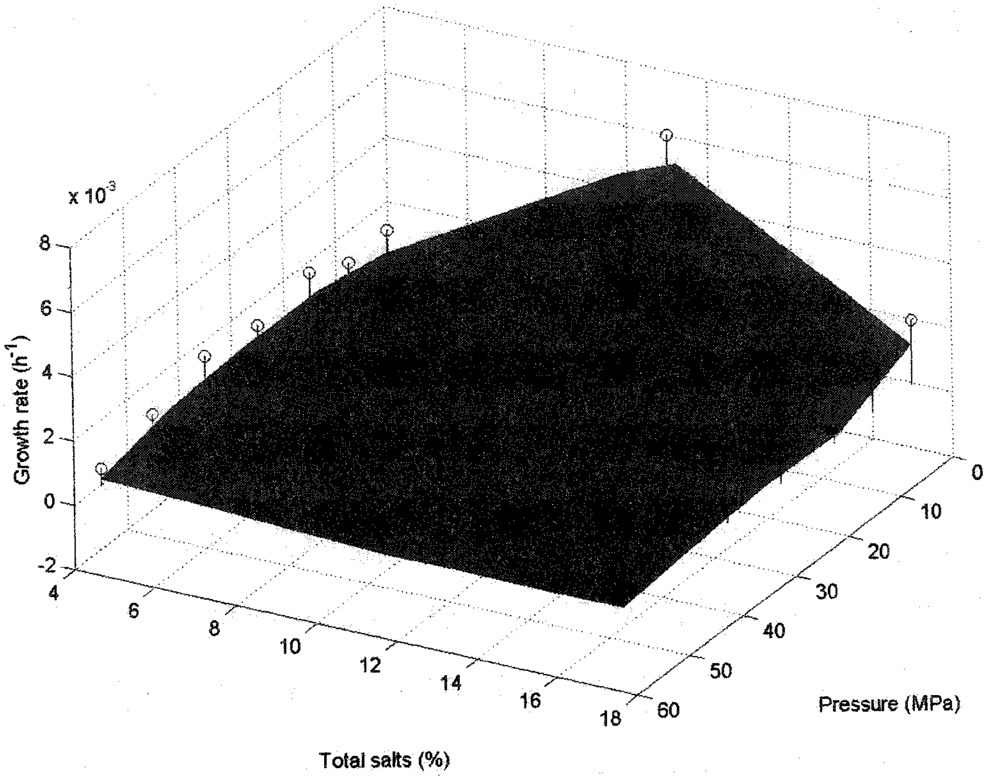

Figure 3.9 Growth of *Halomonas hydrothermalis* at 2°C. Other features as in Fig. 3.1.

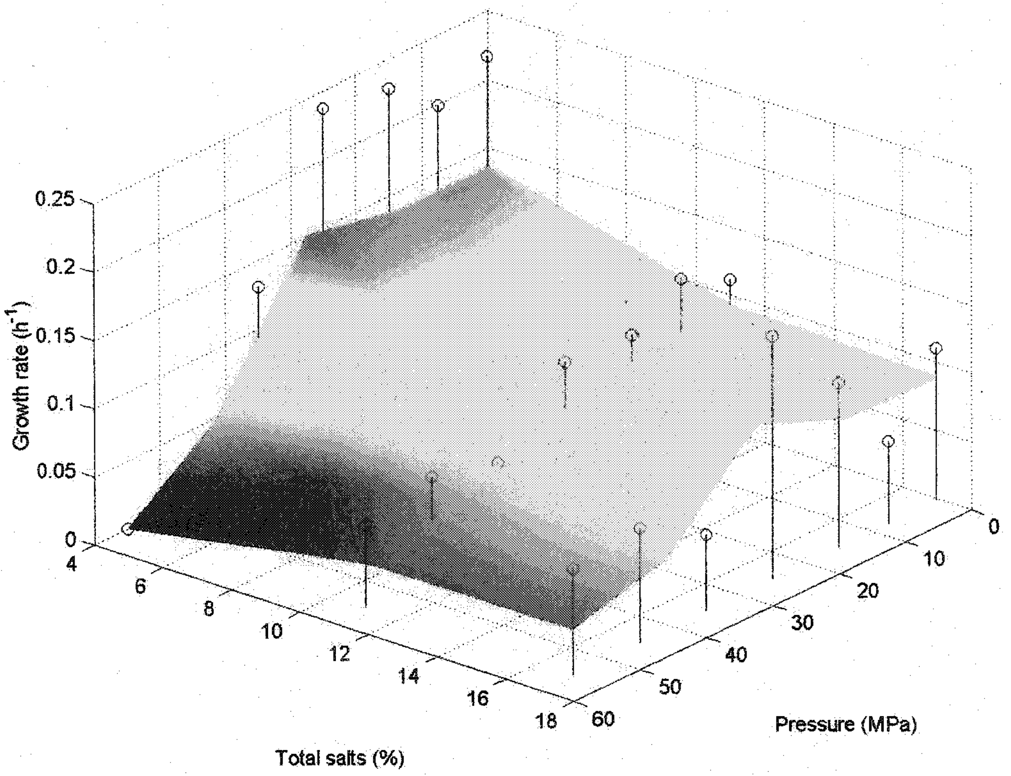

Figure 3.10 Growth of *Halomonas pacifica* at 30°C. Other features as in Fig. 3.1.

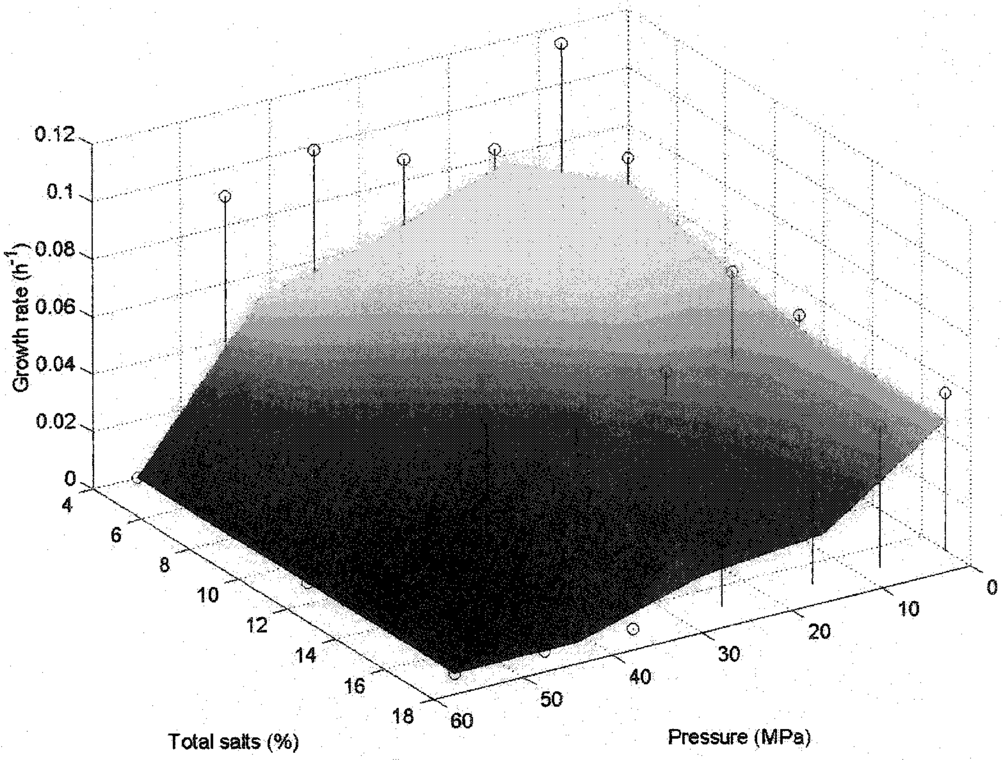

Figure 3.11 Growth of *Halomonas pacifica* at 13°C. Other features as in Fig. 3.1.

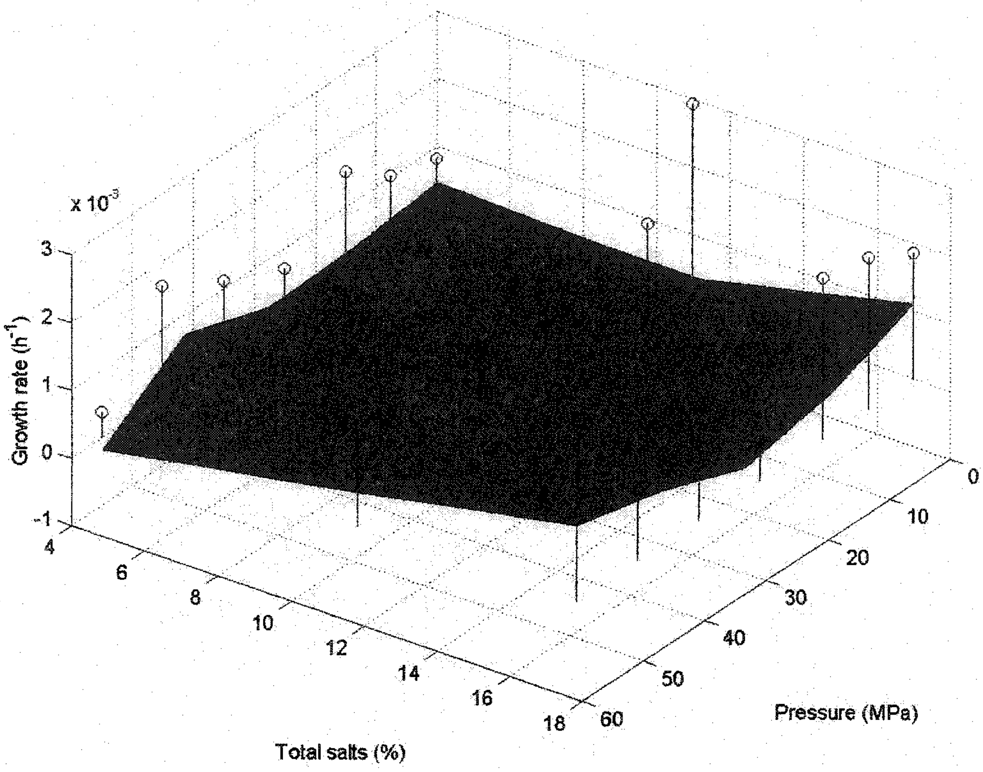

Figure 3.12 Growth of *Halomonas pacifica* at 2°C. Other features as in Fig. 3.1.

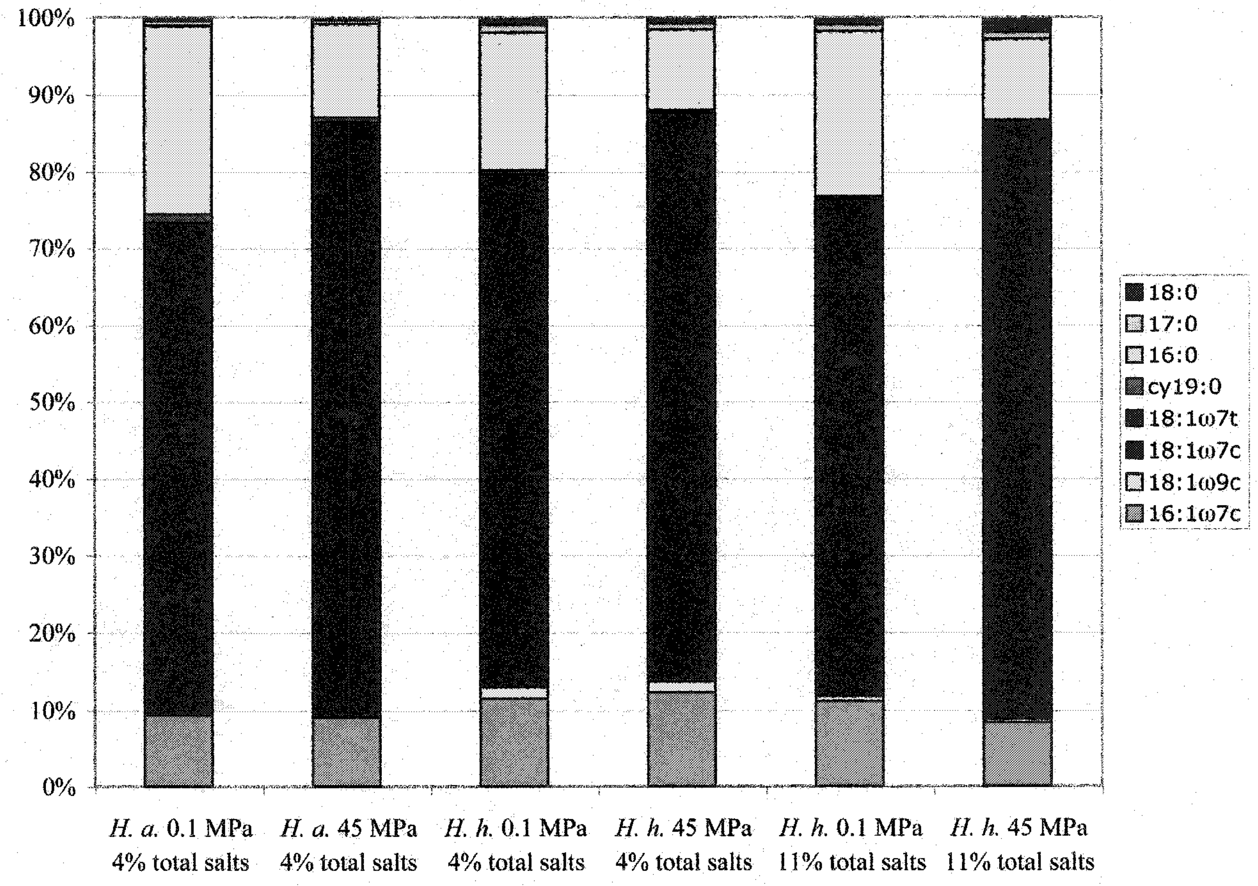

Figure 3.13 Phospholipid fatty acid profiles of *Halomonas axialensis* (*H. a.*) and *H. hydrothermalis* (*H. h.*) grown at 30°C under different hydrostatic-pressure and salinity conditions. Only lipids that comprised ≥0.5% of the total are shown.

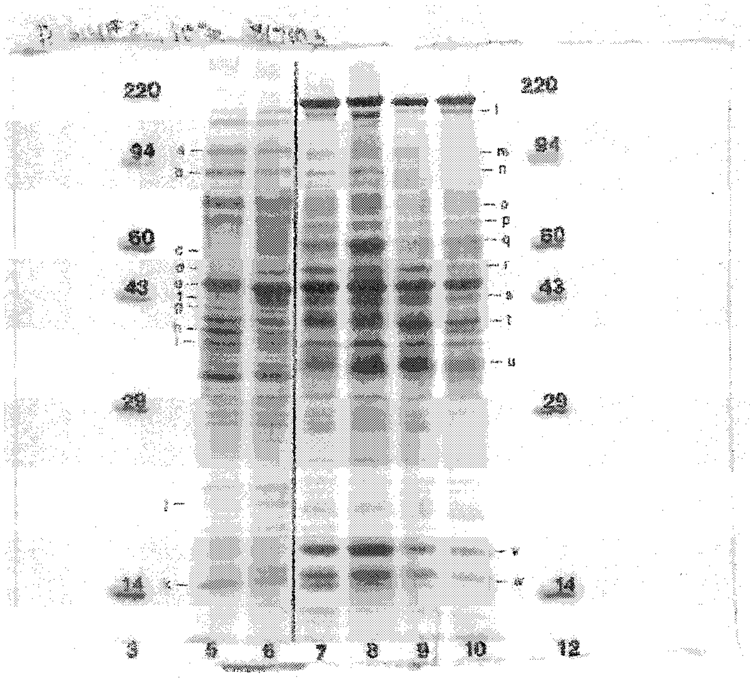

Figure 3.14 Cytosolic-protein patterns. Lanes 3 and 12, molecular weight markers; lanes 5 and 6, *Halomonas axialensis* grown with 4% total salts at 0.1 and 45 MPa, respectively; lanes 7–10, *H. hydrothermalis* grown with 4% total salts (lanes 7 and 8) and 11% total salts (lanes 9 and 10), alternating with 0.1 MPa (lanes 7 and 9) and 45 MPa (lanes 8 and 10). Protein bands that change in intensity noted with letters.

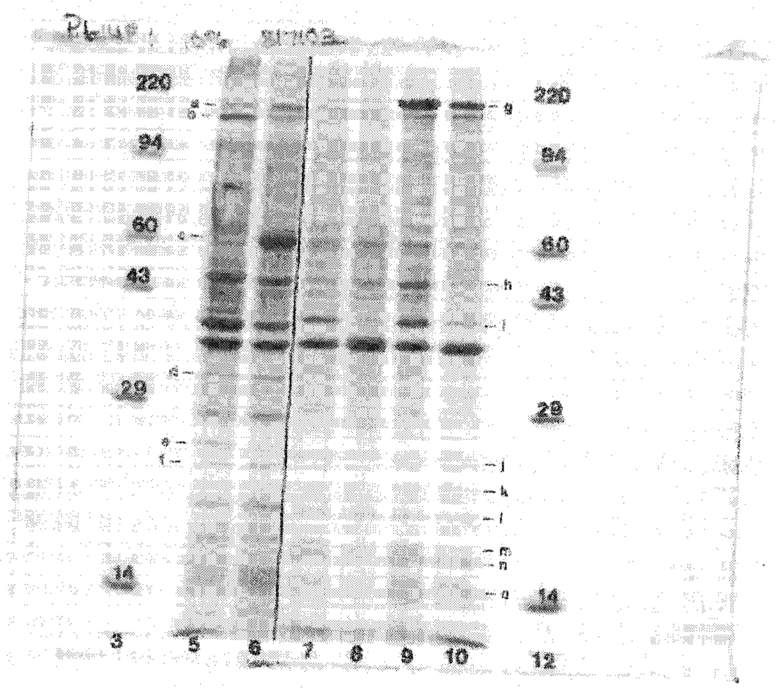

Figure 3.15 Membrane-protein patterns. Other features as in Fig. 3.14.

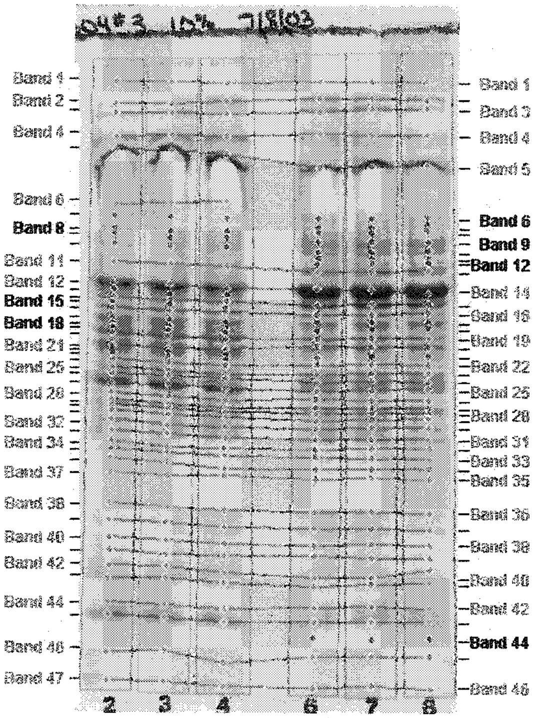

Figure 3.16 Triplicate cytosolic-protein patterns with computerized band matching of *Halomonas axialensis* grown with 4% total salts. Lanes 2–4, 0.1 MPa; lanes 6–8, 45 MPa.

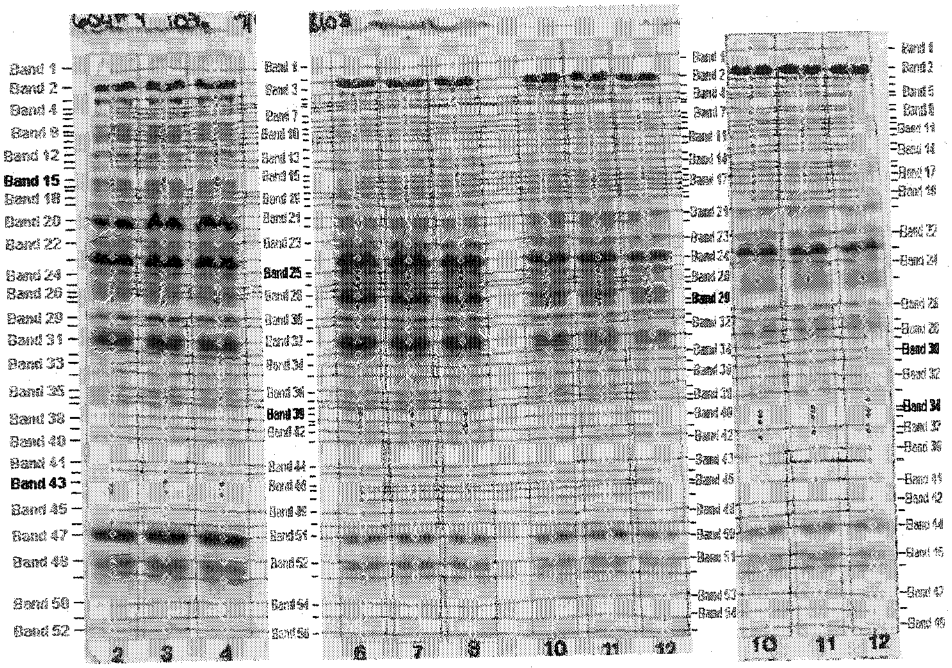

Figure 3.17 Triplicate cytosolic-protein patterns with computerized band matching of *Halomonas hydrothermalis*. Lanes 10–12 (far right), 4% totals salts and 0.1 MPa; lanes 2–4, 4% total salts and 45 MPa; lanes 6–8, 11% total salts and 0.1 MPa; and lanes 10–12 (middle), 11% total salts and 45 MPa.

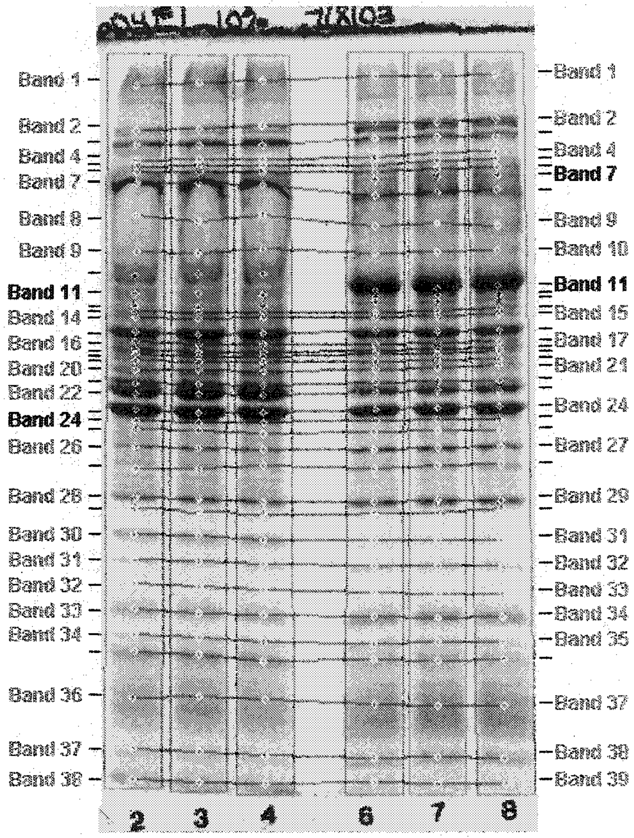

Figure 3.18 Triplicate membrane-protein patterns with computerized band matching of *Halomonas axialensis*. Lanes as in Fig. 3.16.

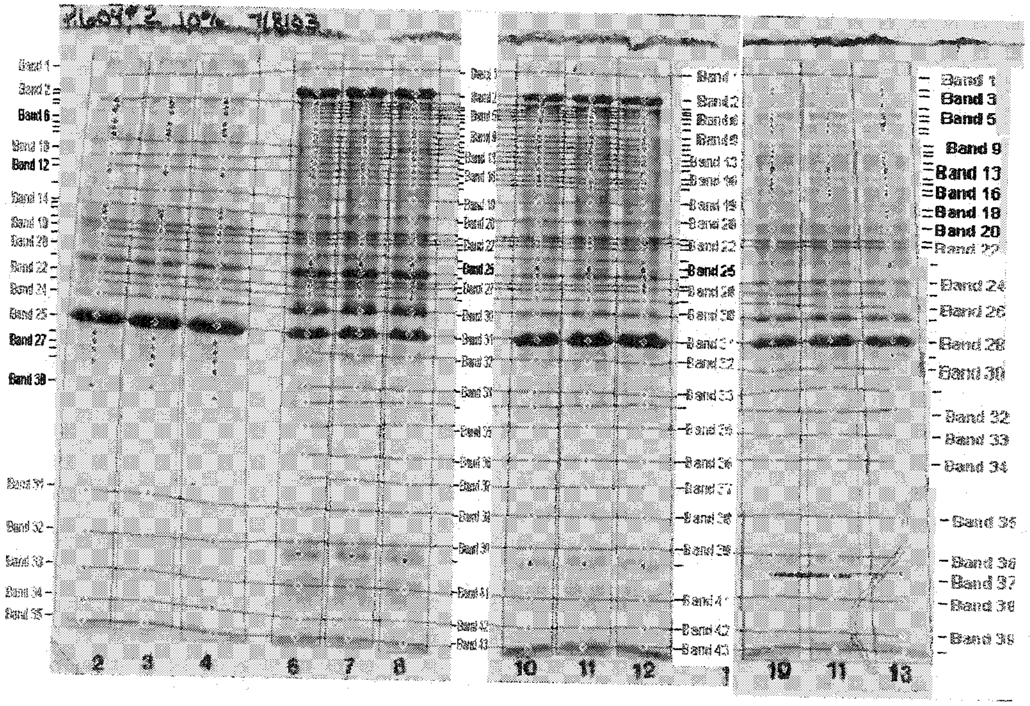

Figure 3.19 Triplicate membrane-protein patterns with computerized band matching of *Halomonas hydrothermalis*. Lanes as in Fig. 3.17, except control lanes at 4% total salts and 0.1 MPa are numbered 10, 11 and 13 (far right).

CHAPTER FOUR: Heavy-metal resistance and precipitation by mesophilic bacteria isolated from hydrothermal-vent environments

Deep-sea hydrothermal vents and associated subseafloor environments at mid-ocean ridges and back-arc basins are among the most metal-enriched natural environments on Earth (235). Massive polymetallic-sulfide deposits accumulate at the seafloor after metal ions are extracted from the oceanic crust and transported by hydrothermal circulation over tens to hundreds of thousands of years (57, 250, 506, 539, 668). Hydrothermal fluids at >250°C contain approximately 0.1–1000 mM Fe and Mn, 1–1000 μM Zn, 1–20 μM Cu, 0.01–10 μM As, 0.1–10 μM Pb, 0.1–1 μM Cd and 0.01–1 μM Co (619). These metal concentrations are reduced by precipitation reactions and dilution with seawater when dropped to temperatures that permit anaerobic hyperthermophiles to grow; they are further reduced in low-temperature hydrothermal-vent fluid emissions (<50°C) where vent fluids mix to an even greater extent with alkaline, oxygenated ambient seawater (92, 93, 123, 322, 463). Conductive cooling of hydrothermal fluids within the seafloor or locally trapped within sulfide structures is also possible, which would maintain metal concentrations near their high-temperature end-member levels.

Metals may act as toxins, nutrients, or electron donors or acceptors in hydrothermal-vent environments (254). Indeed, metal-resistant microorganisms are frequently isolated from these ecosystems. The minimum inhibitory concentration (MIC) for numerous species of *Bacillus*, *Thermococcales* and *Thermotogales* was 0.1–10 mM for Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} (345). The thermophiles *Methanobacterium thermoautotrophicum* and an unnamed methanogenic isolate were reported to tolerate 0.8 mM Cu^{2+}, 0.4 mM Cd^{2+} and 3 mM Ni^{2+} at 60°C (7). Among mesophiles, >50% of 299 strains of heterotrophic microorganisms that were isolated from ground alvinellid worms and their tubes on metal-amended media proved to be resistant to 0.4 mM Cd^{2+}, 0.6 mM Zn^{2+}, 0.6 mM Cu^{2+}, 0.05 mM arsenate ($H_2AsO_4^-$) and 0.05 mM Ag^+ at room temperature, typically with multiple metal resistances for a given strain (279, 280). Euryhaline strains of *Pseudoalteromonas* that were resistant to and capable of reducing tellurite (TeO_3^{2-}) and selenite (SeO_3^{2-}) were recently isolated from hydrothermal plumes, sulfide rock and bottom seawater from the Main Endeavour Field (MEF) on the Endeavour Segment of the Juan de Fuca Ridge (484). In addition, recently characterized euryhaline *Halomonas* spp., isolated from similar samples as well as low-temperature hydrothermal fluids from the MEF and vents along the Southern East Pacific Rise, tolerated 0.05–4.0 mM Cd^{2+} in liquid culture (Chapter 1; 304).

Moderately halophilic and euryhaline bacteria isolated from marine and hypersaline habitats outside of hydrothermal-vent environments are also typically highly metal-resistant. The MIC values for numerous strains of *H. halophila*, *H. elongata* and *Chromohalobacter marismortui* were 1–10 mM for $H_2AsO_4^-$, Cd^{2+}, Co^{2+}, chromate (CrO_4^{2-}), Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, though the same strains were sensitive to Ag^+ and Hg^{2+} (427, 428). An unnamed *Halomonas* sp. also tolerated mM concentrations of uranyl ion (UO_2^-) (190). The moderate halophile *Vibrio costicola* (reclassified as *Salinivibrio costicola* [381]) had a similar resistance pattern to *H. halophila* but was much more resistant to Ni^{2+}, CrO_4^{2-} and $H_2AsO_4^-$ (205). Resistance to 2 M selenate (SeO_4^{2-}) was recently reported for an uncharacterized *Halomonas* strain isolated from a Se-contaminated evaporation pond (135). Lastly, haloalkaliphilic strains of *Halomonas* tolerated 2 mM pertechnetate (TcO_4^-) and reduced it at lower TcO_4^- concentrations (316).

Microorganisms can sequester metal cations and oxyanions and remove them from solution as sulfide, sulfate, phosphate or carbonate minerals, significantly affecting biogeochemical cycling. For example, mineral precipitate crusts on the northern Gorda Ridge were characterized by microbial trace fossils and selective precipitation of Ag-, As- and Cu-sulfide minerals (669). Diverse additional examples include barite ($BaSO_4$) precipitation by *Myxococcus xanthus* (222), accumulation of arsenite (H_3AsO_3) and ferric iron by *Acidithiobacillus ferrooxidans* in acid-mine-drainage environments (162), anaerobic UO_2^{-} precipitation as hydroxide and hydroxyphosphate minerals by a *Halomonas* sp. (190), and SeO_4^{2-} reduction to and intracellular accumulation of Se^0 by sulfide-oxidizing bacteria (418).

Many bacteria isolated from hydrothermal-vent environments that were resistant to multiple metals produced H_2S from cysteine, an extremely efficient way of detoxifying heavy metals (280) that also results in production of metal-sulfide minerals. For example, a vent *Pseudomonas* sp. generated H_2S under oxic conditions, resulting in nearly complete removal of Cd^{2+} from solution via precipitation of CdS (637). *Escherichia coli* has been engineered for bioremediation purposes to express cysteine desulfhydrase as a means to detoxify Cd^{2+} and remove it from solution in the same manner (636). Microbial biofilms, exopolymeric substances and external cell components also accumulate heavy metals from solution (29, 47, 72, 88, 112, 190, 208, 326, 328, 505, 536, 584, 589, 607, 617, 664). It is this property which explains, in part, the functionality of heavy-metal bioremediation bioreactors (52, 543, 582, 608).

Motivated by the ability of a vent *Pseudomonas* sp. to remove >99% of 5 mM Cd^{2+} from solution and precipitate it as CdS (637), we desired to determine if other bacteria isolated from hydrothermal-vent environments could similarly precipitate Cd and other metals under aerobic conditions. In the present study, we determined the Cd^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} MIC patterns of nine mesophilic bacteria belonging to the genera *Halomonas*, *Marinobacter*, *Pseudoalteromonas* and *Vibrio* and two unnamed taxa isolated from hydrothermal plumes, sulfide rock, animal tissue and low-temperature hydrothermal fluids using a citrate medium with elevated concentrations of Fe^{2+} and Mn^{2+} (304). One of the uncharacterized taxa, isolate Eplume4, can oxidize Mn^{2+} (154), and *Marinobacter* sp. Aplume1 can oxidize Fe^{2+} (503). These microorganisms were selected because they originated from environments that cover a range of heavy-metal concentrations; hydrothermal plumes contain very low concentrations of dissolved metals (340), whereas sulfide rock, low-temperature hydrothermal fluids and vent animal tissues are typically enriched with heavy metals (110, 126, 202, 290, 291, 501, 502, 667). The heavy metals chosen for MIC determinations include the more abundant metals (Cu, Zn) present in hydrothermal-vent ecosystems, several of which are important for the mineralogy of massive metal-sulfide deposits. We monitored metal-precipitate formation during growth under agitated and static culture conditions to decipher possible metal-specific detoxification mechanisms and to examine if these bacteria could play a role in production of metal-sulfide or other heavy-metal-containing minerals in hydrothermal-vent environments.

METHODS

Minimum inhibitory concentration (MIC) determinations

H. pacifica ATCC 27122 (isolated from tropical surface seawater [43]) and nine strains of bacteria, representing the genera *Halomonas*, *Marinobacter*, *Pseudoalteromonas*, *Vibrio* and two uncharacterized taxa, isolated from various hydrothermal-vent habitats (304), were grown in the presence of Cd^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} (Fig. 4.1). Organisms were cultured on the solid growth medium described in Chapter 1 (4% total salts, pH 7.0) amended with chloride or sulfate salts of the desired metals at concentration increments of 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 mM. Plates were inoculated in triplicate with a loop or toothpick from cultures freshly grown on metal-free media of the same composition and incubated aerobically for at least three weeks at 20°C. Colonies were transferred to the next three higher concentrations of metals from the highest concentration that initially permitted growth. The concentration that ultimately prevented colony formation (the MIC) is reported.

Precipitate formation

For simplicity during an initial examination of precipitate formation, metal concentrations that permitted strong growth for the less metal-resistant strains during the MIC determinations were chosen to examine whether precipitates formed when cells were grown in static liquid culture. The ten isolates were inoculated into broth with either 0.5 mM Cu^{2+}, 0.1 mM Cd^{2+}, 0.1 mM Co^{2+} or 0.05 mM Zn^{2+} and incubated for two weeks at 20°C (Table 4.1).

H_2S production

Production of H_2S was tested by stabbing cultures into Kligler iron agar (26) using artificial seawater (SSB; Chapter 1) in place of NaCl. The medium was made without metals or amended with 0.5 mM Cu^{2+}, 0.1 mM Cd^{2+}, 0.1 mM Co^{2+} or 0.05 mM Zn^{2+} (Table 4.1). Cultures incubated at 20°C for six weeks.

Growth curves and loss of dissolved metal

H. axialensis, *H. sulfidaeris*, *H. hydrothermalis*, *Pseudoalteromonas* sp. E-twt1 and *Marinobacter* sp. Aplume1 were selected for further metal precipitation analysis because they appeared to catalyze the precipitation of certain metals from solution, tolerated high concentrations of heavy metals and/or derived from presumably metal-enriched environments such as sulfide rock, low-temperature hydrothermal fluids and animal tissue. Strains were grown in static culture with 30–40 ml broth in sealed 50-ml Falcon tubes and while agitated at 100 rpm in 250-ml flasks with foam stoppers and aluminum foil covers starting with metal concentrations a few mM below the MIC values determined on solid media (Table 4.2). Cultures were also grown without metals. As another control, the metal concentration of uninoculated media was simultaneously monitored for abiotic precipitation. Growth was assessed by measuring culture turbidity using a Lambda2S UV/VIS Spectrophotometer (Perkin Elmer) at 600 nm. The pH was checked periodically with test strips (Sigma). The 2–3 ml aliquots used for turbidity measurements were then centrifuged at 13,000 × *g* for 3 min, and the supernatant removed for metal analysis. Supernatant samples with different metals were sometimes pooled together and additionally diluted with deionized water. Samples were frozen at –20°C until analyzed for Cd, Cu, Co and Zn using a 955 AtomComp inductively coupled plasma atomic emission spectrophotometer (ICP-AES; Thermo Jarrell Ash Corp.) in the Chemistry Department at the University of Washington. Standard deviations on triplicate ICP-AES metal analyses were typically <7% for Cd, <13% for Cu, <8% for Co and <13% for Zn, with occasionally higher values on the lowest metal concentrations measured (<0.1 mM).

Electron microscopy

During late-exponential-phase growth, 1 ml of media with precipitate was collected from each static culture and preserved with 25% electron-microscopy-grade glutaraldehyde (2.5% final concentration). Samples were fixed at 2°C for several days, impregnated with Spurr's low viscosity epoxy, thin sectioned, and spotted on copper and nickel Formvar grids. Preliminary analysis of the precipitate was performed with a Sirion XL-30 Schottky Field Emission Gun (FEI) equipped with an Energy Dispersive X-Ray Spectroscopy system (EDS; EDAX) at the Washington Technology Center, University of Washington.

RESULTS

MIC values and preliminary observations of precipitation

All ten bacterial strains showed strong resistance to Cd^{2+}, Co^{2+} and Cu^{2+}, though the isolates were slightly less resistant to Zn^{2+} (Fig. 4.1). The isolates had similar MIC patterns for three metals (3.0–4.0 mM Cd^{2+}, 3.0–6.0 mM Cu^{2+} and 0.1–2 mM Zn^{2+}). *H. pacifica*, γ-proteobacterium strain Elthf1, *Pseudoalteromonas* sp. Etwt1 and *Marinobacter* sp. Aplume1 were inhibited only by 6.0–9.0 mM Co^{2+}, much greater than the 1.0–3.0 mM concentration that inhibited the other strains. No precipitate formed within several weeks of incubation with Co^{2+}, but a bright yellow precipitate formed with each culture in the presence of Cd^{2+} (Table 4.1). Variable responses were found with Cu^{2+} (white, white-brown and brown precipitates) and Zn^{2+} (white precipitate).

H_2S production

All strains produced H_2S (Table 4.1) just below the agar-air interface of metal-free Kligler iron agar stabs in a zone presumably depleted of oxygen. On occasion, the presence of some metals appeared to inhibit H_2S production. In Cu^{2+}-amended Kligler iron media, however, the amount of black coloration in the agar appeared to be enhanced.

Cell growth and measurements of dissolved metal

Metal concentrations were considered to have changed significantly if the standard deviations between any two time points did not overlap. Cell-free controls did not change significantly in metal concentration under static conditions, but during agitation some controls increased in metal concentration due to evaporation (data not shown). The evaporation occurred late in the experiment and does not affect the interpretation of the turbidity measurements or metal precipitation. The pH of cell-free controls remained at 7.0–7.5 over the course of the experiment while the pH of cultures either remained at 7.0–7.5 (Cd^{2+} and Zn^{2+}) or increased to 8.5–9.0 (Co^{2+} and Cu^{2+}).

During static growth, Cd^{2+} was efficiently removed from solution in the presence of *H. axialensis*, *H. sulfidaeris*, *H. hydrothermalis*, *Pseudoalteromonas* sp. Etwt1 and *Marinobacter* sp. Aplume1 when cells had entered late-exponential and stationary-phase growth (Fig. 4.2, 4.4, 4.6, 4.8 and 4.10; Table 4.2). *H. sulfidaeris* and *Pseudoalteromonas* sp. Etwt1 removed less Cd^{2+} than the other strains (54–55% versus 96–98%), with the metal precipitation only occurring during a secondary growth spurt, perhaps by a more metal-resistant sub-population of cells. If these experiments had continued, nearly complete Cd^{2+} removal may have transpired for cultures of *H.*

sulfidaeris and *Pseudoalteromonas* sp. Etwt1. In stark contrast, Cd^{2+} concentrations never changed significantly for any bacterium during agitated culture growth.

Cu^{2+} was removed from solution in only a few instances, and only during stationary-phase growth or after the population had crashed (Fig. 4.2, 4.4, 4.6, 4.8 and 4.10). Similarly, decreases in Co^{2+} concentration (Fig. 4.3, 4.5 and 4.9) coincided with reductions in cell numbers due to cell lysis. It does not appear that any of the bacteria tested are able specifically to precipitate Cu^{2+} or Co^{2+} under any growth condition examined. Cells did not lyse during agitated growth with Cd^{2+}, nor was Cd^{2+} removed from solution under this growth condition.

Zn^{2+} typically displayed a two-phase response (Fig. 4.3, 4.5, 4.7 and 4.11). Zn^{2+} was partially removed from solution (26–48% loss by ~200 h) during late-exponential and early-stationary-phase growth under static conditions for all organisms tested. Subsequently, this metal was further lost from solution (ultimately 59–94% removal), but only after cells began to lyse. Under agitated conditions, cell lysis coincided with Zn^{2+} loss as in the case of Cu^{2+} and Co^{2+}.

Electron microscopy and elemental analysis

Preliminary results from two cultures grown in the presence of Cd^{2+} indicated the presence of Cd and S in material associated with the cell surface (data not shown).

DISCUSSION

Hydrothermal-vent and other environments with elevated concentrations of heavy metals pose a variety of challenges to resident microbial communities. Heavy metals can cause DNA lesions or catalyze the formation of radical oxygen species that effect the same result, and perhaps most importantly, they bind irreversibly to thiol groups in polypeptides, rendering them inoperable (426, 544). The degree of metal toxicity is typically highly correlated with the binding affinity of the metal for H_2S, for which the cellular proxy is the thiol group in proteins (426). Intrinsic metal-resistance mechanisms found among bacteria include modifications of cell walls, production and release of exopolysaccharides, and simply an inability to transport a given dissolved metal inside the cell (201). Extrinsic factors include (i) highly specialized, membrane-bound, energy-dependent, metal-ion efflux systems; (ii) production of cytoplasmic, periplasmic and extracellular ligands and metal-transport proteins; (iii) alteration of membrane-transport activities; and (iv) enzymatic reductive or oxidative transformation of metals to less toxic species (90, 545)

In hydrothermal-vent environments, animal tissues, animal mucous secretions and microbial biofilms concentrate many metals due to passive accumulation and complexation (110, 126, 202, 290, 291, 328, 501, 502, 584, 607, 638, 667) well above the levels found in seawater (436) and low-temperature hydrothermal fluids (619). Bioaccumulation of heavy metals, such as Hg in animal tissues (237), is a widely documented phenomenon that affects marine food chains in polluted environments. In hydrothermal-vent ecosystems, the clam *Vesicomya gigas* from Guaymas Basin was measured to contain remarkably high Cd (115 ppm), Fe (403 ppm), Hg (4.96 ppm), Mn (18 ppm) and Zn (845 ppm) in gill tissue and Cu (29.7 ppm) and Pb (3.67 ppm) in its mantle (510). Another clam, *Calyptogena magnifica* collected from the East Pacific Rise, contained 760 ppm Fe, 148 ppm Cu, 2152 ppm Zn, and >5 ppm Cr, Mn, As, Mo, Ag, Cd, Ba and Pb (dry weight) in its soft body parts (501, 502). Fe, S, Zn, Cu, Cd, Hg, U and As, among other heavy metals, were measured in high concentrations in association with intracellular sulfur granules, symbiosis organs, organic tubes, soft tissues and mucous secretions of alvinellid polychaetes, vestimentiferans and mussels (110, 126, 202, 290, 291, 667). Animal-associated metals, which include those bound in sulfide minerals, indicate biologically mediated, localized metal precipitation and sequestration (126, 290, 667). If the degree of metal resistance of a microorganism is directly correlated with the concentration of metals in the local environment where it is growing, one would expect to find more metal-resistant bacteria and archaea associated with animals, their secretions and microbial biofilms.

The majority of mesophiles and hyperthermophiles enriched from hydrothermal-vent environments that have been exposed to heavy metals has been found to tolerate 0.1–10 mM concentrations of Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} and, to a lesser degree, Ag^{+} and $H_2AsO_4^{-}$ (279, 280, 303, 345, 385). The heterotrophic mesophiles examined in this study (including two from animal tissues) were resistant to similar levels of Cd^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} as the more metal-resistant mesophilic strains isolated from ground *Alvinella pompejana* and *A. caudata* worm specimens and their tube dwellings (279, 280). (One general caveat, however, is that it is difficult to compare exact metal concentrations between studies given the various metal-binding efficiencies and amounts of salts and organic compounds present in the different media employed.) Co^{2+} was less toxic to some species as would be predicted by its lower binding affinity for H_2S, though Zn^{2+} was the most toxic heavy metal for all strains tested, counter to the prediction based on H_2S affinity that Zn^{2+} would only be as toxic as Co^{2+} (426). The hydrothermal-vent *Halomonas* spp. were as metal-resistant as *H. pacifica* (isolated from tropical surface seawater [43]) and other members of the genus (427, 428), indicating that among this group the degree of metal tolerance appears unrelated to the source of isolate. Moreover, the nine hydrothermal-vent bacterial strains in this study showed similar MIC patterns spare for Co^{2+}, such that no correlation was discerned between the degree of metal resistance and the environment of origin within the hydrothermal-vent habitat despite presumably wide variations in metal concentration between hydrothermal plumes, sulfide rock, animal tissues, and low-temperature hydrothermal fluids. It is also possible that the bacteria were locally dispersed from other hydrothermal-vent environments before their capture, rendering correlations between the degree of metal resistance and source of the isolate intractable.

The importance of metal resistance and metal precipitation is challenging to address in an ecological context, for this and many metal-resistance studies. A persistent perplexing question is why bacteria are resistant to levels of metals far above those encountered even in metal-enriched environments given that the degree of metal resistance correlates with the number of metal-resistance genes (426, 544), which are expensive to maintain in the genome if unused. While hydrothermal-vent environments are among the most metal-enriched on Earth, heavy metals are only found in nM to μM concentrations in high-temperature hydrothermal fluids, and even at lower concentrations in low-temperature fluid emissions. Metals may accumulate to μM to mM levels in animal tissues and mucous secretions and microbial biofilms, however (110, 126, 202, 290, 291, 328, 501, 502, 584, 607, 638, 667). While two isolates tested derived from samples of animal tissue, they were only as metal-resistant as those isolated from low-temperature hydrothermal fluids, hydrothermal plumes and surface seawater. Perhaps the bacteria isolated from samples other than animal tissue did inhabit biofilms before their capture, but only those biofilms located in close proximity to metal-enriched habitats would themselves be predicted possibly to contain greatly elevated, millimolar concentrations of heavy metals. The persistent difficulty in this and other studies of correlating the degree of metal resistance with the metal concentration in the sample from which a given bacterium was isolated suggests that metal resistance may actually reflect, in part, an adaptation to another stress, as discussed below.

Analysis of metal loss from solution by ICP-AES during growth of five selected strains revealed metal-specific responses. Removal of Cu^{2+} and Co^{2+} under static and agitated growth conditions, as well as loss of Zn^{2+} during agitated growth and during culture senescence under static growth conditions, can be explained as artifactual metal sequestration due to cell lysis. Protein thiol groups and other intracellular material exposed to the media upon cell lysis may explain the metal loss in these instances. The precipitates initially observed during static growth with Cu^{2+} may be accumulated Cu^{2+}-enriched (in some instances) cell debris.

The role of pH in metal precipitation is key for discerning biologically enhanced from abiotic, artifactual influences on metal solubility. In addition, biologically mediated increases in medium pH may also incidentally enhance metal precipitation. For example, efflux of metals by *Alcaligenes eutrophus* CH34 (reclassified as *Ralstonia eutropha* [654]) was hypothesized to increase the local extracellular pH and cause precipitation of Cd^{2+} and Zn^{2+} from culture media (121). In the present study, the pH of the medium did not measurably increase in Cd^{2+} and Zn^{2+} treatments, both with and without cells. The increase in pH to 8.5–9.0 in Cu^{2+} and Co^{2+} treatments, with and without cells, may have enhanced metal precipitation.

Previous studies have examined the precipitation of Zn^{2+} via formation of ZnS (328) and Cd^{2+} via formation of CdS and $CdHPO_4$, within cells or in the immediate vicinity of the cell surface due, in some cases, to microbially mediated alterations in pH, release of H_2S and localized enrichments of HPO_4^{2-} (8–10, 353, 401). (Some H_2S-producing bacteria also detoxify Hg^{2+} by precipitating it as HgS [8].) Abundant sphalerite (ZnS) deposits were found in biofilms of aerotolerant sulfate-reducing bacteria of the family *Desulfobacteriaceae* in a flooded Pb-Zn ore-mining tunnel (328). A strain of *P. aeruginosa*, isolated from a hydrothermal plume, precipitated >99% of 5 mM Cd^{2+} as CdS during agitated aerobic culture (637). The bright yellow precipitate observed during static growth by all ten bacteria examined in this study strongly suggests formation of CdS, or possibly $CdHPO_4$. All cultures were able to produce H_2S on Kligler iron agar, though the response was eliminated in the presence of Cd^{2+} for *H. neptunia* and *Vibrio* sp. E-limpetgut1. Similarly, the majority of mesophilic bacteria isolated from hydrothermal-vent invertebrate inocula, including those resistant to heavy metals, were able to produce H_2S from cysteine (280, 473, 474).

In this study, Cd^{2+} removal during late-exponential and stationary-phase growth was biologically mediated or biologically enhanced. Cd^{2+} was consistently removed from solution (54–98% loss) under static growth conditions; these cultures all produced H_2S in the presence of Cd^{2+}. (Preliminary SEM-EDS analysis of precipitated material indicated the presence of Cd and S.) The lack of Cd^{2+} precipitation during agitated aerobic growth is intriguing. If the bright yellow precipitate was CdS, a possible explanation is that any H_2S produced was quickly oxidized under highly oxygenated conditions. This is unlikely, however, given that *P. aeruginosa* produced H_2S and precipitated CdS under similar agitation conditions using an identical medium (637). Alternatively, late-exponential and stationary-phase growth in conjunction with oxygen and/or nutrient stress may have induced a generalized stress-response system enabling these bacteria to express genes to produce H_2S as a metal-detoxification mechanism. Certain strains of "*Klebsiella aerogenes*," grown in continuous culture with Cd^{2+}, accumulated CdS to an enhanced degree specifically under glucose limitation (10). In this manner, a stress other than that imposed by high concentrations of heavy metals may induce a general stress response that enables cell to catalyze metal precipitation and to reduce the dissolved metal concentration.

Zn^{2+} was also consistently removed from solution during late-exponential and stationary-phase growth for all strains tested. The initial biologically mediated or biologically enhanced Zn^{2+} removal (26–48% loss after ~200 h) was not as complete as for Cd^{2+}. It is unknown if Zn^{2+} was precipitated as ZnS given the white color of the precipitate initially observed and if the same or a different mechanism as for Cd^{2+} precipitation was employed. Further elemental analysis of the metal-containing precipitates is forthcoming. With both metals, it is impossible within the confines of the current set of experiments to ascertain whether metal precipitation is due to a specific metal-resistance mechanism or merely a byproduct of H_2S production or locally increased extracellular HPO_4^{2-} concentration, for example.

Additional experiments in progress seek to determine which other stress conditions may enhance Cd^{2+} and Zn^{2+} precipitation in order to map out possible underlying cross-regulated stress-response systems. The parameters include oxygen limitation, elevated salinity (17% total salts) and elevated temperature, ~2°C below T_{max} for each strain. Preliminary results indicate increased Zn^{2+} sensitivity and reduced Cd^{2+} precipitation when cells are exposed to other stresses in addition to high concentrations of these metals.

Microbial production of metal-containing compounds, such as CdS, $CdHPO_4$ and ZnS, may augment the abiotic precipitation reactions that dominate hydrothermal-vent environments, as was suggested by microbially mediated, selective accumulations of Ag_3AsS_3, $CuFeS_2$ and other sulfide minerals in microbial trace fossils found in metalliferous sediment on the northern Gorda Ridge (669). These processes may be especially important in metal-enriched microenvironments in association with animal secretions, seafloor and subseafloor microbial biofilms and within sulfide structures, where metals are especially enriched in concentration.

Pore spaces within sulfide structures may contain micron-scale habitats of unknown chemistry, including putative brine pockets as suggested by the presence of haloarchaeal 16S rRNA gene sequences in one sulfide structure examined (577). These results indicate that the evolution of salt- and metal-resistance phenotypes may be linked due to their possible co-occurrence, not to mention that metals will preferentially partition into hypersaline fluids. The persistence of heavy-metal (and salt?) stress in conjunction with the moderate hydrostatic pressures and fluctuating temperatures and oxygen concentrations associated with sulfide structures in particular (and hydrothermal-vent environments in general) suggests that the success of moderately halophilic and euryhaline bacteria, such as members of the genera *Halomonas* and *Marinobacter*, may hinge on multiple resistances and cross-regulated stress responses. A capacity for multiple resistances and possession of a complex stress-response system may enable species from these and other taxa to thrive in a wide variety of marine habitats and may also explain the occurrence of unexpected microbial physiologies (such as extremely high metal resistance) that, at a first glance, do not appear necessarily to fit the niche from which a given microorganism was isolated.

Table 4.1 Precipitate formation during growth in static liquid culture and production of H_2S on Kligler iron agar. The metal concentrations were 0.5 mM Cu^{2+}, 0.1 mM Cd^{2+}, 0.1 mM Co^{2+} and 0.05 mM Zn^{2+}.

Organism	Source	Cd^{2+*}	$Co^{2+\dagger}$	$Cu^{2+\ddagger}$	Zn^{2+}	
		H_2S production	H_2S production	Precipitate	Precipitate	H_2S production
Halomonas neptunia	Hydrothermal plume	–	+	white-brown	–	+
H. sulfidaeris	Sulfide rock	+	–	white-brown	–	+
H. axialensis	Low-T hydrothermal fluid	+	+	white	white	+
H. hydrothermalis	Low-T hydrothermal fluid	+	+	white	white	–
H. pacifica	Surface seawater	+	+	brown	white	–
Mn-oxidizing bacterium Eplume4	Hydrothermal plume	+	+	–	white	+
γ-Proteobacterium strain Elthf1	Low-T hydrothermal fluid	+	+	brown	–	–
Pseudoalteromonas sp. Etwt1	*Ridgeia* trophosome	+	+	white-brown	white	–
Vibrio sp. E-limpetgut1	Limpet gut	–	+	white-brown	white	+
Marinobacter sp. Aplume1	Hydrothermal plume	+	+	brown	–	+

* All strains produced a bright yellow precipitate when grown with Cd^{2+}.

† No precipitates were observed with cells grown in the presence of Co^{2+}.

‡ All bacteria produced H_2S in the presence of Cu^{2+}.

Table 4.2 Metal removal from solution during static and agitated culture growth.

Organism	Growth	Cd^{2+}			Cu^{2+}			Co^{2+}			Zn^{2+}		
		Start (mM)	End (mM)	Loss (%)	Start (mM)	End (mM)	Loss (%)	Start (mM)	End (mM)	Loss (%)	Start (mM)	End (mM)	Loss (%)
Halomonas hydrothermalis	Static	0.48	0.021	96	2.8	2.9	–*	0.50	0.15	71	0.43	0.26	40
	Agitated	0.52	0.50	–	3.0	2.7	–	0.57	0.28	50	0.51	0.017	97
H. sulfidaeris	Static	0.97	0.43	55	1.7	1.9	–	0.93	0.72	22	0.12	0.049	59
	Agitated	1.1	1.1	–	2.3	1.5	34	1.1	0.37	66			
H. axialensis	Static	0.52	0.010	98	3.6	3.5	–				0.47	0.028	94
	Agitated	0.53	0.54	–	4.2	4.0	–				0.54	0.042	92
Marinobacter sp. Aplume1	Static	0.50	0.0080	98	1.0	0.72	28	1.9	1.8	–			
	Agitated	0.50	0.45	–	1.1	0.63	40	2.2	2.0	–			
Pseudoalteromonas sp. Etwt1	Static	1.0	0.46	54	0.99	0.90	–				1.0	0.14	86
	Agitated	1.0	1.1	–	0.97	0.62	36				1.0	0.12	89

* No significant change in metal concentration (<11% change).

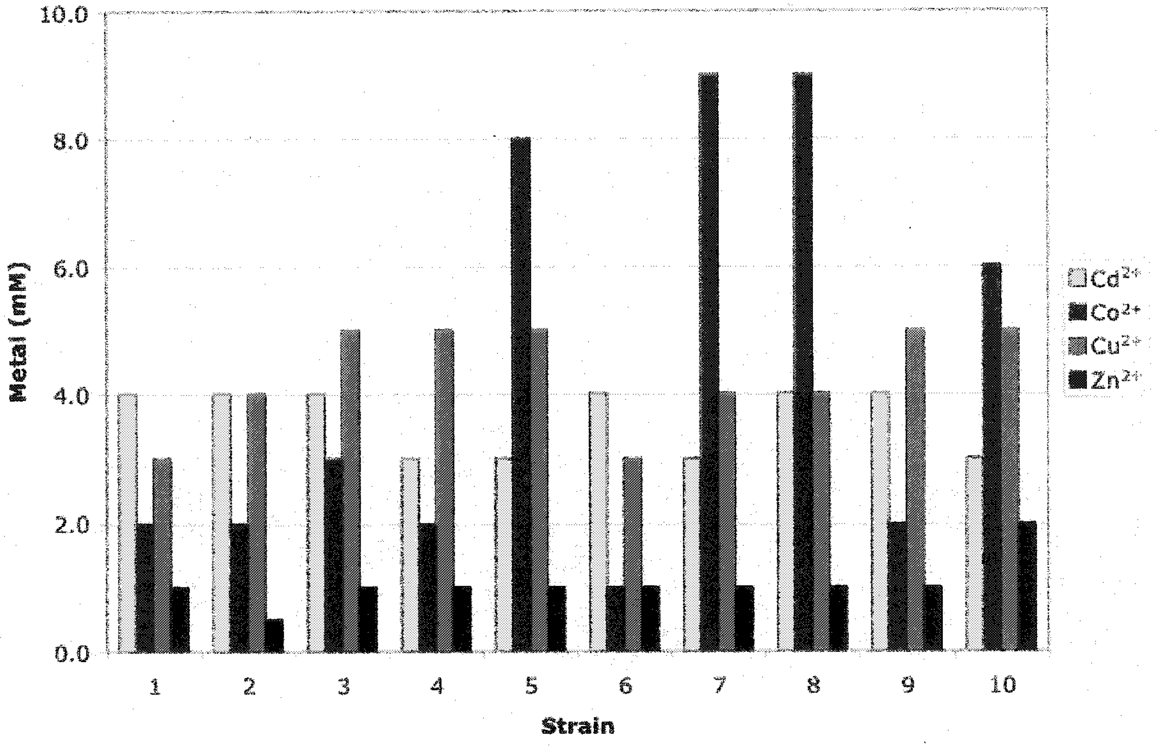

Figure 4.1 Minimum inhibitory concentrations of Cd^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} for: 1, *Halomonas neptunia*; 2, *H. sulfidaeris*; 3, *H. axialensis*; 4, *H. hydrothermalis*; 5, *H. pacifica*; 6, Mn-oxidizing bacterium Eplume4; 7, γ-proteobacterium strain Elthf1; 8, *Pseudoalteromonas* sp. Etwt1; 9, *Vibrio* sp. E-limpetgut1; and 10, *Marinobacter* sp. Aplume1.

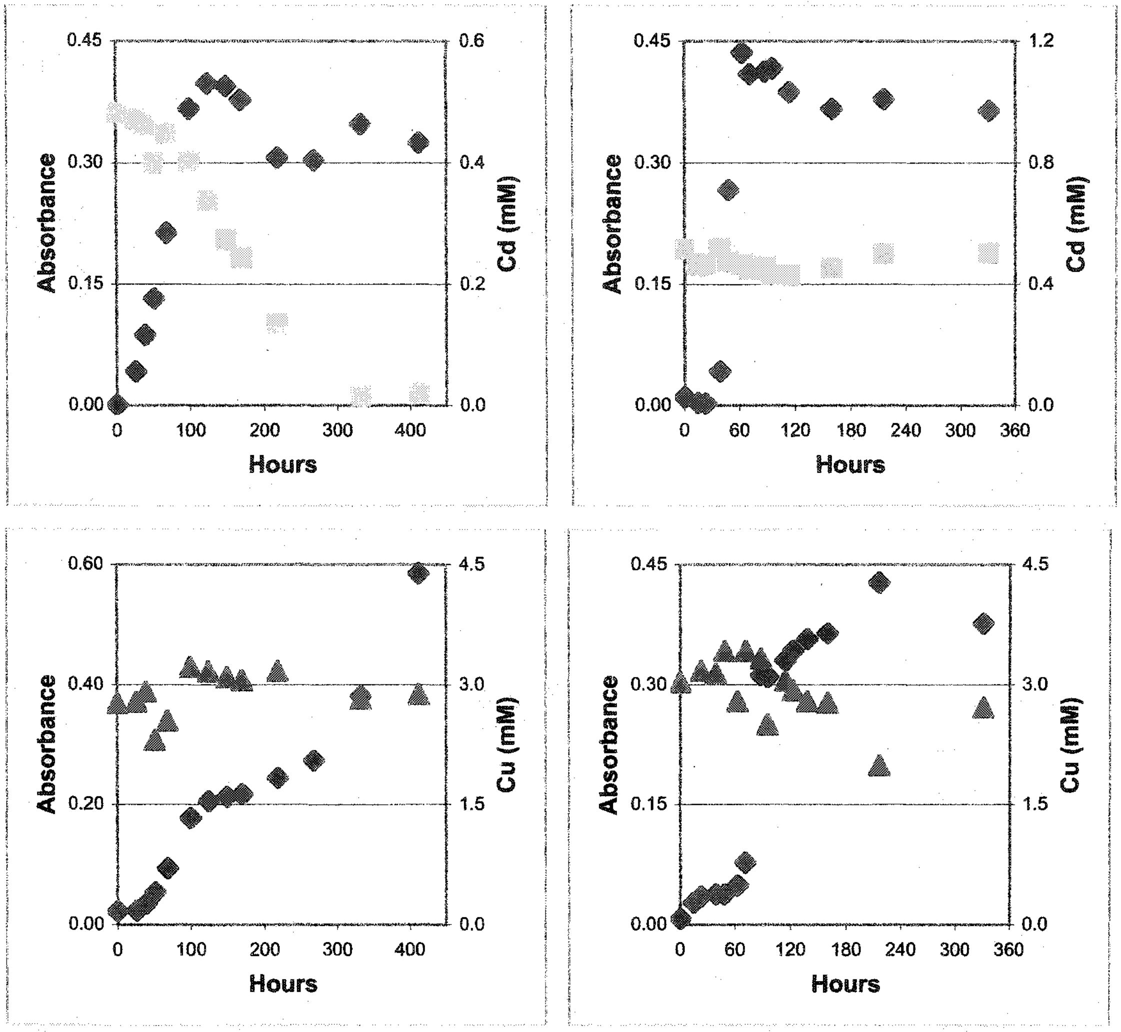

Figure 4.2 *Halomonas hydrothermalis* grown in the presence Cd^{2+} and Cu^{2+}. Cell growth measured by spectrophotometric absorbance at 600 nm (primary *y*-axis; red diamonds). Change in metal concentration measured by ICP-AES (secondary *y*-axis; Cd^{2+}: yellow squares; Cu^{2+}: green triangles). Static cultures on left and agitated cultures on right for each metal.

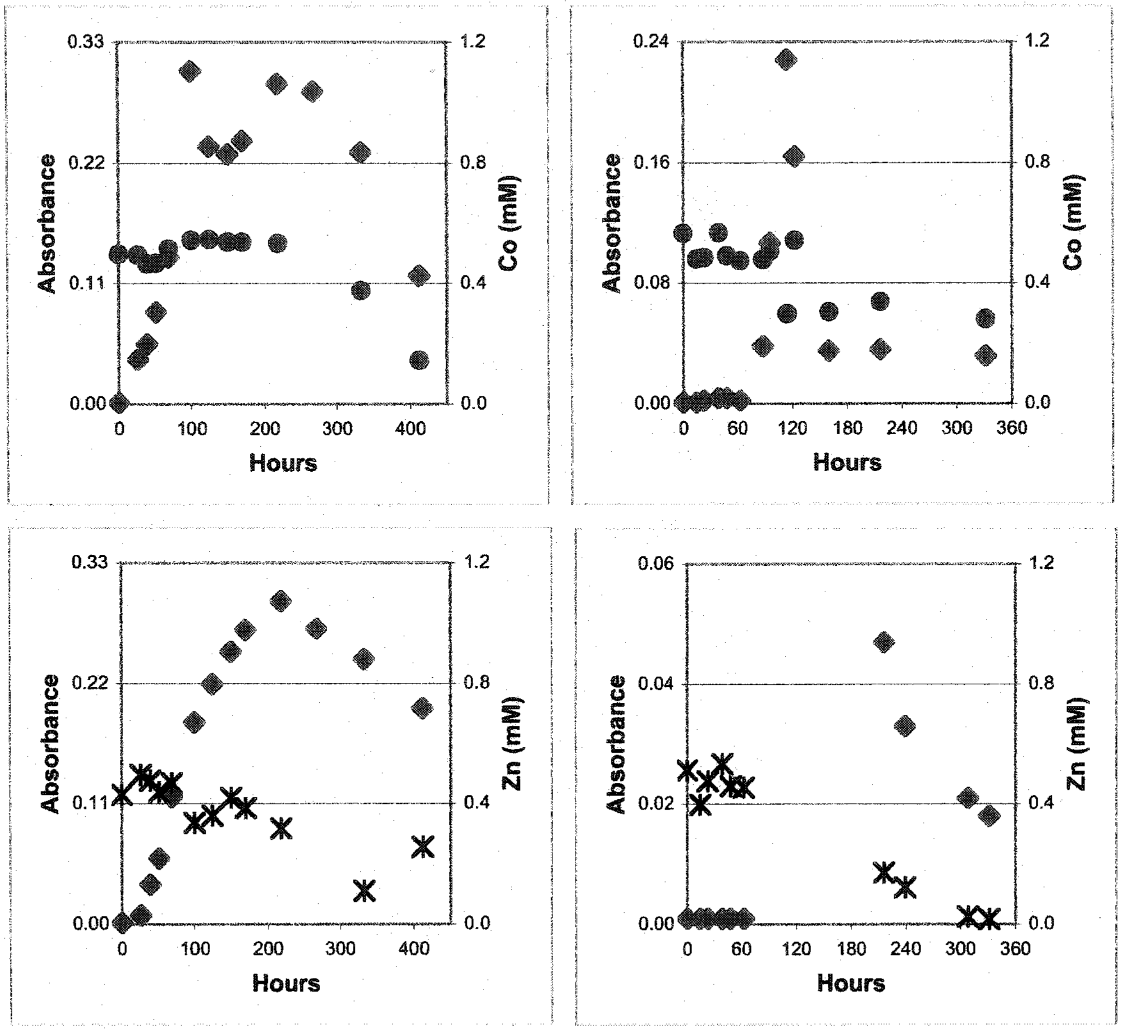

Figure 4.3 *Halomonas hydrothermalis* grown in the presence of Co^{2+} and Zn^{2+}. Change in metal concentration measured by ICP-AES (secondary *y*-axis; Co^{2+}: blue circles; Zn^{2+}: black asterisks). Other features as in Fig. 4.2.

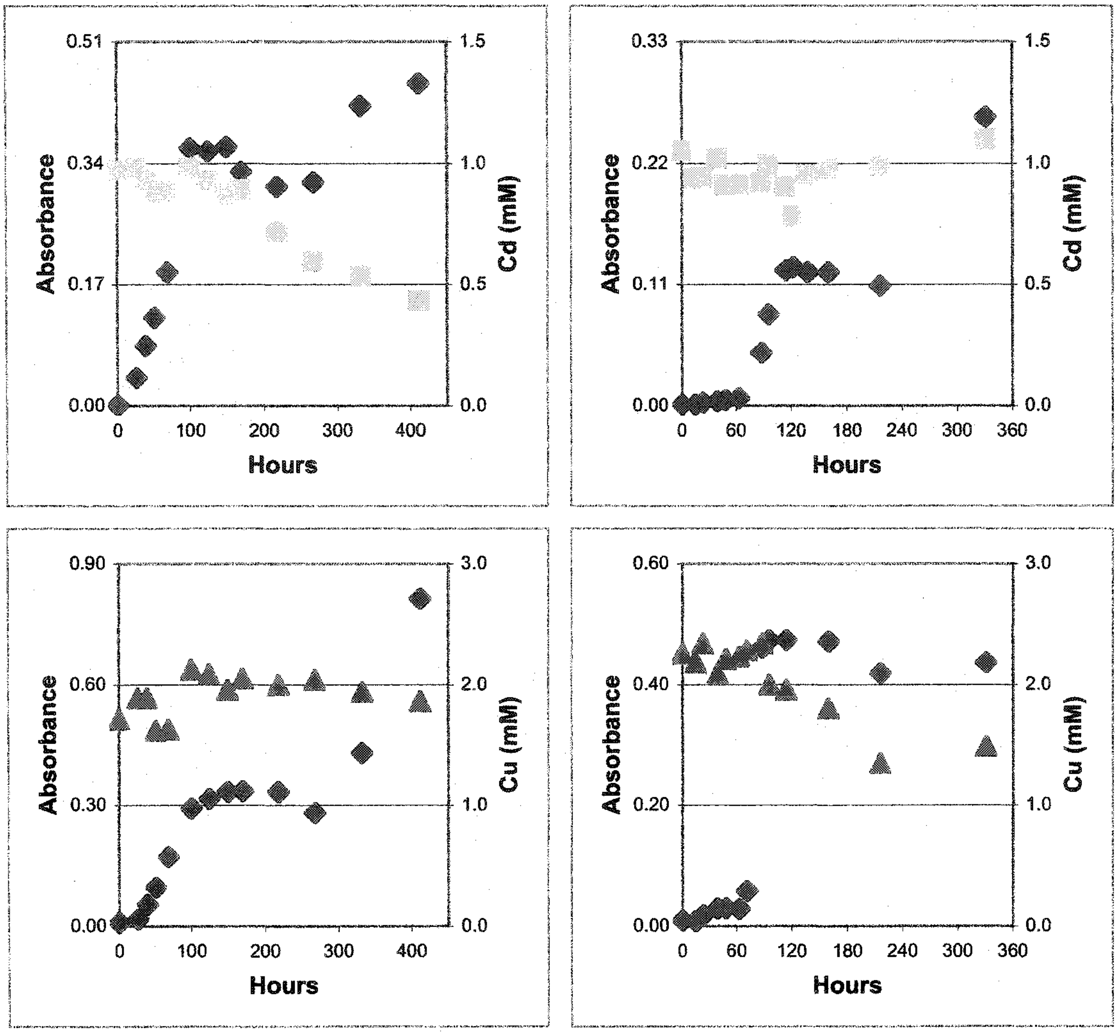

Figure 4.4 *Halomonas sulfidaeris* grown in the presence of Cd^{2+} and Cu^{2+}. Remaining features as in Fig. 4.2.

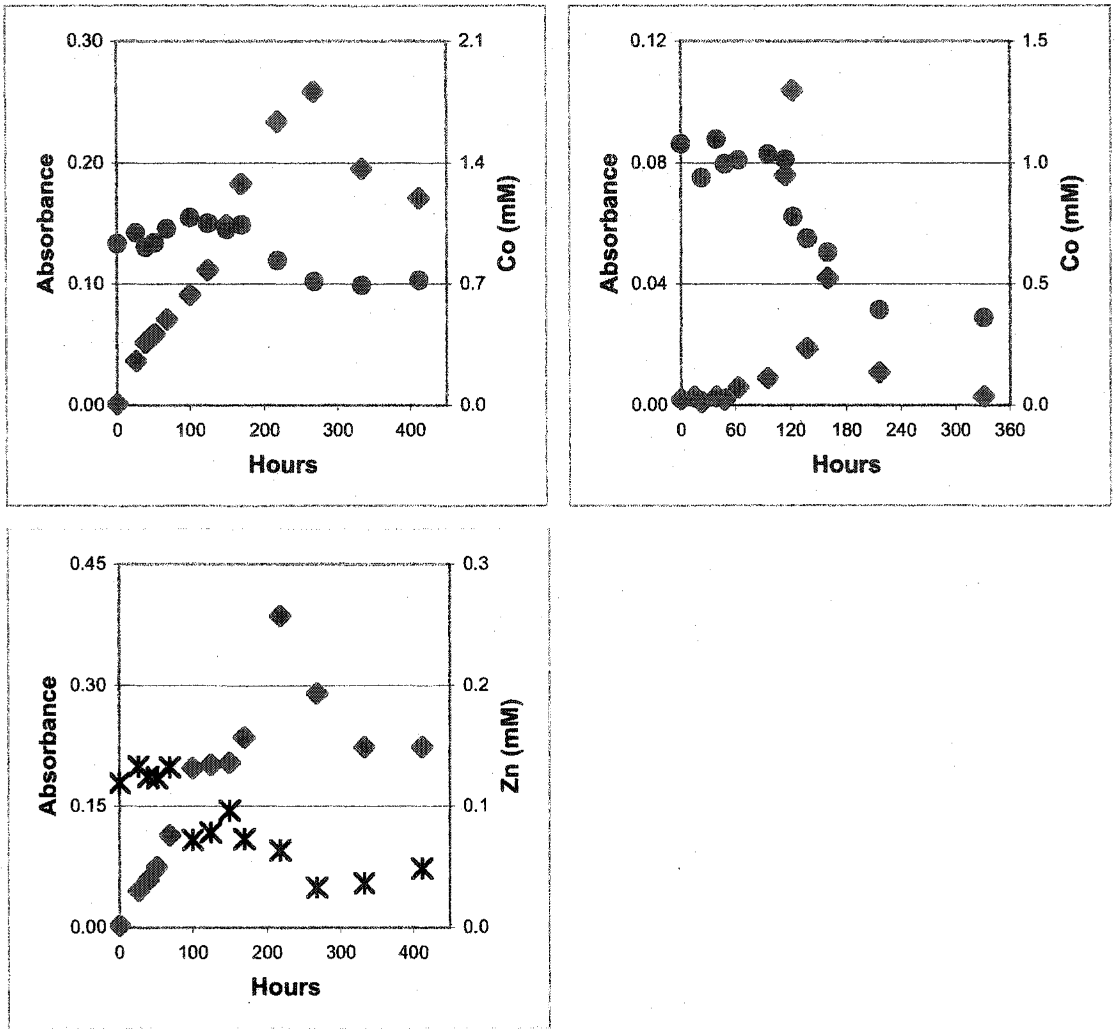

Figure 4.5 *Halomonas sulfidaeris* grown in the presence of Co^{2+} and Zn^{2+}. Remaining features as in Fig. 4.3.

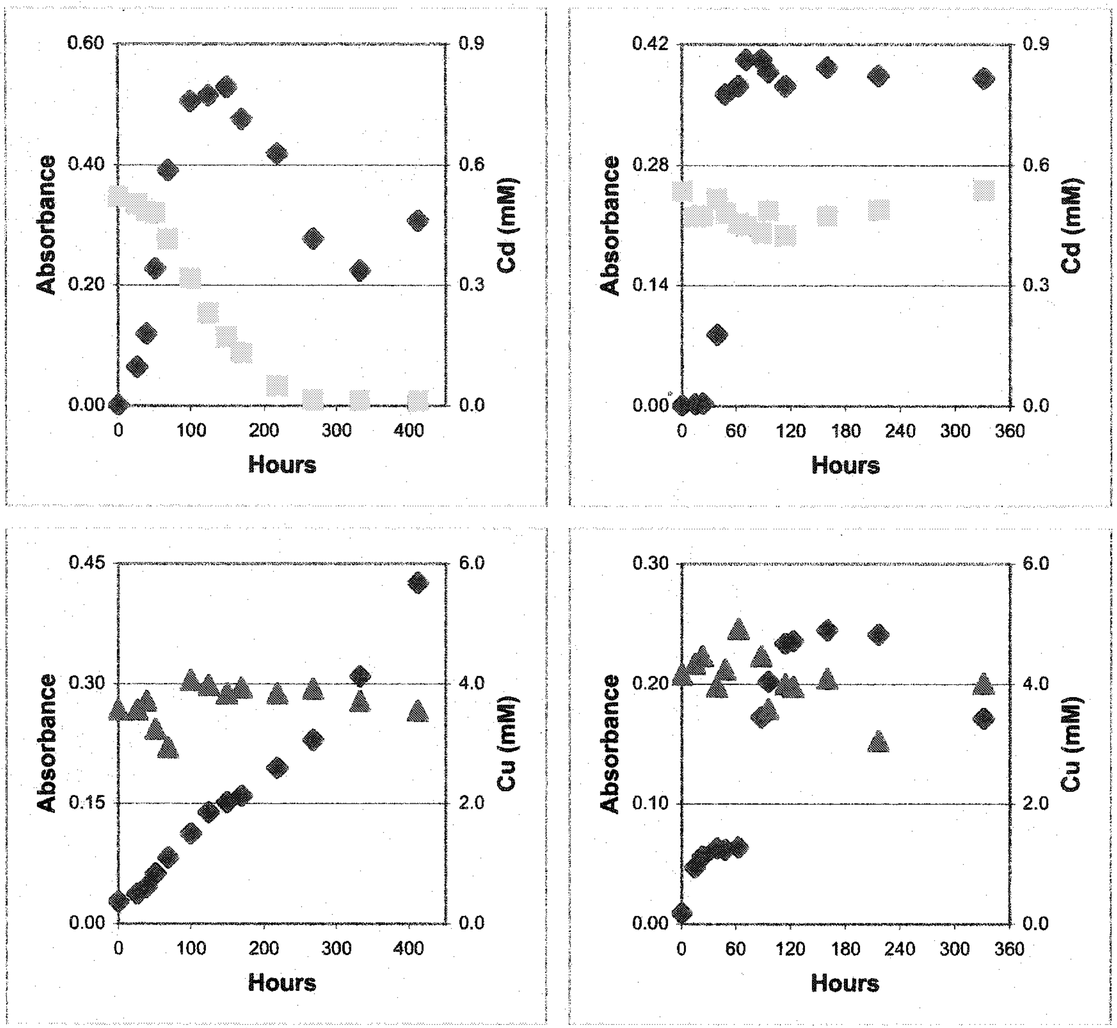

Figure 4.6 *Halomonas axialensis* grown in the presence of Cd^{2+} and Cu^{2+}. Remaining features as in Fig. 4.2.

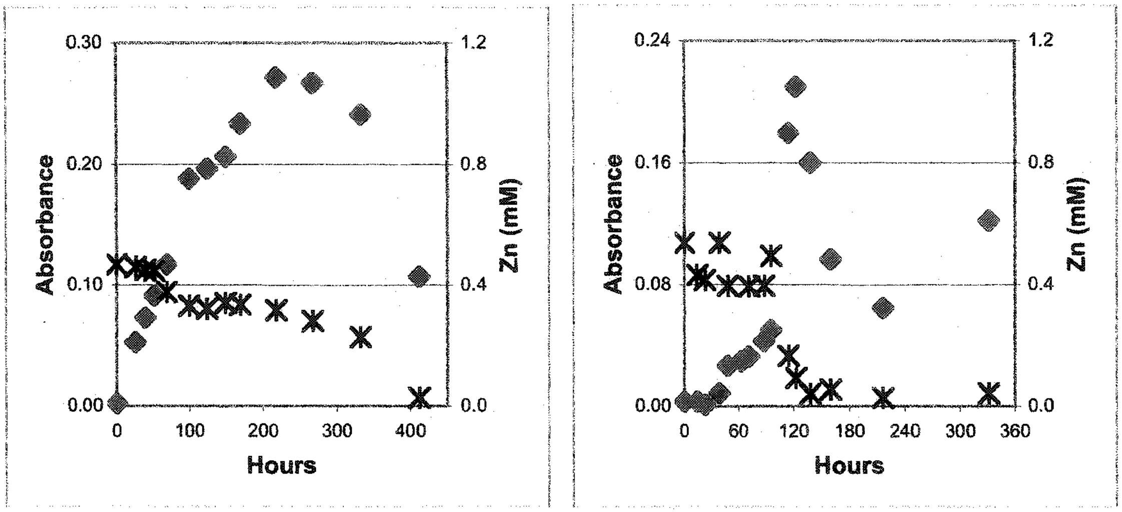

Figure 4.7 *Halomonas axialensis* grown in the presence of Zn^{2+}. Remaining features as in Fig. 4.3.

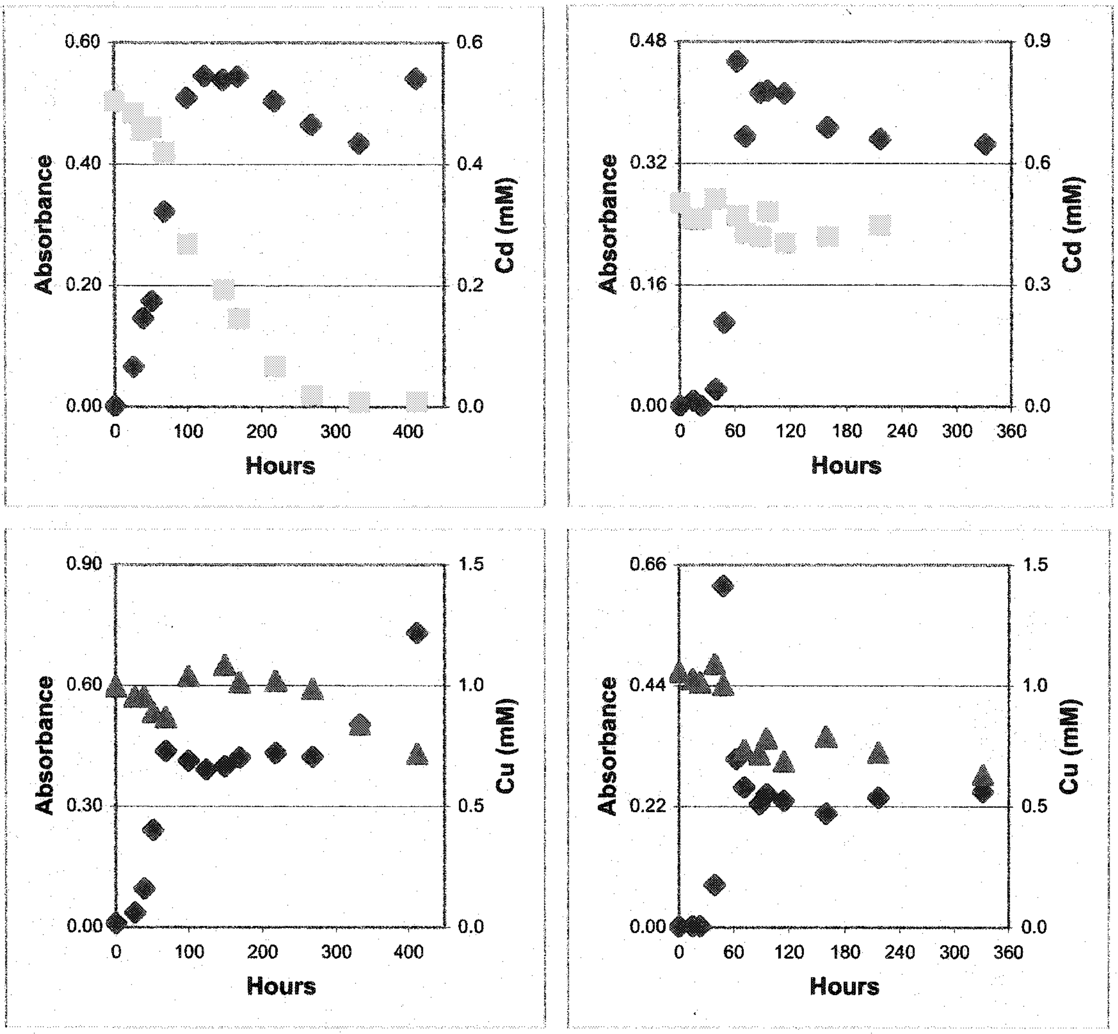

Figure 4.8 *Marinobacter* sp. Aplume1 grown in the presence of Cd^{2+} and Cu^{2+}. Remaining features as in Fig. 4.2.

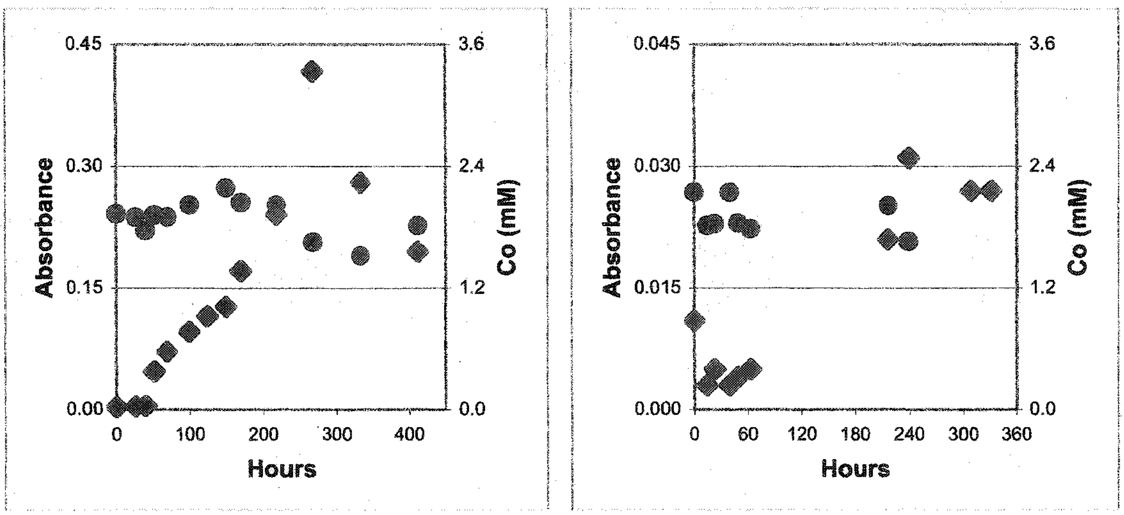

Figure 4.9 *Marinobacter* sp. Aplume1 grown in the presence of Co^{2+}. Remaining features as in Fig. 4.3.

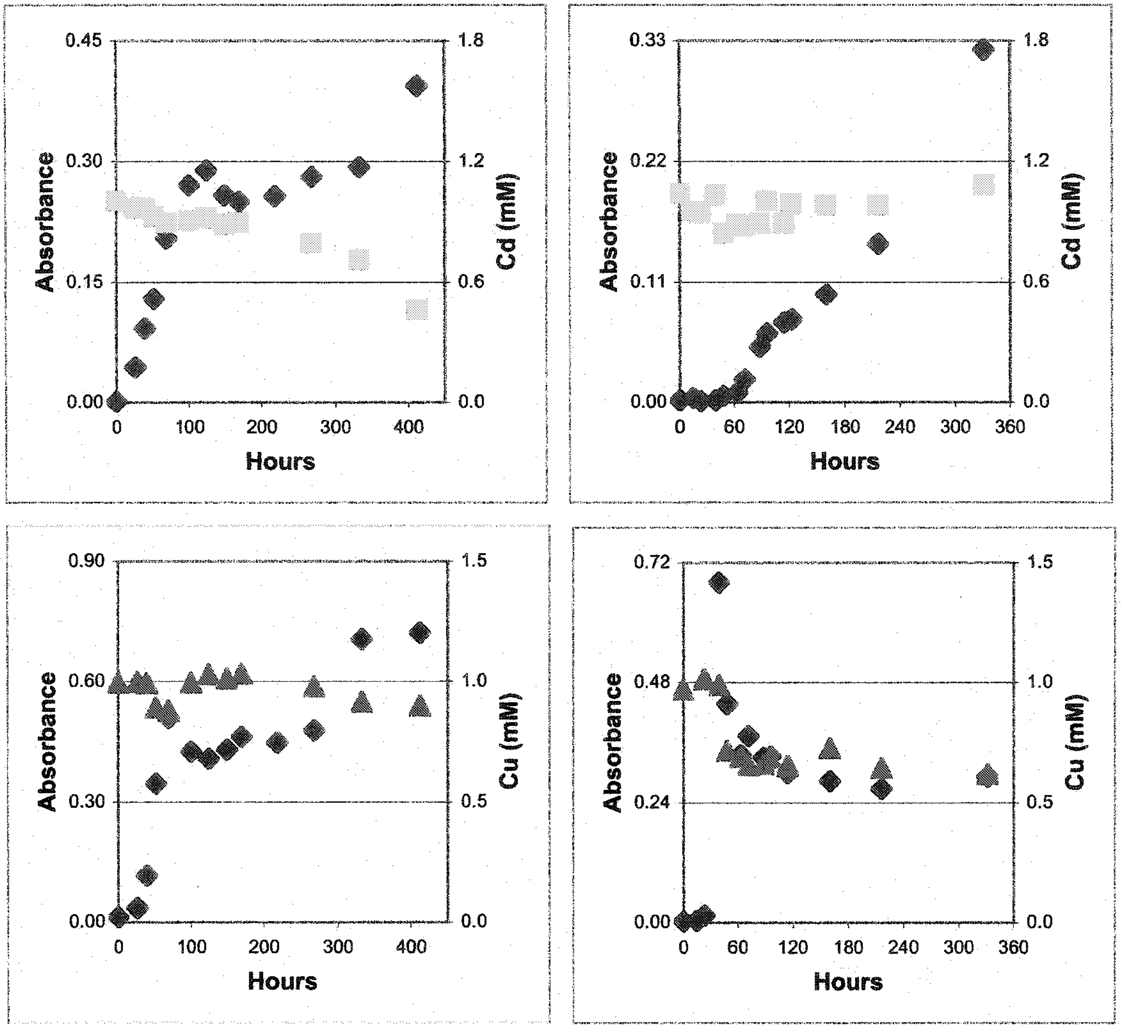

Figure 4.10 *Pseudoalteromonas* sp. Etwt1 grown in the presence of Cd^{2+} and Cu^{2+}. Remaining features as in Fig. 4.2.

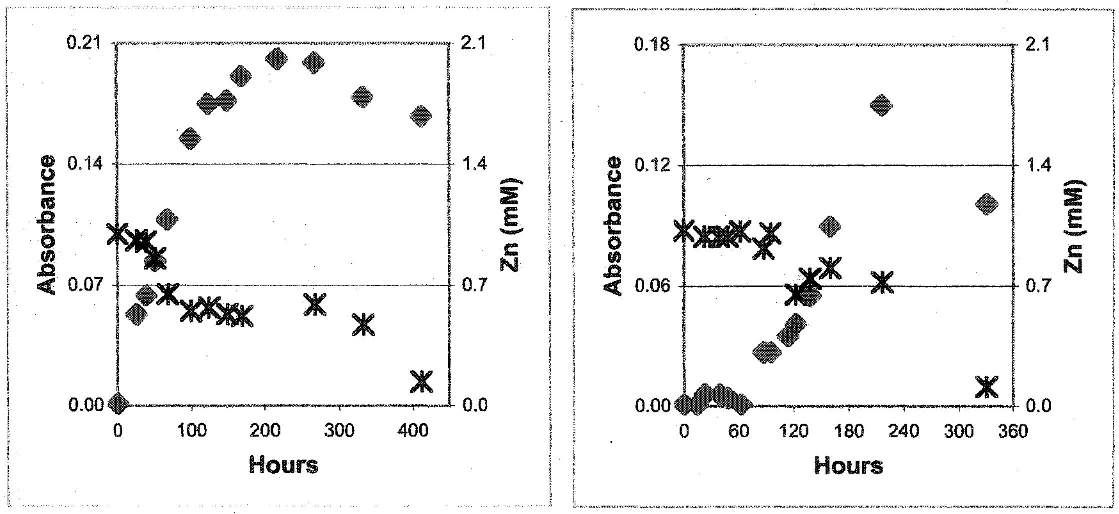

Figure 4.11 *Pseudoalteromonas* sp. Etwt1 grown in the presence of Zn^{2+}. Remaining features as in Fig. 4.3.

SUMMARY

The distribution patterns and physiology of members of the genus *Halomonas* and other moderately halophilic, euryhaline, mesophilic microorganisms raise a variety of questions for marine microbial ecology. The driving force behind this thesis was the discovery that these organisms are abundant and nearly ubiquitous, sometimes comprising >10% of the microbial community in hydrothermal-vent and pelagic environments (304). Beyond their distribution and abundance, very little is known about their growth and physiological response under *in situ* deep-sea conditions, both within and outside of hydrothermal-vent ecosystems.

Characterization of six novel *Halomonas* strains isolated from hydrothermal plumes, low-temperature seafloor vents and sulfide rock highlighted heterotrophic aerobic growth using a wide variety of organic compounds, growth between 0.5 and 22–27% total salts, and resistance to 0.5–4.0 mM Cd^{2+}. In addition, the new isolates grew over a temperature range between –1 to 2°C and 35–40°C and grew anaerobically, albeit slowly on media containing citrate or glucose and NO_3^-. Molecular-phylogenetic characterization revealed that two of the new strains belonged to the species *H. meridiana* and that the remaining isolates were new species, christened *H. neptunia*, *H. sulfidaeris*, *H. axialensis* and *H. hydrothermalis*. Overall, their phenotypic characteristics were consistent with other members of the *Halomonas* group and suggested an ability to grow in metal-enriched, cool to warm hydrothermal-vent and associated subseafloor environments. One significant difference, however, was that the cardinal growth temperatures of the new isolates were shifted cooler by 5–10°C, similar to Antarctic members of the genus (372, 485, 613). Given the synergistic effect of low temperature and high hydrostatic pressure on microbial growth and physiology (39, 658), the cold-shifted growth range and optima indicated a possible adaptation to low-temperature and/or pressurized marine habitats. While psychrotolerant growth and versatile heterotrophy unequivocally implicated success in low-temperature

hydrothermal-vent environments, the ability of the novel *Halomonas* spp. to grow in the cold deep sea (<3°C) revolved around the minimum temperature for growth under deep-sea hydrostatic pressures.

Further phenotypic characterization focused on the ability of the novel *Halomonas* spp. to grow *in situ* and the importance of their hallmark physiological traits, including euryhaline growth and strong metal resistance. Metal-resistance experiments were expanded to include the response of the four new *Halomonas* species, *H. pacifica* (isolated from surface seawater) and five other hydrothermal-vent bacteria (isolated from hydrothermal plumes, low-temperature hydrothermal fluids and animal tissues) to Co^{2+}, Cu^{2+} and Zn^{2+} in addition to Cd^{2+}. Elemental analysis revealed partial Zn^{2+} removal (26–48%) and often nearly complete Cd^{2+} removal (55–98%) from solution, concurrent with late-exponential and stationary-phase growth, but only when cells were not agitated during incubation. Any Co^{2+} or Cu^{2+} removal could be explained as artifactual. The formation of a bright yellow precipitate when cells were grown with Cd^{2+} strongly suggested metal removal via CdS or $CdHPO_4$ production, which in turn implied microbial SO_4^{2-} reduction to H_2S or localized HPO_4^{2-} excretion as either a direct or indirect metal-detoxification mechanism. All strains were strongly metal-resistant to 0.1–8.0 mM concentrations of the metals tested and produced H_2S, but only in presumed low-oxygen regions of Kligler iron agar stabs. Further experiments are required to understand why Cd^{2+} was removed during static but not agitated growth and if the observations reflect an underlying general stress response.

Four *Halomonas* isolates, *H. axialensis*, *H. meridiana* strain Slthf1, *H. hydrothermalis* and *H. pacifica*, were selected for growth curves under an array of conditions that included cold to warm temperatures, sea-surface to abyssal hydrostatic pressures, and seawater to briny salinities. Overall, as expected, the strains showed a mesophilic, piezotolerant and halotolerant response. Little or no growth occurred at 2°C and >15 MPa, suggesting that *Halomonas* spp. are unable to grow in the cold deep sea but would thrive in a warm deep-sea hydrothermal-vent and associated subseafloor habitats. Under certain conditions of low temperature and/or elevated hydrostatic pressure, growth was enhanced with elevated salinity (11% total salts). This synergistic effect on growth indicates that deep-sea brine environments are amenable to the success of *Halomonas*, especially the brine-seawater interface located above brine deeps in the Gulf of Mexico, eastern Mediterranean Sea and Red Sea. The interaction between salinity and hydrostatic pressure on growth also suggests, at a physiological level, that the two stresses may be perceived and accommodated by a singular underlying stress-response system. Accordingly, the euryhaline phenotype of *Halomonas* may actually reflect a hydrostatic-pressure adaptation.

H. axialensis and *H. hydrothermalis* were grown under high and low salinity and hydrostatic-pressure conditions and harvested for phospholipid and protein analyses. Lipid profiles indicated that hydrostatic pressure was by far the dominant force on the degree of membrane-lipid saturation and thus fluidity. Membrane- and cytosolic-protein patterns revealed a variety of responses, including hydrostatic-pressure-induced proteins, salt-induced proteins, and, intriguingly, proteins that were expressed under either salt or hydrostatic-pressure stress, but not with the combination of the two. The hydrostatic-pressure-salinity competition observed at the level of gene expression may also reflect an integrated stress-response system.

To assess the importance of the *Halomonas* isolates that were examined for metal resistance, metal precipitation, and growth under various hydrostatic-pressure, temperature and salinity conditions, selected moderately halophilic and euryhaline constituents of deep-sea and hydrothermal-vent waters were analyzed using a molecular-phylogenetic biogeographic approach. PCR primers designed to amplify members of the family *Halomonadaceae* and the genus *Marinobacter* successfully allowed the detection of these taxa in low-temperature hydrothermal fluids (>3.0-μm fraction) from Axial Seamount and in deep seawater (>0.2-μm fraction) located within a few kilometers of seafloor vents at Axial Seamount, the Main Endeavour Field of the Endeavour Segment of the Juan de Fuca Ridge (JdFR), and on the eastern ridge flank of the JdFR at the Baby Bare outcrop. Distinct populations of *Halomonas* spp. and *Marinobacter* spp. were found to inhabit each site and typically had >99% sequence identity in the 16S rRNA gene with other members of the same clade. Certain characterized species were also found to cluster with environmentally relevant groups. *Halomonas* sub-group 2A, which includes *H. meridiana*, *H. axialensis* and *H. aquamarina*, dominated the clone libraries constructed from low-temperature hydrothermal-vent fluids collected at Axial Seamount, indicating that this group represents an indigenous subseafloor population atop the volcano. Moreover, these libraries derived from the >3.0-µm sample fraction, an indication of a substrate-attached growth life style, which is hypothesized to be a key feature of inhabiting subseafloor environments (260). *Halomonas* sub-group 2B (*H. variabilis*, *H. neptunia* and *H. glaciei*) dominated the 16S rRNA gene sequences from a deep seawater sample obtained near Baby Bare; this group may be adapted to cold habitats found at moderate hydrostatic pressures. *Halomonas* sub-group 2C was the predominant phylogenetic cluster from an on-axis sample taken near the Main Endeavour Field. The sole cultured representative in this group is *H. sulfidaeris*, which was isolated from a sulfide-rock sample at the same location several years earlier. The ecology and physiology of this sub-group may be linked to sulfide structures. The final clone library, which was generated from a deep seawater sample procured near Axial Seamount, contains *Halomonas* sub-groups 2A and 2C and *Marinobacter* 16S rRNA gene sequences. *Marinobacter* spp., which recently have been discovered to grow lithotrophically on Fe^{2+} and iron-sulfide minerals at low temperatures (168), may be capitalizing on inorganic energy sources dispersed via hydrothermal plumes into the water column. These data also suggest that low-temperature hydrothermal vents on Axial Seamount — and presumably elsewhere — are sites of enrichment of certain mesophilic and psychrotolerant microorganisms, including those of *Halomonas* sub-group 2A, that subsequently seed the overlying water column. The biogeography study succeeds in intra-specific mapping of the genus *Halomonas* in deep-sea and hydrothermal-vent habitats.

One of the key points of this dissertation is to be vigilant in the interpretation of microbial physiology in regard to what a phenotype expressed in a laboratory setting means for the behavior of microorganisms in the environment and for environmental characteristics themselves. For example, euryhaline growth of *Halomonas* spp. and *Marinobacter* spp. may in fact reflect a hydrostatic-pressure adaptation. Similarly, strong metal resistance and metal precipitation may also reflect, in part, an adaptation to other physiological stresses. While both euryhaline growth and metal resistance clearly promote success of these bacteria in hypersaline and metal-enriched environments located along coastlines and likely in the brine-seawater interface above localized brine deeps beneath various seas, these physiologies may also manifest as a stress response induced by factors other than salt and heavy metals. The conclusions of this dissertation, while not fully deciphering putative cryptic salt- and heavy-metal-stress responses among moderately halophilic and euryhaline bacteria, signify the importance of investigating both "environmental" and "physiological" hypotheses (as defined in the Introduction) to fully explain how and why certain phenotypes are expressed *in situ*.

REFERENCES

1. **Abe, F., C. Kato, and K. Horikoshi.** 1999. Pressure-regulated metabolism in microorganisms. Trends Microbiol. **7:**447–453.

2. **Addy, S. K., and E. W. Behrens.** 1980. Time of accumulation of hypersaline anoxic brine in Orca Basin (Gulf of Mexico). Mar. Geol. **37:**241–252.

3. **Adiwidjaja, G., K. Friese, K.-H. Klaska, and J. Schlüter.** 1997. The crystal structure of gordaite $NaZn_4(SO_4)(OH)_6Cl\cdot 6H_2O$. Z. Kristallogr. **212:**704–707.

4. **Adkins, J. P., L. A. Cornell, and R. S. Tanner.** 1992. Microbial composition of carbonate petroleum reservoir fluids. Geomicrobiol. J. **10:**87–97.

5. **Adkins, J. P., M. T. Madigan, L. Mandelco, C. R. Woese, and R. S. Tanner.** 1993. *Arhodomonas aquaeolei* gen. nov., sp. nov., an aerobic, halophilic bacterium isolated from a subterranean brine. Int. J. Syst. Bacteriol. **43:**514–520.

6. **Aertsen, A., K. Hauben, and C. Michiels.** 2001. Multiple-stress resistance in pressure resistant mutants of *Escherichia coli* (abstract). Abstr. Gen. Meet. Am. Soc. Microbiol. **101:**566.

7. **Ahring, B. K., and P. Westermann.** 1985. Sensitivity of thermophilic methanogenic bacteria to heavy metals. Curr. Microbiol. **12:**273–276.

8. **Aiking, H., H. Govers, and J. van 't Riet.** 1985. Detoxification of mercury, cadmium, and lead in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. Appl. Environ. Microbiol. **50:**1262–1267.

9. **Aiking, H., K. Kok, H. van Heerikhuizen, and J. van 't Riet.** 1982. Adaptation to cadmium by *Klebsiella aerogenes* growing in continuous culture proceeds mainly via formation of cadmium sulfide. Appl. Environ. Microbiol. **44:**938–944.

10. **Aiking, H., A. Stijnman, C. van Garderen, H. van Heerikhuizen, and J. van 't Riet.** 1984. Inorganic phosphate accumulation and cadmium detoxification in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. Appl. Environ. Microbiol. **47:**374–377.

11. **Alain, K., M. Olagnon, D. Desbruyères, A. Pagé, G. Barbier, S. K. Juniper, J. Quérellou, and M.-A. Cambon-Bonavita.** 2002. Phylogenetic characterization of the bacterial assemblage associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis*. FEMS Microbiol. Ecol. **42:**463–476.

12. **Alain, K., J. Querellou, F. Lesongeur, P. Pignet, P. Crassous, G. Raguénès, V. Cueff, and M.-A. Cambon-Bonavita.** 2002. *Caminibacter hydrogeniphilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. Int. J. Syst. Evol. Microbiol. **52:**1317–1323.

13. **Allen, E. E., and D. H. Bartlett.** 2000. FabF is required for piezoregulation of *cis*-vaccenic acid levels and piezophilic growth of the deep-sea bacterium *Photobacterium profundum* strain SS9. J. Bacteriol. **182:**1264–1271.

14. **Allen, E. E., D. Facciotti, and D. H. Bartlett.** 1999. Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature. Appl. Environ. Microbiol. **65:**1710–1720.

15. **Alpas, H., N. Kalchayanand, F. Bozoglu, and B. Ray.** 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. Int. J. Food Microbiol. **60:**33–42.

16. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.

17. **Andrews, S. C., A. K. Robinson, and F. Rodríguez-Quiñones.** 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:**215–237.

18. **Antoine, E., V. Cilia, J. R. Meunier, J. Guezennec, F. Lesongeur, and G. Barbier.** 1997. *Thermosipho melanesiensis* sp. nov., a new thermophilic anaerobic bacterium belonging to the order *Thermotogales*, isolated from deep-sea hydrothermal vents in the southwestern Pacific Ocean. Int. J. Syst. Bacteriol. **47:**1118–1123.

19. **Antón, J., E. Llobet-Brossa, F. Rodríguez-Valera, and R. Amann.** 1999. Fluorescence *in situ* hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environ. Microbiol. **1:**517–523.

20. **Antón, J., R. Rosselló-Mora, F. Rodríguez-Valera, and R. Amann.** 2000. Extremely halophilic *Bacteria* in crystallizer ponds from solar salterns. Appl. Environ. Microbiol. **66:**3052–3057.

21. **Antunes, A., W. Eder, P. Fareleira, H. Santos, and R. Huber.** 2003. *Salinisphaera shabanensis* gen. nov., sp. nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea. Extremophiles **7:**29–34.

22. **Arahal, D. R., A. M. Castillo, W. Ludwig, K. H. Schleifer, and A. Ventosa.** 2002. Proposal of *Cobetia marina* gen. nov., comb. nov., within the family *Halomonadaceae* to include the species *Halomonas marina*. Syst. Appl. Microbiol. **25:**207–211.

23. **Arahal, D. R., M. C. Gutiérrez, B. E. Volcani, and A. Ventosa.** 2000. Taxonomic analyses of extremely halophilic archaea isolated from 56-years-old Dead Sea brine samples. Syst. Appl. Microbiol. **23:**376–385.

24. **Arahal, D. R., W. Ludwig, K. H. Schleifer, and A. Ventosa.** 2002. Phylogeny of the family *Halomonadaceae* based on 23S and 16S rDNA sequence analyses. Int. J. Syst. Evol. Microbiol. **52:**241–249.

25. **Argandoña, M., F. Martínez-Checa, I. Llamas, E. Quesada, and A. del Moral.** 2003. Megaplasmids in Gram-negative, moderately halophilic bacteria. FEMS Microbiol. Lett. **227:**81–86.

26. **Atlas, R. M.** 1993. Handbook of microbiological media. CRC Press, Boca Raton, Fla.

27. **Azam, F.** 1998. Microbial control of oceanic carbon flux: the plot thickens. Science **280:**694–696.

28. **Azam, F., D. C. Smith, G. F. Steward, and Å. Hagström.** 1993. Bacteria-organic matter coupling and its significance for oceanic carbon cycling. Microb. Ecol. **28:**167–179.

29. **Bae, W., C. H. Wu, J. Kostal, A. Mulchandani, and W. Chen.** 2003. Enhanced mercury biosorption by bacterial cells with surface-displayed MerR. Appl. Environ. Microbiol. **69:**3176–3180.

30. **Baker, B. J., and J. F. Banfield.** 2003. Microbial communities in acid mine drainage. FEMS Microbiol. Ecol. **44:**139–152.

31. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane-sulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:**781–791.

32. **Barkay, T., S. M. Miller, and A. O. Summers.** 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. **27:**355–384.

33. **Baross, J. A.** 1993. Isolation and cultivation of hyperthermophilic bacteria from marine and freshwater habitats, p. 21–30. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, Fla.

34. **Baross, J. A., F. J. Hanus, and R. Y. Morita.** 1975. Survival of human enteric and other sewage microorganisms under simulated deep-sea conditions. Appl. Microbiol. **30:**309–318.

35. **Baross, J. A., F. J. Hanus, and R. Y. Morita.** Unpublished manuscript. Synergetic temperature-salinity effects on amino acid uptake and growth of *Vibrio marinus* MP-1.

36. **Baross, J. A., M. Summit, and J. F. Holden.** 1997. Is there a microbial biosphere in the subseafloor? JOI/USSAC News. **10:**8–11.

37. **Barth, S., M. Huhn, B. Matthey, A. Klimka, E. A. Galinski, and A. Engert.** 2000. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl. Environ. Microbiol. **66:**1572–1579.

38. **Bartlett, D., M. Wright, A. A. Yayanos, and M. Silverman.** 1989. Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium. Nature **342:**572–574.

39. **Bartlett, D. H.** 2002. Pressure effects on in vivo microbial processes. Biochim. Biophys. Acta **1595:**367–381.

40. **Bartlett, D. H., C. Kato, and K. Horikoshi.** 1995. High pressure influences on gene and protein expression. Res. Microbiol. **146:**697–706.

41. **Battista, J. R.** 1998. DNA repair in *Deinococcus radiodurans*, p. 287–303. *In* J. A. Nickoloff and M. F. Hoekstra (ed.), DNA damage and repair, vol. 1: DNA repair in prokaryotes and lower eukaryotes. Humana Press, Totowa, N.J.

42. **Baumann, L., P. Baumann, M. Mandel, and R. D. Allen.** 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. **110:**402–429.

43. **Baumann, L., R. D. Bowditch, and P. Baumann.** 1983. Description of *Deleya* gen. nov. created to accommodate the marine species *Alcaligenes aestus*, *A. pacificus*, *A. cupidus*, *A. venustus*, and *Pseudomonas marina*. Int. J. Syst. Bacteriol. **33:**793–802.

44. **Baumann, P., L. Baumann, and M. Mandel.** 1971. Taxonomy of marine bacteria: the genus *Beneckea*. J. Bacteriol. **107:**268–294.

45. **Becker, L. A., M. S. Çetin, R. W. Hutkins, and A. K. Benson.** 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. **180:**4547–4554.

46. **Becker, L. A., S. N. Evans, R. W. Hutkins, and A. K. Benson.** 2000. Role of σ^B in adaptation of *Listeria monocytogenes* to growth at low temperature. J. Bacteriol. **182:**7083–7087.

47. **Bencheikh-Latmani, R., and J. O. Leckie.** 2003. Association of uranyl with the cell wall of *Pseudomonas fluorescens* inhibits metabolism. Geochim. Cosmochim. Acta **67:**4057–4066.

48. **Benito, A., V. Georgia, M. Casadei, T. Robinson, and B. Mackey.** 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Appl. Environ. Microbiol. **65:**1564–1569.

49. **Berendes, F., G. Gottschalk, E. Heine-Dobbernack, E. R. B. Moore, and B. J. Tindall.** 1996. *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. Syst. Appl. Microbiol. **19:**158–167.

50. **Bernhardt, G., R. Jaenicke, H.-D. Lüdemann, H. König, and K. O. Stetter.** 1988. High pressure enhances the growth rate of the thermophilic archaebacterium *Methanococcus thermolithotrophicus* without extending its temperature range. Appl. Environ. Microbiol. **54:**1258–1261.

51. **Bertone, S., M. Giacomini, C. Ruggiero, C. Piccarolo, and L. Calegari.** 1996. Automated systems for identification of heterotrophic marine bacteria on the basis of their fatty acid composition. Appl. Environ. Microbiol. **62:**2122–2132.

52. **Beveridge, T. J.** 1989. Role of cellular design in bacterial metal accumulation and mineralization. Annu. Rev. Microbiol. **43:**147–171.

53. **Bhupathiraju, V. K., M. J. McInerney, C. R. Woese, and R. S. Tanner.** 1999. *Haloanaerobium kushneri* sp. nov., an obligately halophilic, anaerobic bacterium from an oil brine. Int. J. Syst. Bacteriol. **49:**953–960.

54. **Bidle, K. A., and D. H. Bartlett.** 1999. RecD function is required for high-pressure growth of a deep-sea bacterium. J. Bacteriol. **181:**2330–2337.

55. **Bignell, R. D.** 1976. Climate and rifting related to metallogenesis of the Red Sea precipitates. Geol. Assoc. Can., Spec. Pap. **14:**181–184.

56. **Bignell, R. D., D. S. Cronan, and J. S. Tooms.** 1976. Red Sea metalliferous brine precipitates. Geol. Assoc. Can., Spec. Pap. **14:**147–179.

57. **Bischoff, J. L., and F. T. Manheim.** 1969. Economic potential of the Red Sea heavy metal deposits, p. 535–541. *In* E. T. Degens and D. A. Ross (ed.), Hot

brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

58. **Bischoff, J. L., and R. J. Rosenbauer.** 1984. The critical point and two-phase boundary of seawater, 200–500°C. Earth Planet. Sci. Lett. **68:**172–180.

59. **Bischoff, J. L., and R. J. Rosenbauer.** 1985. An empirical equation of state for hydrothermal seawater (3.2 percent NaCl). Am. J. Sci. **285:**725–763.

60. **Bischoff, J. L., and R. J. Rosenbauer.** 1987. Phase separation in seafloor geothermal systems: an experimental study of the effects on metal transport. Am. J. Sci. **287:**953–978.

61. **Bischoff, J. L., and R. J. Rosenbauer.** 1988. Liquid-vapor relations in the critical region of the system NaCl–H_2O from 380 to 415°C: a refined determination of the critical point and two-phase boundary of seawater. Geochim. Cosmochim. Acta **52:**2121–2126.

62. **Bischoff, J. L., and R. J. Rosenbauer.** 1989. Salinity variations in submarine hydrothermal systems by layered double-diffusive convection. J. Geol. **97:**613–623.

63. **Bjedov, I., O. Tenaillon, B. Gérard, V. Souza, E. Denamur, M. Radman, F. Taddei, and I. Matic.** 2003. Stress-induced mutagenesis in bacteria. Science **300:**1404–1409.

64. **Blencowe, D. K., and A. P. Morby.** 2003. Zn(II) metabolism in prokaryotes. FEMS Microbiol. Rev. **27:**291–311.

65. **Boldrin, A., and S. Rabitti.** 1990. Hydrography of the brines in the Bannock and Tyro anoxic basins (eastern Mediterranean). Mar. Chem. **31:**21–33.

66. **Bonatti, E.** 1985. Punctiform initiation of seafloor spreading in the Red Sea during transition from a continental to an oceanic rift. Nature **316:**33–37.

67. **Bonch-Osmolovskaya, E. A., M. L. Miroshnichenko, A. V. Lebedinsky, N. A. Chernyh, T. N. Nazina, V. S. Ivoilov, S. S. Belyaev, E. S. Boulygina, Y. P. Lysov, A. N. Perov, A. D. Mirzabekov, H. Hippe, E. Stackebrandt, S. L'Haridon, and C. Jeanthon.** 2003. Radioisotopic, culture-based, and oligonucleotide microchip analyses of thermophilic microbial communities in a continental high-temperature petroleum reservoir. Appl. Environ. Microbiol. **69:**6143–6151.

68. **Boonyaratanakornkit, B. B., C. B. Park, and D. S. Clark.** 2002. Pressure effects on intra- and intermolecular interactions within proteins. Biochim. Biophys. Acta **1595:**235–249.

69. **Bouchotroch, S., E. Quesada, A. del Moral, I. Llamas, and V. Béjar.** 2001. *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. Int. J. Syst. Evol. Microbiol. **51:**1625–1632.

70. **Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin.** 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. **63:**3068–3078.

71. **Bowman, J. P., and R. D. McCuaig.** 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. Appl. Environ. Microbiol. **69:**2463–2483.

72. **Boyanov, M. I., S. D. Kelly, K. M. Kemner, B. A. Bunker, J. B. Fein, and D. A. Fowle.** 2003. Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: a pH-dependent X-ray absorption fine structure spectroscopy study. Geochim. Cosmochim. Acta **67:**3299–3311.

73. **Braun, V.** 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. Biol. Chem. **378:**779–786.

74. **Bregant, D., G. Catalano, G. Civitarese, and A. Luchetta.** 1990. Some chemical characteristics of the brines in Bannock and Tyro Basins: salinity, sulphur compounds, Ca^{2+}, F^{-}, pH, A_t, PO_4^{3-}, SiO_2, NH_3. Mar. Chem. **31:**35–62.

75. **Brett, R., H. T. Evans, Jr., E. K. Gibson, Jr., J. W. Hedenquist, M.-V. Wandless, and M. A. Sommer.** 1987. Mineralogical studies of sulfide samples and volatile concentrations of basalt glasses from the southern Juan de Fuca Ridge. J. Geophys. Res. **92:**11,373–11,379.

76. **Brewer, P. G., C. D. Densmore, R. Munns, and R. J. Stanley.** 1969. Hydrography of the Red Sea brines, p. 138–147. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

77. **Brewer, P. G., and D. W. Spencer.** 1969. A note on the chemical composition of the Red Sea brines, p. 174–179. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

78. **Bright, T. J., P. A. LaRock, R. D. Lauer, and J. M. Brooks.** 1980. A brine seep at the East Flower Garden Bank, northwestern Gulf of Mexico. Int. Rev. gesamten Hydrobiol. **65:**535–549.

79. **Brigulla, M., T. Hoffmann, A. Krisp, A. Völker, E. Bremer, and U. Völker.** 2003. Chill induction of the SigB-dependent general stress response in *Bacillus*

subtilis and its contribution to low-temperature adaptation. J. Bacteriol. **185:**4305–4314.

80. **Brim, H., A. Venkateswaran, H. M. Kostandarithes, J. K. Fredrickson, and M. J. Daly.** 2003. Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. Appl. Environ. Microbiol. **69:**4575–4582.

81. **Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke.** 2003. Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl. Environ. Microbiol. **69:**6610–6619.

82. **Brinkoff, T., G. Muyzer, C. O. Wirsen, and J. Kuever.** 1999. *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a *Thioploca* mat. Int. J. Syst. Bacteriol. **49:**875–879.

83. **Brisbarre, N., M.-L. Fardeau, V. Cueff, J.-L. Cayol, G. Barbier, V. Cilia, G. Ravot, P. Thomas, J.-L. Garcia, and B. Ollivier.** 2003. *Clostridium caminithermale* sp. nov., a slightly halophilic and moderately thermophilic bacterium isolated from an Atlantic deep-sea hydrothermal chimney. Int. J. Syst. Evol. Microbiol. **53:**1043–1049.

84. **Brooks, R. R., I. R. Kaplan, and M. N. A. Peterson.** 1969. Trace element composition of Red Sea geothermal brine and interstitial water, p. 180–203. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

85. **Brown, M. V., and J. P. Bowman.** 2001. A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiol. Ecol. **35:**267–275.

86. **Bull, H. B., and K. Breese.** 1974. Surface tension of amino acid solutions: a hydrophobicity scale of the amino acid residues. Arch. Biochem. Biophys. **161:**665–670.

87. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J.-F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H.-P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058–1073.

88. **Burke, B. E., K. W. Tsang, and R. M. Pfister.** 1991. Cadmium sorption by bacteria and freshwater sediment. J. Ind. Microbiol. **8:**201–208.

89. **Burton, N. F., M. J. Day, and A. T. Bull.** 1982. Distribution of bacterial plasmids in clean and polluted sites in a South Wales river. Appl. Environ. Microbiol. **44:**1026–1029.

90. **Busenlehner, L. S., M. A. Pennella, and D. P. Giedroc.** 2003. The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. FEMS Microbiol. Rev. **27:**131–143.

91. **Butterfield, D. A.** 2002. Personal communication.

92. **Butterfield, D. A., I. R. Jonasson, G. J. Massoth, R. A. Feely, K. K. Roe, R. E. Embley, J. F. Holden, R. E. McDuff, M. D. Lilley, and J. R. Delaney.** 1997. Seafloor eruptions and evolution of hydrothermal fluid chemistry. Phil. Trans. R. Soc. Lond. A **355:**369–386.

93. **Butterfield, D. A., and G. J. Massoth.** 1994. Geochemistry of north Cleft vent fluids: temporal changes in chlorinity and their possible relation to recent volcanism. J. Geophys. Res. **99:**4951–4968.

94. **Butterfield, D. A., G. J. Massoth, R. E. McDuff, J. E. Lupton, and M. D. Lilley.** 1990. Geochemistry of hydrothermal fluids from Axial Seamount Hydrothermal Emissions Study vent field, Juan de Fuca Ridge: subseafloor boiling and subsequent fluid-rock interaction. J. Geophys. Res. **95:**12,895–12,921.

95. **Butterfield, D. A., R. E. McDuff, M. J. Mottl, M. D. Lilley, J. E. Lupton, and G. J. Massoth.** 1994. Gradients in the composition of hydrothermal fluids from the Endeavour segment vent field: phase separation and brine loss. J. Geophys. Res. **99:**9561–9583.

96. **Button, D. K., B. R. Robertson, P. W. Lepp, and T. M. Schmidt.** 1998. A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. Appl. Environ. Microbiol. **64:**4467–4476.

97. **Cairns, J., J. Overbaugh, and S. Miller.** 1988. The origin of mutants. Nature **335:**142–145.

98. **Camerlenghi, A.** 1990. Anoxic basins of the eastern Mediterranean: geological framework. Mar. Chem. **31:**1–19.

99. **Campbell, B. J., C. Jeanthon, J. E. Kostka, G. W. Luther, III, and S. C. Cary.** 2001. Growth and phylogenetic properties of novel bacteria belonging to the epsilon subdivision of the *Proteobacteria* enriched from *Alvinella pompejana* and deep-sea hydrothermal vents. Appl. Environ. Microbiol. **67:**4566–4572.

100. **Canganella, F., J. M. Gonzalez, M. Yanagibayashi, C. Kato, and K. Horikoshi.** 1997. Pressure and temperature effects on growth and viability of the hyperthermophilic archaeon *Thermococcus peptonophilus*. Arch. Microbiol. **168:**1–7.

101. **Carmel-Harel, O., and G. Storz.** 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu. Rev. Microbiol. **54:**439–461.

102. **Cary, S. C., M. T. Cottrell, J. L. Stein, F. Camacho, and D. Desbruyères.** 1997. Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid *Alvinella pompejana*. Appl. Environ. Microbiol. **63:**1124–1130.

103. **Cases, I., V. de Lorenzo, and C. A. Ouzounis.** 2003. Transcription regulation and environmental adaptation in bacteria. Trends Microbiol. **11:**248–253.

104. **Cavet, J. S., G. P. M. Borrelly, and N. J. Robinson.** 2003. Zn, Cu and Co in cyanobacteria: selective control of metal availability. FEMS Microbiol. Rev. **27:**165–181.

105. **Cayley, S., B. A. Lewis, and M. T. Record, Jr.** 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J. Bacteriol. **174:**1586–1595.

106. **Cayol, J.-L., M.-L. Fardeau, J.-L. Garcia, and B. Ollivier.** 2002. Evidence of interspecies hydrogen transfer from glycerol in saline environments. Extremophiles **6:**131–134.

107. **Chan, K.-y., and O. C. Leung.** 1979. Nutrition and growth of the moderately halophilic bacteria *Micrococcus morrhuae* K-17 and *Micrococcus luteus* K-15. Microbios **25:**71–84.

108. **Changela, A., K. Chen, Y. Xue, J. Holschen, C. E. Outten, T. V. O'Halloran, and A. Mondragón.** 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science **301:**1383–1387.

109. **Charlou, J. L., J. P. Donval, T. Zitter, N. Roy, P. Jean-Baptiste, J. P. Foucher, J. Woodside, and the MEDINAUT Scientific Party.** 2003. Evidence

of methane venting and geochemistry of brines on mud volcanoes of the eastern Mediterranean Sea. Deep-Sea Res. I **50:**941–958.

110. **Chassard-Bouchaud, C., A. Fiala-Medioni, and P. Galle.** 1986. Étude microanalytique de *Bathymodiolus* sp. (Mollusque Lamellibranche Mytilidae) provenant des sources hydrothermales de la Ride du Pacifique oriental. Données préliminaires. C. R. Acad. Sci., Sér. III, Sci. Vie **302:**117–124.

111. **Chattopadhyay, M. K.** 2002. The cryoprotective effects of glycine betaine on bacteria. Trends Microbiol. **10:**311.

112. **Chen, J.-H., L. W. Lion, W. C. Ghiorse, and M. L. Shuler.** 1995. Mobilization of adsorbed cadmium and lead in aquifer material by bacterial extracellular polymers. Water Res. **29:**421–430.

113. **Chi, E., and D. H. Bartlett.** 1993. Use of a reporter gene to follow high-pressure signal transduction in the deep-sea bacterium *Photobacterium* sp. strain SS9. J. Bacteriol. **175:**7533–7540.

114. **Chi, E., and D. H. Bartlett.** 1995. An *rpoE*-like locus controls outer membrane protein synthesis and growth at cold temperatures and high pressures in the deep-sea bacterium *Photobacterium* sp. strain SS9. Mol. Microbiol. **17:**713–726.

115. **Chilukuri, L. N., D. H. Bartlett, and P. A. G. Fortes.** 2002. Comparison of high pressure-induced dissociation of single-stranded DNA-binding protein (SSB) from high pressure-sensitive and high pressure-adapted marine *Shewanella* species. Extremophiles **6:**377–383.

116. **Ciulla, R. A., S. Burggraf, K. O. Stetter, and M. F. Roberts.** 1994. Occurrence and role of di-*myo*-inositol-1,1'-phosphate in *Methanococcus igneus*. Appl. Environ. Microbiol. **60:**3660–3664.

117. **Cline, J. S., and R. J. Bodnar.** 1991. Can economic porphyry copper mineralization be generated by a typical calc-alkaline melt? J. Geophys. Res. **96:**8113–8126.

118. **Cobet, A. B., C. Wirsen, Jr., and G. E. Jones.** 1970. The effect of nickel on a marine bacterium, *Arthrobacter marinus* sp. nov. J. Gen. Microbiol. **62:**159–169.

119. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.

120. **Cole, T. G.** 1988. The nature and origin of smectite in the Atlantis II Deep, Red Sea. Can. Mineral. **26:**755–763.

121. **Collard, J. M., P. Corbisier, L. Diels, Q. Dong, C. Jeanthon, M. Mergeay, S. Taghavi, D. van der Lelie, A. Wilmotte, and S. Wuertz.** 1994. Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: mechanisms and applications. FEMS Microbiol. Rev. **14:**405–414.

122. **Connon, S. A., and S. J. Giovannoni.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. **68:**3878–3885.

123. **Cooper, M. J., H. Elderfield, and A. Schultz.** 2000. Diffuse hydrothermal fluids from Lucky Strike hydrothermal vent field: evidence for a shallow conductively heated system. J. Geophys. Res. **105:**19,369–19,375.

124. **Corliss, J. B.** 1976. Sea water, seafloor spreading, subduction, and ore deposits. Geol. Assoc. Can., Spec. Pap. **14:**145–146.

125. **Corre, E., A.-L. Reysenbach, and D. Prieur.** 2001. ε-Proteobacterial diversity from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. FEMS Microbiol. Lett. **205:**329–335.

126. **Cosson-Mannevy, M.-A., R. P. Cosson, F. Gaill, and L. Laubier.** 1988. Transfert, accumulation et régulation des éléments minéraux chez les organismes des sources hydrothermales. Oceanol. Acta **SP8:**219–226.

127. **Cowen, J. P., M. A. Bertram, S. G. Wakeham, R. E. Thomson, J. W. Lavelle, E. T. Baker, and R. A. Feely.** 2001. Ascending and descending particle flux from hydrothermal plumes at Endeavour Segment, Juan de Fuca Ridge. Deep-Sea Res. I **48:**1093–1120.

128. **Cox, G. F. N., and W. F. Weeks.** 1983. Equations for determining the gas and brine volumes in sea-ice samples. J. Glaciol. **29:**306–316.

129. **D'Souza, S. E., W. Altekar, and S. F. D'Souza.** 1997. Adaptive response of *Haloferax mediterranei* to low concentrations of NaCl (<20%) in the growth medium. Arch. Microbiol. **168:**68–71.

130. **da Costa, M. S.** 2001. Are there thermophilic and hyperthermophilic halophilic organisms? (abstract). Halophiles 2001 International Conference on Halophilic Microorganisms:L5.

131. **de Lange, G. J., G. Catalano, G. P. Klinkhammer, and G. W. Luther, III.** 1990. The interface between oxic seawater and the anoxic Bannock brine; its

sharpness and the consequences for the redox-related cycling of Mn and Ba. Mar. Chem. **31:**205–217.

132. **de Lange, G. J., J. J. Middelburg, C. H. van der Weijden, G. Catalano, G. W. Luther, III, D. J. Hydes, J. R. W. Woittiez, and G. P. Klinkhammer.** 1990. Composition of anoxic hypersaline brines in the Tyro and Bannock Basins, eastern Mediterranean. Mar. Chem. **31:**63–88.

133. **de Lange, G. J., and H. L. ten Haven.** 1983. Recent sapropel formation in the eastern Mediterranean. Nature **305:**797–798.

134. **De Ley, J., and R. Tijtgat.** 1970. Evaluation of membrane filter methods for DNA-DNA hybridization. Antonie Leeuwenhoek **36:**461–474.

135. **de Souza, M. P., A. Amini, M. A. Dojka, I. J. Pickering, S. C. Dawson, N. R. Pace, and N. Terry.** 2001. Identification and characterization of bacteria in a selenium-contaminated hypersaline evaporation pond. Appl. Environ. Microbiol. **67:**3785–3794.

136. **Dean-Ross, D., and A. L. Mills.** 1989. Bacterial community structure and function along a heavy metal gradient. Appl. Environ. Microbiol. **55:**2002–2009.

137. **Degens, E. T., and D. A. Ross (ed.).** 1969. Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

138. **Delaney, J. R., D. S. Kelley, M. D. Lilley, D. A. Butterfield, R. E. McDuff, J. A. Baross, J. W. Deming, H. P. Johnson, and V. Robigou.** 1997. The Endeavour hydrothermal system I: cellular circulation above an active cracking front yields large sulfide structures, “fresh” vent water, and hyperthermophilic Archaea. RIDGE Events **8:**11–19.

139. **Delaney, J. R., D. S. Kelley, E. A. Mathez, D. R. Yoerger, J. Baross, M. O. Schrenk, M. K. Tivey, J. Kaye, and V. Robigou.** 2001. “Edifice Rex” sulfide recovery project: analysis of submarine hydrothermal, microbial habitat. Eos, Trans. AGU **82:**67, 72–73.

140. **Delaney, J. R., D. W. Mogk, and M. J. Mottl.** 1987. Quartz-cemented breccias from the Mid-Atlantic Ridge: samples of a high-salinity hydrothermal upflow zone. J. Geophys. Res. **92:**9175–9192.

141. **Delaney, J. R., V. Robigou, R. E. McDuff, and M. K. Tivey.** 1992. Geology of a vigorous hydrothermal system on the Endeavour Segment, Juan de Fuca Ridge. J. Geophys. Res. **97:**19,663–19,682.

142. **DeLong, E. F., D. G. Franks, and A. A. Yayanos.** 1997. Evolutionary relationships of cultivated psychrophilic and barophilic deep-sea bacteria. Appl. Environ. Microbiol. **63:**2105–2108.

143. **DeLong, E. F., and N. R. Pace.** 2001. Environmental diversity of Bacteria and Archaea. Syst. Biol. **50:**470–478.

144. **DeLong, E. F., and A. A. Yayanos.** 1985. Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. Science **228:**1101–1103.

145. **DeLong, E. F., and A. A. Yayanos.** 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. Appl. Environ. Microbiol. **51:**730–737.

146. **Deming, J. W.** 1986. Ecological strategies of barophilic bacteria in the deep ocean. Microbiol. Sci. **3:**205–211.

147. **Deming, J. W.** 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. **5:**301–309.

148. **Deming, J. W., and J. A. Baross.** 1993. Deep-sea smokers: window to a subsurface biosphere? Geochim. Cosmochim. Acta **57:**3219–3230.

149. **Deming, J. W., and J. A. Baross.** 2000. Survival, dormancy, and nonculturable cells in extreme deep-sea environments, p. 147–197. *In* R. R. Colwell and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. American Society for Microbiology, Washington, D.C.

150. **Deming, J. W., and R. R. Colwell.** 1985. Observations of barophilic microbial activity in samples of sediment and intercepted particulates from the Demerara abyssal plain. Appl. Environ. Microbiol. **50:**1002–1006.

151. **Deming, J. W., L. K. Somers, W. L. Straube, D. G. Swartz, and M. T. MacDonell.** 1988. Isolation of an obligately barophilic bacterium and description of a new genus, *Colwellia* gen. nov. Syst. Appl. Microbiol. **10:**152–160.

152. **Dennis, P. P., and L. C. Shimmin.** 1997. Evolutionary divergence and salinity-mediated selection in halophilic archaea. Microbiol. Mol. Biol. Rev. **61:**90–104.

153. **Díaz-Raviña, M., and E. Bååth.** 1996. Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. Appl. Environ. Microbiol. **62:**2970–2977.

154. **Dick, G., and B. M. Tebo.** 2002. Personal communication.

155. **Ding, K., and W. E. Seyfried, Jr.** 1992. Determination of Fe-Cl complexing in the low pressure supercritical region (NaCl fluid): iron solubility constraints on pH of subseafloor hydrothermal fluids. Geochim. Cosmochim. Acta **56:**3681–3692.

156. **Dobbs, F. C., and K. A. Selph.** 1997. Thermophilic bacterial activity in a deep-sea sediment from the Pacific Ocean. Aquat. Microb. Ecol. **13:**209–212.

157. **Dobson, S. J., S. R. James, P. D. Franzmann, and T. A. McMeekin.** 1990. Emended description of *Halomonas halmophila* (NCMB 1971^T). Int. J. Syst. Bacteriol. **40:**462–463.

158. **Dobson, S. J., T. A. McMeekin, and P. D. Franzmann.** 1993. Phylogenetic relationships between some members of the genera *Deleya*, *Halomonas*, and *Halovibrio*. Int. J. Syst. Bacteriol. **43:**665–673.

159. **Duché, O., F. Trémoulet, A. Namane, The European *Listeria* Genome Consortium, and J. Labadie.** 2002. A proteomic analysis of the salt stress response of *Listeria monocytogenes*. FEMS Microbiol. Lett. **215:**183–188.

160. **Duckworth, A. W., W. D. Grant, B. E. Jones, D. Meijer, M. C. Márquez, and A. Ventosa.** 2000. *Halomonas magadii* sp. nov., a new member of the genus *Halomonas*, isolated from a soda lake of the East African Rift Valley. Extremophiles **4:**53–60.

161. **Duckworth, A. W., W. D. Grant, B. E. Jones, and R. van Steenbergen.** 1996. Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol. Ecol. **19:**181–191.

162. **Duquesne, K., S. Lebrun, C. Casiot, O. Bruneel, J.-C. Personné, M. Leblanc, F. Elbaz-Poulichet, G. Morin, and V. Bonnefoy.** 2003. Immobilization of arsenite and ferric iron by *Acidithiobacillus ferrooxidans* and its relevance to acid mine drainage. Appl. Environ. Microbiol. **69:**6165–6173.

163. **Durand, P., A.-L. Reysenbach, D. Prieur, and N. Pace.** 1993. Isolation and characterization of *Thiobacillus hydrothermalis* sp. nov., a mesophilic obligately chemolithotrophic bacterium isolated from a deep-sea hydrothermal vent in Fiji Basin. Arch. Microbiol. **159:**39–44.

164. **Eardly, D. F., M. W. Carton, J. M. Gallagher, and J. W. Patching.** 2001. Bacterial abundance and activity in deep-sea sediments from the eastern North Atlantic. Prog. Oceanogr. **50:**245–259.

165. **Eder, W., L. L. Jahnke, M. Schmidt, and R. Huber.** 2001. Microbial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea, studied via 16S

rRNA gene sequences and cultivation methods. Appl. Environ. Microbiol. **67:**3077–3085.

166. **Eder, W., W. Ludwig, and R. Huber.** 1999. Novel 16S rRNA gene sequences retrieved from highly saline brine sediments of Kebrit Deep, Red Sea. Arch. Microbiol. **172:**213–218.

167. **Edwards, K. J., T. M. McCollom, H. Konishi, and P. R. Buseck.** 2003. Seafloor bioalteration of sulfide minerals: results from in situ incubation studies. Geochim. Cosmochim. Acta **67:**2843–2856.

168. **Edwards, K. J., D. R. Rogers, C. O. Wirsen, and T. M. McCollom.** 2003. Isolation and characterization of novel psychrophilic, neutrophilic, Fe-oxidizing, chemolithoautotrophic α- and γ-*Proteobacteria* from the deep sea. Appl. Environ. Microbiol. **69:**2906–2913.

169. **Ehrlich, H. L.** 2002. Geomicrobiology, 4th ed. Marcel Dekker, New York.

170. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.

171. **Ellis, R. J., P. Morgan, A. J. Weightman, and J. C. Fry.** 2003. Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl. Environ. Microbiol. **69:**3223–3230.

172. **Emerson, D., and C. Moyer.** 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Appl. Environ. Microbiol. **63:**4784–4792.

173. **Emerson, D., and C. L. Moyer.** 2002. Neutrophilic Fe-oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. Appl. Environ. Microbiol. **68:**3085–3093.

174. **Ermolenko, D. N., and G. I. Makhatadze.** 2002. Bacterial cold-shock proteins. Cell. Mol. Life Sci. **59:**1902–1913.

175. **Fahey, R. C., W. C. Brown, W. B. Adams, and M. B. Worsham.** 1978. Occurrence of glutathione in bacteria. J. Bacteriol. **133:**1126–1129.

176. **Fang, J., M. J. Barcelona, Y. Nogi, and C. Kato.** 2000. Biochemical implications and geochemical significance of novel phospholipids of the extremely barophilic bacteria from the Marianas Trench at 11,000 m. Deep-Sea Res. I **47:**1173–1182.

177. **Fenchel, T.** 2003. Biogeography for bacteria. Science **301:**925–926.

178. **Fendrich, C.** 1988. *Halovibrio variabilis* gen. nov. sp. nov., *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid Eubacterium from Great Salt Lake, Utah, USA. Syst. Appl. Microbiol. **11:**36–43.

179. **Fendrich, C., and B. Schink.** 1988. Degradation of glucose, glycerol and acetate by aerobic bacteria in surface water of Great Salt Lake, Utah, USA. Syst. Appl. Microbiol. **11:**94–96.

180. **Ferguson, G. P.** 1999. Protective mechanisms against toxic electrophiles in *Escherichia coli*. Trends Microbiol. **7:**242–247.

181. **Fernandez-Castillo, R., C. Vargas, J. J. Nieto, A. Ventosa, and F. Ruíz-Berraquero.** 1992. Characterization of a plasmid from moderately halophilic eubacteria. J. Gen. Microbiol. **138:**1133–1137.

182. **Fiala, G., C. R. Woese, T. A. Langworthy, and K. O. Stetter.** 1990. *Flexistipes sinusarabici*, a novel genus and species of eubacteria occurring in the Atlantis II Deep brines of the Red Sea. Arch. Microbiol. **154:**120–126.

183. **Finney, L. A., and T. V. O'Halloran.** 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science **300:**931–936.

184. **Fish, S. A., T. J. Shepherd, T. J. McGenity, and W. D. Grant.** 2002. Recovery of 16S ribosomal RNA gene fragments from ancient halite. Nature **417:**432–436.

185. **Flahaut, S., A. Benachour, J.-C. Giard, P. Boutibonnes, and Y. Auffray.** 1996. Defense against lethal treatments and de novo protein synthesis induced by NaCl in *Enterococcus faecalis* ATCC 19433. Arch. Microbiol. **165:**317–324.

186. **Forsyth, M. P., D. B. Shindler, M. B. Gochnauer, and D. J. Kushner.** 1971. Salt tolerance of intertidal marine bacteria. Can. J. Microbiol. **17:**825–828.

187. **Foster, P. L.** 1999. Mechanisms of stationary phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. **33:**57–88.

188. **Fournier, R. O.** 1987. Conceptual models of brine evolution in magmatic-hydrothermal systems, p. 1487–1506. *In* R. W. Decker, T. L. Wright, and P. H. Stauffer (ed.), U.S. Geological Survey professional paper 1350, Volcanism in Hawaii. United States Government Printing Office, Washington, D.C.

189. **Fox, C. G.** 1990. Consequences of phase separation on the distribution of hydrothermal fluids at ASHES vent field, Axial Volcano, Juan de Fuca Ridge. J. Geophys. Res. **95:**12,923–12,926.

190. **Francis, A. J., C. J. Dodge, J. B. Gillow, and H. W. Papenguth.** 2000. Biotransformation of uranium compounds in high ionic strength brine by a

halophilic bacterium under denitrifying conditions. Environ. Sci. Technol. **34:**2311–2317.

191. **Franke, S., G. Grass, C. Rensing, and D. H. Nies.** 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. **185:**3804–3812.

192. **Franzmann, P. D., H. R. Burton, and T. A. McMeekin.** 1987. *Halomonas subglaciescola*, a new species of halotolerant bacteria isolated from Antarctica. Int. J. Syst. Bacteriol. **37:**27–34.

193. **Franzmann, P. D., P. P. Deprez, H. R. Burton, and J. van den Hoff.** 1987. Limnology of Organic Lake, Antarctica, a meromictic lake that contains high concentrations of dimethyl sulfide. Aust. J. Mar. Freshwater Res. **38:**409–417.

194. **Franzmann, P. D., and B. J. Tindall.** 1990. A chemotaxonomic study of members of the family *Halomonadaceae*. Syst. Appl. Microbiol. **13:**142–147.

195. **Fritz, I.** 2000. Das Bakterioplankton im Westlichen Mittelmeer. Analyse der taxonomischen Struktur freilebender und partikelgebundener bakterieller Lebensgemeinschaften mit mikrobiologischen und molekularbiologischen Methoden. Ph.D. thesis. Technische Universität Carolo-Wilhelmina Braunschweig, Braunschweig, Germany.

196. **Frostegård, Å., A. Tunlid, and E. Bååth.** 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl. Environ. Microbiol. **59:**3605–3617.

197. **Fuhrman, J. A., and A. A. Davis.** 1997. Widespread Archaea and novel Bacteria from the deep sea as shown by 16S rRNA gene sequences. Mar. Ecol. Prog. Ser. **150:**275–285.

198. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. **59:**1294–1302.

199. **Fujii, S., K. Nakasone, and K. Horikoshi.** 2000. Cloning of the *rpoD* gene encoding the principal sigma factor of deep-sea barophilic *Shewanella violacea* strain DSS12. JAMSTEC J. Deep Sea Res. **40:**21–23.

200. **Fusi, N., G. Aloisi de Larderel, A. Borello, O. Amelio, D. Castradori, A. Negri, B. Rimoldi, R. Sanvoisin, P. Tarbini, and M. B. Cita.** 1996. Marine geology of the Medriff corridor, Mediterranean Ridge. The Island Arc **5:**420–439.

201. **Gadd, G. M.** 1992. Molecular biology and biotechnology of microbial interactions with organic and inorganic heavy metal compounds, p. 225–257. *In* R. A. Herbert and R. J. Sharp (ed.), Molecular biology and biotechnology of extremophiles. Chapman and Hall, New York.

202. **Gaill, F., S. Halpern, C. Quintana, and D. Desbruyères.** 1984. Présence intracellulaire d'arsenic et de zinc associés au soufre chez une Polychète des sources hydrothermales (*Alvinella pompejana*). C. R. Acad. Sci., Sér. III, Sci. Vie **298:**331–336.

203. **Galinski, E. A.** 1995. Osmoadaptation in bacteria. Adv. Microb. Physiol. **37:**273–328.

204. **Galinski, E. A., H.-P. Pfeiffer, and H. G. Trüper.** 1985. 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. Eur. J. Biochem. **149:**135–139.

205. **García, M. T., J. J. Nieto, A. Ventosa, and F. Ruíz-Berraquero.** 1987. The susceptibility of the moderate halophile *Vibrio costicola* to heavy metals. J. Appl. Bacteriol. **63:**63–66.

206. **García, M. T., A. Ventosa, F. Ruíz-Berraquero, and M. Kocur.** 1987. Taxonomic study and amended description of *Vibrio costicola*. Int. J. Syst. Bacteriol. **37:**251–256.

207. **Gardan, R., P. Cossart, The European *Listeria* Genome Consortium, and J. Labadie.** 2003. Identification of *Listeria monocytogenes* genes involved in salt and alkaline-pH tolerance. Appl. Environ. Microbiol. **69:**3137–3143.

208. **Gauthier, M. J., R. L. Clément, G. N. Flatau, and J.-C. Amiard.** 1986. Accumulation du cadmium par les bactéries marines à Gram négatif selon leur sensibilité au métal et leur type respiratoire. Oceanol. Acta **9:**333–337.

209. **Gauthier, M. J., B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J.-C. Bertrand.** 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int. J. Syst. Bacteriol. **42:**568–576.

210. **Ghiorse, W. C., and H. L. Ehrlich.** 1992. Microbial biomineralization of iron and manganese, p. 75–99. *In* H. C. W. Skinner and R. W. Fitzpatrick (ed.), Biomineralization: processes of iron and manganese — modern and ancient environments. CATENA, Cremlingen-Destedt, Germany.

211. **Giard, J.-C., A. Hartke, S. Flahaut, A. Benachour, P. Boutibonnes, and Y. Auffray.** 1996. Starvation-induced multiresistance in *Enterococcus faecalis* JH2-2. Curr. Microbiol. **32:**264–271.

212. **Gibbons, N. E., and J. I. Payne.** 1961. Relation of temperature and sodium chloride concentration to growth and morphology of some halophilic bacteria. Can. J. Microbiol. **7:**483–489.

213. **Gillett, M. B., J. R. Suko, F. O. Santoso, and P. H. Yancey.** 1997. Elevated levels of trimethylamine oxide in muscles of deep-sea gadiform teleosts: a high-pressure adaptation? J. Exp. Zool. **279:**386–391.

214. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345:**60–63.

215. **Giovannoni, S. J., M. S. Rappé, D. Gordon, E. Urbach, M. Suzuki, and K. G. Field.** 1996. Ribosomal RNA and the evolution of bacterial diversity, p. 63–85. *In* D. M. Roberts, P. Sharp, G. Alderson, and M. Collins (ed.), Evolution of microbial life. Cambridge University Press, New York.

216. **Gittings, S. R., T. J. Bright, and E. N. Powell.** 1984. Hard-bottom macrofauna of the East Flower Garden brine seep: impact of a long-term, sulfurous brine discharge. Contrib. Mar. Sci. **27:**105–125.

217. **Gobeil, C., N. Silverberg, B. Sundby, and D. Cossa.** 1987. Cadmium diagenesis in Laurentian Trough sediments. Geochim. Cosmochim. Acta **51:**589–596.

218. **Goldfarb, M. S., and J. R. Delaney.** 1988. Response of two-phase fluids to fracture configurations within submarine hydrothermal systems. J. Geophys. Res. **93:**4585–4594.

219. **Goldman, M., R. H. Deibel, and C. F. Niven, Jr.** 1963. Interrelationship between temperature and sodium chloride on growth of lactic acid bacteria isolated from meat-curing brines. J. Bacteriol. **85:**1017–1021.

220. **Göller, K., and E. A. Galinski.** 1999. Protection of a model enzyme (lactate dehydrogenase) against heat, urea and freeze-thaw treatment by compatible solute additives. J. Mol. Catal., B Enzym. **7:**37–45.

221. **Gonçalves, L. G., R. Huber, M. S. da Costa, and H. Santos.** 2003. A variant of the hyperthermophile *Archaeoglobus fulgidus* adapted to grow at high salinity. FEMS Microbiol. Lett. **218:**239–244.

222. **González-Muñoz, M. T., B. Fernández-Luque, F. Martínez-Ruiz, K. Ben Chekroun, J. M. Arias, M. Rodríguez-Gallego, M. Martínez-Cañamero, C.**

de Linares, and A. Paytan. 2003. Precipitation of barite by *Myxococcus xanthus*: possible implications for the biogeochemical cycle of barium. Appl. Environ. Microbiol. **69:**5722–5725.

223. **Gordon, A. S.** 1998. Microbial control of trace metals in the oceans. Am. Soc. Microbiol. News **64:**79–83.

224. **Gould, S. J., and R. C. Lewontin.** 1979. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. Proc. R. Soc. Lond. B **205:**581–598.

225. **Grassia, G. S., K. M. McLean, P. Glénat, J. Bauld, and A. J. Sheehy.** 1996. A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. FEMS Microbiol. Ecol. **21:**47–58.

226. **Graur, D., and T. Pupko.** 2001. The Permian bacterium that isn't. Mol. Biol. Evol. **18:**1143–1146.

227. **Greenberg, A. E., L. S. Clescen, and A. D. Eaton.** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.

228. **Guérin-Faublée, V., L. Rosso, M. Vigneulle, and J.-P. Flandrois.** 1995. The effect of incubation temperature and sodium chloride concentration on the growth kinetics of *Vibrio anguillarum* and *Vibrio anguillarum*-related organisms. J. Appl. Bacteriol. **78:**621–629.

229. **Gupta, A., B. A. Whitton, A. P. Morby, J. W. Huckle, and N. J. Robinson.** 1992. Amplification and rearrangement of a prokaryotic metallothionein locus *smt* in *Synechococcus* PCC 6301 selected for tolerance to cadmium. Proc. R. Soc. Lond. B **248:**273–281.

230. **Halgasova, N., G. Bukovska, J. Ugorcakova, J. Timko, and J. Kormanec.** 2002. The *Brevibacterium flavum* sigma factor SigB has a role in the environmental stress response. FEMS Microbiol. Lett. **216:**77–84.

231. **Hall, B. G.** 1988. Adaptive evolution that requires multiple spontaneous mutations. I. Mutations involving an insertion sequence. Genetics **120:**887–897.

232. **Hall, B. G.** 1990. Spontaneous point mutations that occur more often when advantageous than when neutral. Genetics **126:**5–16.

233. **Hall, B. G.** 1991. Increased rates of advantageous mutations in response to environmental challenges. Am. Soc. Microbiol. News **57:**82–86.

234. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.

235. **Hannington, M. D., I. R. Jonasson, P. M. Herzig, and S. Peterson.** 1995. Physical and chemical processes of seafloor mineralization at mid-ocean ridges, p. 115–157. *In* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union, Washington, D.C.

236. **Hao, M. V., M. Kocur, and K. Komagata.** 1984. *Marinococcus* gen. nov., a new genus for motile cocci with meso-diaminopimelic acid in the cell wall; and *Marinococcus albus* sp. nov. and *Marinococcus halophilus* (Novitsky and Kushner) comb. nov. J. Gen. Appl. Microbiol. **30:**449–459.

237. **Harris, H. H., I. J. Pickering, and G. N. George.** 2003. The chemical form of mercury in fish. Science **301:**1203.

238. **Hartmann, M., J. C. Scholten, and P. Stoffers.** 1998. Hydrographic structure of brine-filled deeps in the Red Sea: correction of Atlantis II Deep temperatures. Mar. Geol. **144:**331–332.

239. **Hartmann, M., J. C. Scholten, P. Stoffers, and F. Wehner.** 1998. Hydrographic structure of brine-filled deeps in the Red Sea — new results from the Shaban, Kebrit, Atlantis II, and Discovery Deep. Mar. Geol. **144:**311–330.

240. **Hasegawa, M., H. Kishino, and T.-a. Yano.** 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:**160–174.

241. **Hauben, K. J. A., D. H. Bartlett, C. C. F. Soontjens, K. Cornelis, E. Y. Wuytack, and C. W. Michiels.** 1997. *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl. Environ. Microbiol. **63:**945–950.

242. **Hauben, K. J. A., K. Bernaerts, and C. W. Michiels.** 1998. Protective effect of calcium on inactivation of *Escherichia coli* by high hydrostatic pressure. J. Appl. Microbiol. **85:**678–684.

243. **Hazel, J. R., and E. E. Williams.** 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog. Lipid Res. **29:**167–227.

244. **Hebert, A. M., and R. H. Vreeland.** 1987. Phenotypic comparison of halotolerant bacteria: *Halomonas halodurans* sp. nov., nom. rev., comb. nov. Int. J. Syst. Bacteriol. **37:**347–350.

245. **Hecker, M., and U. Völker.** 1998. Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the σ^B regulon. Mol. Microbiol. **29:**1129–1136.

246. **Hecker, M., and U. Völker.** 2001. General stress response of *Bacillus subtilis* and other bacteria. Adv. Microb. Physiol. **44:**35–91.

247. **Hei, D. J., and D. S. Clark.** 1994. Pressure stabilization of proteins from extreme thermophiles. Appl. Environ. Microbiol. **60:**932–939.

248. **Hengge-Aronis, R.** 1996. Back to log phase: σ^S as a global regulator in the osmotic control of gene expression in *Escherichia coli.* Mol. Microbiol. **21:**887–893.

249. **Hensel, R., and H. König.** 1988. Thermoadaptation of methanogenic bacteria by intracellular ion concentration. FEMS Microbiol. Lett. **49:**75–79.

250. **Herzig, P. M., and M. D. Hannington.** 2000. Polymetallic massive sulfides and gold mineralization at mid-ocean ridges and in subduction-related environments, p. 347–368. *In* D. S. Cronan (ed.), Handbook of marine mineral deposits. CRC Press, Boca Raton, Fla.

251. **Heyrman, J., A. Balcaen, P. De Vos, and J. Swings.** 2002. *Halomonas muralis* sp. nov., isolated from microbial biofilms colonizing the walls and murals of the Saint-Catherine chapel (Castle Herberstein, Austria). Int. J. Syst. Evol. Microbiol. **52:**2049–2054.

252. **Hiramatsu, T., Y. Ohno, H. Hara, I. Yano, and M. Masui.** 1980. Effects of NaCl concentration on the envelope components in a moderately halophilic bacterium, *Pseudomonas halosaccharolytica*, p. 189–200. *In* H. Morisita and M. Masui (ed.), Saline Environment: Physiological and Biochemical Adaptation in Halophilic Microorganisms. Nakanishi Printing, Kyoto.

253. **Hoeft, S. E., F. Lucas, J. T. Hollibaugh, and R. S. Oremland.** 2002. Characterization of microbial arsenate reduction in the anoxic bottom waters of Mono Lake, California. Geomicrobiol. J. **19:**23–40.

254. **Holden, J. F., and M. W. W. Adams.** 2003. Microbe-metal interactions in marine hydrothermal environments. Curr. Opin. Chem. Biol. **7:**160–165.

255. **Holden, J. F., and J. A. Baross.** 1993. Enhanced thermotolerance and temperature-induced changes in protein composition in the hyperthermophilic Archaeon ES4. J. Bacteriol. **175:**2839–2843.

256. **Holden, J. F., and J. A. Baross.** 1995. Enhanced thermotolerance by hydrostatic pressure in the deep-sea hyperthermophile *Pyrococcus* strain ES4. FEMS Microbiol. Ecol. **18:**27–34.

257. **Holden, J. F., M. Summit, and J. A. Baross.** 1998. Thermophilic and hyperthermophilic microorganisms in 3–30°C hydrothermal fluids following a deep-sea volcanic eruption. FEMS Microbiol. Ecol. **25:**33–41.

258. **Hornor, S. G., and B. A. Hilt.** 1985. Distribution of zinc-tolerant bacteria in stream sediments. Hydrobiologia **128:**155–160.

259. **Houba, C., and J. Remacle.** 1980. Composition of the saprophytic bacterial communities in freshwater systems contaminated by heavy metals. Microb. Ecol. **6:**55–69.

260. **Huber, J. A., D. A. Butterfield, and J. A. Baross.** 2002. Temporal changes in archaeal diversity and chemistry in a mid-ocean ridge subseafloor habitat. Appl. Environ. Microbiol. **68:**1585–1594.

261. **Huber, J. A., D. A. Butterfield, and J. A. Baross.** 2003. Bacterial diversity in a subseafloor habitat following a deep-sea volcanic eruption. FEMS Microbiol. Ecol. **43:**393–409.

262. **Hugenholtz, P., and T. Huber.** 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int. J. Syst. Evol. Microbiol. **53:**289–293.

263. **Hülsmann, A., T. M. Rosche, I.-S. Kong, H. M. Hassan, D. M. Beam, and J. D. Oliver.** 2003. RpoS-dependent stress response and exoenzyme production in *Vibrio vulnificus*. Appl. Environ. Microbiol. **69:**6114–6120.

264. **Ikegami, A., K. Nakasone, C. Kato, Y. Nakamura, I. Yoshikawa, R. Usami, and K. Horikoshi.** 2000. Glutamine synthetase gene expression at elevated hydrostatic pressure in a deep-sea piezophilic *Shewanella violacea*. FEMS Microbiol. Lett. **192:**91–95.

265. **Inagaki, F., M. Suzuki, K. Takai, and K. Horikoshi.** Unpublished data from the National Center for Biotechnology Information website.

266. **Ingram, M.** 1957. Micro-organisms resisting high concentrations of sugars or salts. Symp. Soc. Gen. Microbiol. **7:**90–133.

267. **Isaksen, M. F., F. Bak, and B. B. Jørgensen.** 1994. Thermophilic sulfate-reducing bacteria in cold marine sediment. FEMS Microbiol. Ecol. **14:**1–8.

268. **Ishibashi, M., T. Arakawa, J. S. Philo, K. Sakashita, Y. Yonezawa, H. Tokunaga, and M. Tokunaga.** 2002. Secondary and quaternary structural

transition of the halophilic archaeon nucleoside diphosphate kinase under high- and low-salt conditions. FEMS Microbiol. Lett. **216:**235–241.

269. **Ishida, Y.** 1970. Growth behavior of halobacteria in relation to concentration of NaCl and temperature of environments. Bull. Japan. Soc. Sci. Fish. **36:**397–401.

270. **Ishida, Y., A. Nakayama, and H. Kadota.** 1974. Temperature-salinity effects upon the growth of marine bacteria, p. 80–91. *In* R. R. Colwell and R. Y. Morita (ed.), Effect of the ocean environment on microbial activities. University Park Press, Baltimore, Md.

271. **Jaenicke, R., G. Bernhardt, H.-D. Lüdemann, and K. O. Stetter.** 1988. Pressure-induced alterations in the protein pattern of the thermophilic archaebacterium *Methanococcus thermolithotrophicus*. Appl. Environ. Microbiol. **54:**2375–2380.

272. **Jambor, J. L., E. S. Grew, and A. C. Roberts.** 1998. New mineral names. Am. Mineral. **83:**185–189.

273. **James, S. R., S. J. Dobson, P. D. Franzmann, and T. A. McMeekin.** 1990. *Halomonas meridiana*, a new species of extremely halotolerant bacteria isolated from Antarctic saline lakes. Syst. Appl. Microbiol. **13:**270–278.

274. **Jannasch, H. W., and M. J. Mottl.** 1985. Geomicrobiology of deep-sea hydrothermal vents. Science **229:**717–725.

275. **Jannasch, H. W., D. C. Nelson, and C. O. Wirsen.** 1989. Massive natural occurrence of unusually large bacteria (*Beggiatoa* sp.) at a hydrothermal deep-sea vent site. Nature **342:**834–836.

276. **Jannasch, H. W., C. O. Wirsen, S. J. Molyneaux, and T. A. Langworthy.** 1992. Comparative physiological studies on hyperthermophilic Archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* strain GB-D. Appl. Environ. Microbiol. **58:**3472–3481.

277. **Jarrell, K. F., G. D. Sprott, and A. T. Matheson.** 1984. Intracellular potassium concentration and relative acidity of the ribosomal proteins of methanogenic bacteria. Can. J. Microbiol. **30:**663–668.

278. **Javor, B.** 1989. Hypersaline environments: microbiology and biogeochemistry. Springer-Verlag, Berlin.

279. **Jeanthon, C., and D. Prieur.** 1990. Resistance to heavy metals of heterotrophic bacteria isolated from the deep-sea hydrothermal vent polychaete, *Alvinella pompejana*. Prog. Oceanogr. **24:**81–88.

280. **Jeanthon, C., and D. Prieur.** 1990. Susceptibility to heavy metals and characterization of heterotrophic bacteria isolated from two hydrothermal vent polychaete annelids, *Alvinella pompejana* and *Alvinella caudata*. Appl. Environ. Microbiol. **56:**3308–3314.

281. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:**2779–2781.

282. **Johnson, H. P., and R. W. Embley.** 1990. Axial Seamount: an active ridge axis volcano on the central Juan de Fuca Ridge. J. Geophys. Res. **95:**12,689–12,696.

283. **Johnson, J. L.** 1994. Similarity analysis of DNAs, p. 655–681. *In* P. Gerhardt (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.

284. **Jones, S. L., P. Drouin, B. J. Wilkinson, and P. D. Morse, II.** 2002. Correlation of long-range membrane order with temperature-dependent growth characteristics of parent and a cold-sensitive, branched-chain-fatty-acid-deficient mutant of *Listeria monocytogenes*. Arch. Microbiol. **177:**217–222.

285. **Jongsma, D., A. R. Fortuin, W. Huson, S. R. Troelstra, G. T. Klaver, J. M. Peters, D. van Harten, G. J. de Lange, and L. ten Haven.** 1983. Discovery of an anoxic basin within the Strabo Trench, eastern Mediterranean. Nature **305:**795–797.

286. **Joux, F., P. Lebaron, and M. Troussellier.** 1997. Changes in cellular states of the marine bacterium *Deleya aquamarina* under starvation conditions. Appl. Environ. Microbiol. **63:**2686–2694.

287. **Juneja, V. K., H. M. Marks, and T. Mohr.** 2003. Predictive thermal inactivation model for effects of temperature, sodium lactate, NaCl, and sodium pyrophosphate on *Salmonella* serotypes in ground beef. Appl. Environ. Microbiol. **69:**5138–5156.

288. **Jung, K., B. Tjaden, and K. Altendorf.** 1997. Purification, reconstitution, and characterization of KdpD, the turgor sensor of *Escherichia coli*. J. Biol. Chem. **272:**10,847–10,852.

289. **Jung, K., M. Veen, and K. Altendorf.** 2000. K^+ and ionic strength directly influence the autophosphorylation activity of the putative turgor sensor KdpD of *Escherichia coli*. J. Biol. Chem. **275:**40,142–40,147.

290. **Juniper, S. K.** 1988. Géochimie et écologie d'un microenvironnement hydrothermal: les sécrétions de mucus de *Paralvinella palmiformis*. Oceanol. Acta **SP8:**167–172.

291. **Juniper, S. K., and P. Martineu.** 1995. Alvinellids and sulfides at hydrothermal vents of the Eastern Pacific: a review. Am. Zool. **35:**174–185.

292. **Kaeberlein, T., K. Lewis, and S. S. Epstein.** 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science **296:**1127–1129.

293. **Kaneko, H., H. Takami, A. Inoue, and K. Horikoshi.** 2000. Effects of hydrostatic pressure and temperature on growth and lipid composition of the inner membrane of barotolerant *Pseudomonas* sp. BT1 isolated from the deep-sea. Biosci. Biotechnol. Biochem. **64:**72–79.

294. **Karamanou, S., and P. Katinakis.** 1988. Heat shock proteins in the moderately halophilic bacterium *Deleya halophila*: protective effect of high salt concentration against thermal shock. Ann. Inst. Pasteur/Microbiol. **139:**505–514.

295. **Karl, D. M.** 1995. Ecology of free-living, hydrothermal vent microbial communities, p. 35–124. *In* D. M. Karl (ed.), The microbiology of deep-sea hydrothermal vents. CRC Press, Boca Raton, Fla.

296. **Karl, D. M.** 2002. Nutrient dynamics in the deep blue sea. Trends Microbiol. **10:**410–418.

297. **Kashefi, K., D. E. Holmes, J. A. Baross, and D. R. Lovley.** 2003. Thermophily in the *Geobacteraceae*: *Geothermobacter ehrlichii* gen. nov., sp. nov., a novel thermophilic member of the *Geobacteraceae* from the "Bag City" hydrothermal vent. Appl. Environ. Microbiol. **69:**2985–2993.

298. **Kashefi, K., and D. R. Lovley.** 2003. Extending the upper temperature limit for life. Science **301:**934.

299. **Kato, C., and D. H. Bartlett.** 1997. The molecular biology of barophilic bacteria. Extremophiles **1:**111–116.

300. **Kato, C., L. Li, J. Tamaoka, and K. Horikoshi.** 1997. Molecular analyses of the sediment of the 11000-m deep Mariana Trench. Extremophiles **1:**117–123.

301. **Kato, C., and Y. Nogi.** 2001. Correlation between phylogenetic structure and function: examples from deep-sea *Shewanella*. FEMS Microbiol. Ecol. **35:**223–230.

302. **Kato, C., and M. H. Qureshi.** 1999. Pressure response in deep-sea piezophilic bacteria. J. Mol. Microbiol. Biotechnol. **1:**87–92.

303. **Kaye, J. Z., and J. A. Baross.** 1998. Salt-tolerant microbes isolated from hydrothermal-vent environments (abstract). Eos, Trans. AGU, Suppl. **79:**59.

304. **Kaye, J. Z., and J. A. Baross.** 2000. High incidence of halotolerant bacteria in Pacific hydrothermal-vent and pelagic environments. FEMS Microbiol. Ecol. **32:**249–260.

305. **Kaye, J. Z., and J. A. Baross.** Unpublished data.

306. **Kaye, J. Z., M. C. Márquez, A. Ventosa, and J. A. Baross.** Unpublished data.

307. **Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann.** 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. J. Bacteriol. **185:**5722–5734.

308. **Kehres, D. G., and M. E. Maguire.** 2003. Emerging themes in manganese transport, biochemistry and pathogenesis in bacteria. FEMS Microbiol. Rev. **27:**263–290.

309. **Kelley, D. S.** 1997. Fluid evolution in slow-spreading environments, p. 399–415. *In* J. A. Karson, M. Cannat, D. J. Miller, and D. Elthon (ed.), Proceedings of the Ocean Drilling Program, scientific results, vol. 153. Ocean Drilling Program, College Station, Tex.

310. **Kelley, D. S., and J. R. Delaney.** 1987. Two-phase separation and fracturing in mid-ocean ridge gabbros at temperatures greater than 700°C. Earth Planet. Sci. Lett. **83:**53–66.

311. **Kelley, D. S., J. R. Delaney, and D. R. Yoerger.** 2001. Geology and venting characteristics of the Mothra hydrothermal field, Endeavour Segment, Juan de Fuca Ridge. Geology **29:**959–962.

312. **Kelley, D. S., K. M. Gillis, and G. Thompson.** 1993. Fluid evolution in submarine magma-hydrothermal systems at the Mid-Atlantic Ridge. J. Geophys. Res. **98:**19,579–19,596.

313. **Kelley, D. S., and P. T. Robinson.** 1990. Development of a brine-dominated hydrothermal system at temperatures of 400–500°C in the upper level plutonic sequence, Troodos ophiolite, Cyprus. Geochim. Cosmochim. Acta **54:**653–661.

314. **Kelley, D. S., P. T. Robinson, and J. G. Malpas.** 1992. Processes of brine generation and circulation in the oceanic crust: fluid inclusion evidence from the Troodos ophiolite, Cyprus. J. Geophys. Res. **97:**9307–9322.

315. **Kelly, R. H., and P. H. Yancey.** 1999. High contents of trimethylamine oxide correlating with depth in deep-sea teleost fishes, skates, and decapod crustaceans. Biol. Bull. **196:**18–25.

316. **Khijniak, T. V., N. N. Medvedeva-Lyalikova, and M. Simonoff.** 2003. Reduction of pertechnetate by haloalkaliphilic strains of *Halomonas*. FEMS Microbiol. Ecol. **44:**109–115.

317. **Kivisaar, M.** 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environ. Microbiol. **5:**814–827.

318. **Knapp, S., R. Ladenstein, and E. A. Galinski.** 1999. Extrinsic protein stabilization by the naturally occurring osmolytes β-hydroxyectoine and betaine. Extremophiles **3:**191–198.

319. **Knight, B. P., S. P. McGrath, and A. M. Chaudri.** 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol. **63:**39–43.

320. **Knight, V. K., I. Nijenhuis, L. J. Kerkhof, and M. M. Häggblom.** 2002. Degradation of aromatic compounds coupled to selenate reduction. Geomicrobiol. J. **19:**77–86.

321. **Konisky, J., P. C. Michels, and D. S. Clark.** 1995. Pressure stabilization is not a general property of thermophilic enzymes: the adenylate kinases of *Methanococcus voltae*, *Methanococcus maripaludis*, *Methanococcus thermolithotrophicus*, and *Methanococcus jannaschii*. Appl. Environ. Microbiol. **61:**2762–2764.

322. **Koschinsky, A., R. Seifert, P. Halbach, M. Bau, S. Brasse, L. M. de Carvalho, and N. M. Fonseca.** 2002. Geochemistry of diffuse low-temperature hydrothermal fluids in the North Fiji Basin. Geochim. Cosmochim. Acta **66:**1409–1427.

323. **Kottmeier, S. T., and C. W. Sullivan.** 1988. Sea ice microbial communities (SIMCO). Polar Biol. **8:**293–304.

324. **Kraegeloh, A., and H. J. Kunte.** 2002. Novel insights into the role of potassium for osmoregulation in *Halomonas elongata*. Extremophiles **6:**453–462.

325. **Krembs, C., H. Eicken, K. Junge, and J. W. Deming.** 2002. High concentrations of exopolymeric substances in Arctic winter sea ice: implications for the polar ocean carbon cycle and cryoprotection of diatoms. Deep-Sea Res. I **49:**2163–2181.

326. **Kurek, E., A. J. Francis, and J.-M. Bollag.** 1991. Immobilization of cadmium by microbial extracellular products. Arch. Environ. Contam. Toxicol. **20:**106–111.

327. **Kushner, D. J.** 1978. Life at high salt and solute concentrations: halophilic bacteria, p. 317–368. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, London.

328. **Labrenz, M., G. K. Druschel, T. Thomsen-Ebert, B. Gilbert, S. A. Welch, K. M. Kemner, G. A. Logan, R. E. Summons, G. De Stasio, P. L. Bond, B. Lai, S. D. Kelly, and J. F. Banfield.** 2000. Formation of sphalerite (ZnS) deposits in natural biofilms of sulfate-reducing bacteria. Science **290:**1744–1747.

329. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.

330. **Lai, M.-C., K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus.** 1991. Distribution of compatible solutes in the halophilic methanogenic Archaebacteria. J. Bacteriol. **173:**5352–5358.

331. **Lamosa, P., L. O. Martins, M. S. da Costa, and H. Santos.** 1998. Effects of temperature, salinity, and medium composition on compatible solute accumulation by *Thermococcus* spp. Appl. Environ. Microbiol. **64:**3591–3598.

332. **Landau, J. V., and D. H. Pope.** 1980. Recent advances in the area of barotolerant protein synthesis in bacteria and implications concerning barotolerant and barophilic growth. Adv. Aquat. Microbiol. **2:**49–76.

333. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

334. **Lanyi, J. K.** 1974. Salt-dependent properties of proteins from extremely halophilic bacteria. Bacteriol. Rev. **38:**272–290.

335. **LaRock, P. A., R. Lauer, T. Bright, and E. Powell.** 1987. Bacterial sulfur formation in an anoxic brine (abstract). Abstr. Gen. Meet. Am. Soc. Microbiol. **87:**254.

336. **LaRock, P. A., R. D. Lauer, J. R. Schwarz, K. K. Watanabe, and D. A. Wiesenburg.** 1979. Microbial biomass and activity distribution in an anoxic, hypersaline basin. Appl. Environ. Microbiol. **37:**466–470.

337. **Le Pichon, X., J. Angelier, J. Aubouin, N. Lyberis, S. Monti, V. Renard, H. Got, K. Hsü, Y. Mart, J. Mascle, D. Matthews, D. Mitropoulos, P. Tsoflias,**

and G. Chronis. 1979. From subduction to transform motion: a Seabeam survey of the Hellenic trench system. Earth Planet. Sci. Lett. **44:**441–450.

338. **Leite, O., and J. Mascle.** 1982. Geological structures on the South Cretan continental margin and Hellenic Trench (eastern Mediterranean). Mar. Geol. **49:**199–223.

339. **Li, Y., J. Hugenholtz, T. Abee, and D. Molenaar.** 2003. Glutathione protects *Lactococcus lactis* against oxidative stress. Appl. Environ. Microbiol. **69:**5739–5745.

340. **Lilley, M. D., R. A. Feely, and J. H. Trefry.** 1995. Chemical and biochemical transformations in hydrothermal plumes, p. 369–391. *In* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union, Washington, D.C.

341. **Lippert, K., and E. A. Galinski.** 1992. Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotechnol. **37:**61–65.

342. **Litchfield, C. D., and P. M. Gillevet.** 2002. Microbial diversity and complexity in hypersaline environments: a preliminary assessment. J. Ind. Microbiol. Biotechnol. **28:**48–55.

343. **Ljunger, C.** 1962. Introductory investigations of ions and thermal resistance. Physiol. Plant. **15:**148–160.

344. **Llamas, I., M. J. Sánchez, M. Argandoña, V. Béjar, E. Quesada, and A. del Moral.** 2002. Analysis of the genome of the moderate halophile *Halomonas eurihalina*. Curr. Microbiol. **45:**233–239.

345. **Llanos, J., C. Capasso, E. Parisi, D. Prieur, and C. Jeanthon.** 2000. Susceptibility to heavy metals and cadmium accumulation in aerobic and anaerobic thermophilic microorganisms isolated from deep-sea hydrothermal vents. Curr. Microbiol. **41:**201–205.

346. **Lloyd, J. R.** 2003. Microbial reduction of metals and radionuclides. FEMS Microbiol. Rev. **27:**411–425.

347. **Lloyd, J. R., J. Chesnes, S. Glasauer, D. J. Bunker, F. R. Livens, and D. R. Lovley.** 2002. Reduction of actinides and fission products by Fe(III)-reducing bacteria. Geomicrobiol. J. **19:**103–120.

348. **López-García, P., F. Gaill, and D. Moreira.** 2002. Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*. Environ. Microbiol. **4**:204–215.

349. **Lovley, D. R. (ed.).** 2000. Environmental microbe-metal interactions. American Society for Microbiology, Washington, D.C.

350. **Lowe, S. E., M. K. Jain, and J. G. Zeikus.** 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. **57:**451–509.

351. **Lowell, R. P., and W. Xu.** 2000. Sub-critical two-phase seawater convection near a dike. Earth Planet. Sci. Lett. **174:**385–396.

352. **Macario, A. J. L., M. Lange, B. K. Ahring, and E. C. de Macario.** 1999. Stress genes and proteins in the Archaea. Microbiol. Mol. Biol. Rev. **63:**923–967.

353. **Macaskie, L. E., and A. C. R. Dean.** 1984. Cadmium accumulation by a *Citrobacter* sp. J. Gen. Microbiol. **130:**53–62.

354. **MacDonell, M. T., and R. R. Colwell.** 1985. Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. Syst. Appl. Microbiol. **6:**171–182.

355. **Madden, T. L., R. L. Tatusov, and J. Zhang.** 1996. Applications of network BLAST server. Methods Enzymol. **266:**131–141.

356. **Manheim, F. T.** 1974. Red Sea geochemistry, p. 975–998. *In* R. B. Whitmarsh, O. E. Weser, D. A. Ross, *et al.* (ed.), Initial reports of the Deep Sea Drilling Project, vol. 23. U. S. Government Printing Office, Washington, D.C.

357. **Marceau, A., M. Zagorec, and M.-C. Champomier-Vergès.** 2003. Positive effects of growth at suboptimal temperature and high salt concentration on long-term survival of *Lactobacillus sakei*. Res. Microbiol. **154:**37–42.

358. **Marguet, E., and P. Forterre.** 1998. Protection of DNA by salts against thermodegradation at temperatures typical for hyperthermophiles. Extremophiles **2:**115–122.

359. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218.

360. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5:**109–118.

361. **Marquis, R. E., and C. E. ZoBell.** 1971. Magnesium and calcium ions enhance barotolerance of streptococci. Arch. Mikrobiol. **79:**80–92.

362. **Marteinsson, V. T., J.-L. Birrien, C. Jeanthon, and D. Prieur.** 1996. Numerical taxonomic study of thermophilic *Bacillus* isolated from three geographically separated deep-sea hydrothermal vents. FEMS Microbiol. Ecol. **21:**255–266.

363. **Marteinsson, V. T., P. Moulin, J.-L. Birrien, A. Gambacorta, M. Vernet, and D. Prieur.** 1997. Physiological responses to stress conditions and barophilic behavior of the hyperthermophilic vent archaeon *Pyrococcus abyssi*. Appl. Environ. Microbiol. **63:**1230–1236.

364. **Marteinsson, V. T., A.-L. Reysenbach, J.-L. Birrien, and D. Prieur.** 1999. A stress protein is induced in the deep-sea barophilic hyperthermophile *Thermococcus barophilus* when grown under atmospheric pressure. Extremophiles **3:**277–282.

365. **Martin, D. D., D. H. Bartlett, and M. F. Roberts.** 2002. Solute accumulation in the deep-sea bacterium *Photobacterium profundum*. Extremophiles **6:**507–514.

366. **Martin, D. D., R. A. Ciulla, and M. F. Roberts.** 1999. Osmoadaptation in Archaea. Appl. Environ. Microbiol. **65:**1815–1825.

367. **Martín, S., M. C. Márquez, C. Sánchez-Porro, E. Mellado, D. R. Arahal, and A. Ventosa.** 2003. *Marinobacter lipolyticus* sp. nov., a novel moderate halophile with lipolytic activity. Int. J. Syst. Evol. Microbiol. **53:**1383–1387.

368. **Martins, L. O., L. S. Carreto, M. S. da Costa, and H. Santos.** 1996. New compatible solutes related to di-*myo*-inositol phosphate in members of the order *Thermotogales*. J. Bacteriol. **178:**5644–5651.

369. **Martins, L. O., R. Huber, H. Huber, K. O. Stetter, M. S. da Costa, and H. Santos.** 1997. Organic solutes in hyperthermophilic *Archaea*. Appl. Environ. Microbiol. **63:**896–902.

370. **Martins, L. O., and H. Santos.** 1995. Accumulation of mannosylglycerate and di-*myo*-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl. Environ. Microbiol. **61:**3299–3303.

371. **Massoth, G. J., D. A. Butterfield, J. E. Lupton, R. E. McDuff, M. D. Lilley, and I. R. Jonasson.** 1989. Submarine venting of phase-separated hydrothermal fluids at Axial Volcano, Juan de Fuca Ridge. Nature **340:**702–705.

372. **Mata, J. A., J. Martínez-Cánovas, E. Quesada, and V. Béjar.** 2002. A detailed phenotypic characterisation of the type strains of *Halomonas* species. Syst. Appl. Microbiol. **25:**360–375.

373. **McEwan, A. G., J. P. Ridge, C. A. McDevitt, and P. Hugenholtz.** 2002. The DMSO reductase family of microbial molybdenum enzymes; molecular properties and role in the dissimilatory reduction of toxic elements. Geomicrobiol. J. **19:**3–21.

374. **McGrath, S. P., B. Knight, K. Killham, S. Preston, and G. I. Paton.** 1999. Assessment of the toxicity of metals in soils amended with sewage sludge using a chemical speciation technique and a *lux*-based biosensor. Environ. Toxicol. Chem. **18:**659–663.

375. **McMeekin, T. A., and P. D. Franzmann.** 1988. Effect of temperature on the growth rates of halotolerant and halophilic bacteria isolated from Antarctic saline lakes. Polar Biol. **8:**281–285.

376. **McMeekin, T. A., P. D. Nichols, D. S. Nichols, A. Juhasz, and P. D. Franzmann.** 1993. Biology and biotechnological potential of halotolerant bacteria from Antarctic saline lakes. Experientia **49:**1042–1046.

377. **MEDRIFF Consortium.** 1995. Three brine lakes discovered in the seafloor of the eastern Mediterranean. Eos, Trans. AGU **76:**313, 318.

378. **Mehta, M. P., D. A. Butterfield, and J. A. Baross.** 2003. Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. Appl. Environ. Microbiol. **69:**960–970.

379. **Mellado, E., M. T. García, E. Roldán, J. J. Nieto, and A. Ventosa.** 1998. Analysis of the genome of the gram-negative moderate halophiles *Halomonas* and *Chromohalobacter* by using pulsed-field gel electrophoresis. Extremophiles **2:**435–438.

380. **Mellado, E., E. R. B. Moore, J. J. Nieto, and A. Ventosa.** 1995. Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui*, *Volcaniella eurihalina*, and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina* comb. nov. Int. J. Syst. Bacteriol. **45:**712–716.

381. **Mellado, E., E. R. B. Moore, J. J. Nieto, and A. Ventosa.** 1996. Analysis of 16S rRNA gene sequences of *Vibrio costicola* strains: description of *Salinivibrio costicola* gen. nov., comb. nov. Int. J. Syst. Bacteriol. **46:**817–821.

382. **Menaia, J. A. G. F., J. C. Duarte, and D. R. Boone.** 1993. Osmotic adaptation of moderately halophilic methanogenic Archaeobacteria, and detection of cytosolic *N,N*-dimethylglycine. Experientia **49:**1047–1054.

383. **Mendum, M. L., and L. T. Smith.** 2002. Characterization of glycine betaine porter I from *Listeria monocytogenes* and its roles in salt and chill tolerance. Appl. Environ. Microbiol. **68:**813–819.

384. **Mergaert, J., A. Verhelst, M. C. Cnockaert, T.-L. Tan, and J. Swings.** 2001. Characterization of facultative oligotrophic bacteria from polar seas by analysis of their fatty acids and 16S rDNA sequences. Syst. Appl. Microbiol. **24:**98–107.

385. **Mergeay, M., N. Glansdorff, and C. Jeanthon.** 2002. Nickel-tolerant mesophiles from deep-sea hydrothermal sources of the Eastern Pacific Rise (12°45'N, 103°59'W). Cah. Biol. Mar. **43:**417–419.

386. **Mergeay, M., S. Monchy, T. Vallaeys, V. Auquier, A. Benotmane, P. Bertin, S. Taghavi, J. Dunn, D. van der Lelie, and R. Wattiez.** 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. FEMS Microbiol. Rev. **27:**385–410.

387. **Michaelis, W., A. Jenisch, and H. H. Richnow.** 1990. Hydrothermal petroleum generation in Red Sea sediments from the Kebrit and Shaban Deeps. Appl. Geochem. **5:**103–114.

388. **Michels, P. C., and D. S. Clark.** 1997. Pressure-enhanced activity and stability of a hyperthermophilic protease from a deep-sea methanogen. Appl. Environ. Microbiol. **63:**3985–3991.

389. **Miller, A. R., C. D. Densmore, E. T. Degens, J. C. Hathaway, F. T. Manheim, P. F. McFarlin, R. Pocklington, and A. Jokela.** 1966. Hot brines and recent iron deposits in deeps of the Red Sea. Geochim. Cosmochim. Acta **30:**341–359.

390. **Miller, J. F., N. N. Shah, C. M. Nelson, J. M. Ludlow, and D. S. Clark.** 1988. Pressure and temperature effects on growth and methane production of the extreme thermophile *Methanococcus jannaschii*. Appl. Environ. Microbiol. **54:**3039–3042.

391. **Mills, A. L., and R. R. Colwell.** 1977. Microbiological effects of metal ions in Chesapeake Bay water and sediments. Bull. Environ. Contam. Toxicol. **18:**99–103.

392. **Minor, E. C., S. G. Wakeham, and C. Lee.** 2003. Changes in the molecular-level characteristics of sinking marine particles with water column depth. Geochim. Cosmochim. Acta **67:**4277–4288.

393. **Miroshnichenko, M. L., N. A. Kostrikina, N. A. Chernyh, N. V. Pimenov, T. P. Tourova, A. N. Antipov, S. Spring, E. Stackebrandt, and E. A. Bonch-Osmolovskaya.** 2003. *Caldithrix abyssi* gen. nov., sp. nov., a nitrate-reducing, thermophilic, anaerobic bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent, represents a novel bacterial lineage. Int. J. Syst. Evol. Microbiol. **53:**323–329.

394. **Miroshnichenko, M. L., N. A. Kostrikina, S. L'Haridon, C. Jeanthon, H. Hippe, E. Stackebrandt, and E. A. Bonch-Osmolovskaya.** 2002. *Nautilia lithotrophica* gen. nov., sp. nov., a thermophilic sulfur-reducing ε-proteobacterium isolated from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. **52:**1299–1304.

395. **Mojica, F. J. M., E. Cisneros, C. Ferrer, F. Rodríguez-Valera, and G. Juez.** 1997. Osmotically induced response in representatives of halophilic prokaryotes: the bacterium *Halomonas elongata* and the archaeon *Haloferax volcanii*. J. Bacteriol. **179:**5471–5481.

396. **Monteoliva-Sanchez, M., M. R. Ferrer, A. Ramos-Cormenzana, E. Quesada, and M. Monteoliva.** 1988. Cellular fatty acid composition of *Deleya halophila*: effect of growth temperature and salt concentration. J. Gen. Microbiol. **134:**199–203.

397. **Monteoliva-Sanchez, M., and A. Ramos-Cormenzana.** 1986. Effect of growth temperature and salt concentration on the fatty acid composition of *Flavobacterium halmephilum* CCM2831. FEMS Microbiol. Lett. **33:**51–54.

398. **Monteoliva-Sanchez, M., and A. Ramos-Cormenzana.** 1987. Cellular fatty acid composition in moderately halophilic Gram-negative rods. J. Appl. Bacteriol. **62:**361–366.

399. **Monteoliva-Sanchez, M., and A. Ramos-Cormenzana.** 1987. Cellular fatty acid composition of *Planococcus halophilus* NRCC 14033 as affected by growth temperature and salt concentration. Curr. Microbiol. **15:**133–136.

400. **Monteoliva-Sanchez, M., A. Ramos-Cormenzana, and N. J. Russell.** 1993. The effect of salinity and compatible solutes on the biosynthesis of cyclopropane fatty acids in *Pseudomonas halosaccharolytica*. J. Gen. Microbiol. **139:**1877–1884.

401. **Montgomery, D. M., A. C. R. Dean, P. Wiffen, and L. E. Macaskie.** 1995. Phosphatase production and activity in *Citrobacter freundii* and a naturally occurring, heavy-metal-accumulating *Citrobacter* sp. Microbiol. **141:**2433–2441.

402. **Morel, F. M. M., and N. M. Price.** 2003. The biogeochemical cycles of trace metals in the oceans. Science **300:**944–947.

403. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1–23. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.

404. **Morita, R. Y., L. P. Jones, R. P. Griffiths, and T. E. Staley.** 1973. Salinity and temperature interactions and their relationship to the microbiology of the estuarine environment, p. 221–232. *In* L. H. Stevenson and R. R. Colwell (ed.), Estuarine microbial ecology. University of South Carolina Press, Columbia, S.C.

405. **Mormile, M. R., M. F. Romine, M. T. Garcia, A. Ventosa, T. J. Bailey, and B. M. Peyton.** 1999. *Halomonas campisalis* sp. nov., a denitrifying, moderately haloalkaliphilic bacterium. Syst. Appl. Microbiol. **22:**551–558.

406. **Mottl, M. J., G. Wheat, E. Baker, N. Becker, E. Davis, R. Feely, A. Grehan, D. Kadko, M. Lilley, M. G., C. Moyer, and F. Sansone.** 1998. Warm springs discovered on 3.5 Ma oceanic crust, eastern flank of the Juan de Fuca Ridge. Geology **26:**51–54.

407. **Mouné, S., P. Caumette, R. Matheron, and J. C. Willison.** 2003. Molecular sequence analysis of prokaryotic diversity in the anoxic sediments underlying cyanobacterial mats of two hypersaline ponds in Mediterranean salterns. FEMS Microbiol. Ecol. **44:**117–130.

408. **Moyer, C. L., F. C. Dobbs, and D. M. Karl.** 1995. Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl. Environ. Microbiol. **61:**1555–1562.

409. **Mullins, T. D., T. B. Britschgi, R. L. Krest, and S. J. Giovannoni.** 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol. Oceanogr. **40:**148–158.

410. **Mulrooney, S. B., and R. P. Hausinger.** 2003. Nickel uptake and utilization by microorganisms. FEMS Microbiol. Rev. **27:**239–261.

411. **Naganuma, T., and K. Horikoshi.** 1993. Extremely halotolerant *Halomonas* sp. isolated from a tar ball and its cellular fatty acid composition. J. Mar. Biotechnol. **1:**151–157.

412. **Nakagawa, S., K. Takai, K. Horikoshi, and Y. Sako.** 2003. *Persephonella hydrogeniphila* sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium from a deep-sea hydrothermal vent chimney. Int. J. Syst. Evol. Microbiol. **53:**863–869.

413. **Nakasone, K., A. Ikegami, S. Fujii, C. Kato, and K. Horikoshi.** 2000. Isolation and piezoresponse of the *rpoA* gene encoding the RNA polymerase α subunit from the deep-sea piezophilic bacterium *Shewanella violacea*. FEMS Microbiol. Lett. **193:**261–268.

414. **Nakasone, K., A. Ikegami, C. Kato, R. Usami, and K. Horikoshi.** 1998. Mechanisms of gene expression controlled by pressure in deep-sea microorganisms. Extremophiles **2:**149–154.

415. **Nasdala, L., T. Witzke, B. Ullrich, and R. Brett.** 1998. Gordaite [$Zn_4Na(OH)_6(SO_4)Cl\cdot 6H_2O$]: second occurrence in the Juan de Fuca Ridge, and new data. Am. Mineral. **83:**1111–1116.

416. **Nehlig, P.** 1993. Interactions between magma chambers and hydrothermal systems: oceanic and ophiolitic constraints. J. Geophys. Res. **98:**19,621–19,633.

417. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Sunderland, Mass.

418. **Nelson, D. C., W. H. Casey, J. D. Sison, E. E. Mack, A. Ahmad, and J. S. Pollack.** 1996. Selenium uptake by sulfur-accumulating bacteria. Geochim. Cosmochim. Acta **60:**3531–3539.

419. **Nelson, D. C., C. O. Wirsen, and H. W. Jannasch.** 1989. Characterization of large, autotrophic *Beggiatoa* spp. abundant at hydrothermal vents of the Guaymas Basin. Appl. Environ. Microbiol. **55:**2909–2917.

420. **Newby, D. T., K. L. Josephson, and I. L. Pepper.** 2000. Detection and characterization of plasmid pJP4 transfer to indigenous soil bacteria. Appl. Environ. Microbiol. **66:**290–296.

421. **Newman, D. K., and J. F. Banfield.** 2002. Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. Science **296:**1071–1077.

422. **Nguyen, B. H., E. B. M. Denner, T. C. H. Dang, G. Wanner, and H. Stan-Lotter.** 1999. *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. Int. J. Syst. Bacteriol. **49:**367–375.

423. **Nichols, D. S.** 2003. Prokaryotes and the input of polyunsaturated fatty acids to the marine food web. FEMS Microbiol. Lett. **219:**1–7.

424. **Nichols, D. S., J. Olley, H. Garda, R. R. Brenner, and T. A. McMeekin.** 2000. Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*. Appl. Environ. Microbiol. **66:**2422–2429.

425. **Nickle, D. C., G. H. Learn, M. W. Rain, J. I. Mullins, and J. E. Mittler.** 2002. Curiously modern DNA for a "250 million-year-old" bacterium. J. Mol. Evol. **54:**134–137.

426. **Nies, D. H.** 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. **27:**313–339.

427. **Nieto, J. J.** 1991. The response of halophilic bacteria to heavy metals, p. 173–179. *In* F. Rodríguez-Valera (ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York.

428. **Nieto, J. J., R. Fernández-Castillo, M. C. Márquez, A. Ventosa, E. Quesada, and F. Ruíz-Berraquero.** 1989. Survey of metal tolerance in moderately halophilic eubacteria. Appl. Environ. Microbiol. **55:**2385–2390.

429. **Nogi, Y., C. Kato, and K. Horikoshi.** 1998. *Moritella japonica* sp. nov., a novel barophilic bacterium isolated from a Japan Trench sediment. J. Gen. Appl. Microbiol. **44:**289–295.

430. **Nogi, Y., C. Kato, and K. Horikoshi.** 1998. Taxonomic studies of deep-sea barophilic *Shewanella* strains and description of *Shewanella violacea* sp. nov. Arch. Microbiol. **170:**331–338.

431. **Nogi, Y., N. Masui, and C. Kato.** 1998. *Photobacterium profundum* sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment. Extremophiles **2:**1–7.

432. **Noma, S., and I. Hayakawa.** 2002. Barotolerance of *Staphylococcus aureus* is increased by incubation at below 0°C prior to hydrostatic pressure treatment. Int. J. Food Microbiol. **80:**261–264.

433. **Novitsky, J. A., and R. Y. Morita.** 1978. Starvation-induced barotolerance as a survival mechanism of a psychrophilic marine vibrio in the waters of the Antarctic convergence. Mar. Biol. **49:**7–10.

434. **Novitsky, T. J., and D. J. Kushner.** 1975. Influence of temperature and salt concentration on the growth of a facultatively halophilic "*Micrococcus*" sp. Can. J. Microbiol. **21:**107–110.

435. **Novitsky, T. J., and D. J. Kushner.** 1976. *Planococcus halophilus* sp. nov., a facultatively halophilic coccus. Int. J. Syst. Bacteriol. **26:**53–57.

436. **Nozaki, Y.** 1997. A fresh look at element distribution in the North Pacific Ocean. Eos, Trans. AGU **78:**221.

437. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.

438. **O'Toole, R., and H. D. Williams.** 2003. Universal stress proteins and *Mycobacterium tuberculosis*. Res. Microbiol. **154:**387–392.

439. **Obraztsova, A. Y., C. A. Francis, and B. M. Tebo.** 2002. Sulfur disproportionation by the facultative anaerobe *Pantoea agglomerans* SP1 as a mechanism for chromium(VI) reduction. Geomicrobiol. J. **19:**121–132.

440. **Ochsenreiter, T., F. Pfeifer, and C. Schleper.** 2002. Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. Extremophiles **6:**267–274.

441. **Ohno, Y., I. Yano, and M. Masui.** 1979. Effect of NaCl concentration and temperature on the phospholipid and fatty acid compositions of a moderately halophilic bacterium, *Pseudomonas halosaccharolytica*. J. Biochem. **85:**413–421.

442. **Okamoto, T.** 2003. Personal communication.

443. **Okamoto, T., K. Fujioka, and T. Naganuma.** 2001. Phylogenetic similarity of aerobic gram-negative halophilic bacteria from a deep-sea hydrothermal mound and Antarctic habitats. Polar Biosci. **14:**1–9.

444. **Okamoto, T., and T. Naganuma.** Unpublished data from the National Center for Biotechnology Information website.

445. **Okamoto, T., H. Taguchi, K. Nakamura, H. Ikenaga, H. Kuraishi, and K. Yamasato.** 1993. *Zymobacter palmae* gen. nov., sp. nov., a new ethanol-fermenting peritrichous bacterium isolated from palm sap. Arch. Microbiol. **160:**333–337.

446. **Oliveira, A. C., L. P. Gaspar, A. T. Da Poian, and J. L. Silva.** 1994. Arc repressor will not denature under pressure in the absence of water. J. Mol. Biol. **240:**184–187.

447. **Ollivier, B., P. Caumette, J.-L. Garcia, and R. A. Mah.** 1994. Anaerobic bacteria from hypersaline environments. Microbiol. Rev. **58:**27–38.

448. **Olson, B. H., T. Barkay, and R. R. Colwell.** 1979. Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. Appl. Environ. Microbiol. **38:**478–485.

449. **Oren, A.** 1986. Intracellular salt concentrations of the anaerobic halophilic eubacteria *Haloanaerobium praevalens* and *Halobacteroides halobius*. Can. J. Microbiol. **32:**4–9.

450. **Oren, A.** 1999. Bioenergetic aspects of halophilism. Microbiol. Mol. Biol. Rev. **63:**334–348.

451. **Oren, A.** 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J. Ind. Microbiol. Biotechnol. **28:**56–63.

452. **Oren, A.** 2002. Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiol. Ecol. **39:**1–7.

453. **Oren, A., M. Heldal, and S. Norland.** 1997. X-ray microanalysis of intracellular ions in the anaerobic halophilic eubacterium *Haloanaerobium praevalens*. Can. J. Microbiol. **43:**588–592.

454. **Oren, A., M. Heldal, S. Norland, and E. A. Galinski.** 2002. Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*. Extremophiles **6:**491–498.

455. **Oren, A., and L. Mana.** 2002. Amino acid composition of bulk protein and salt relationships of selected enzymes of *Salinibacter ruber*, an extremely halophilic bacterium. Extremophiles **6:**217–223.

456. **Ortigosa, M., E. Garay, and M.-J. Pujalte.** 1994. Numerical taxonomy of aerobic, Gram-negative bacteria associated with oysters and surrounding seawater of the Mediterranean coast. Syst. Appl. Microbiol. **17:**589–600.

457. **Outten, C. E., and T. V. O'Halloran.** 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science **292:**2488–2492.

458. **Owen, R. J., and L. R. Hill.** 1979. The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids, p. 217–296. *In* D. W. Lovelock (ed.), Identification methods for microbiologists, 2nd ed. Academic Press, London.

459. **Owen, R. J., and D. Pitcher.** 1985. Current methods for estimating DNA base composition and levels of DNA-DNA hybridization, p. 67–93. *In* E. Minnikin (ed.), Chemical methods in bacterial systematics. Academic Press, London.

460. **Page, R. D. M.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.

461. **Palmer, D. S., and L. J. Albright.** 1970. Salinity effects on the maximum hydrostatic pressure for growth of the marine psychrophilic bacterium, *Vibrio marinus*. Limnol. Oceanogr. **15:**343–347.

462. **Papke, R. T., N. B. Ramsing, M. M. Bateson, and D. M. Ward.** 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. **5:**650–659.

463. **Pascoe, A. R., and J. R. Cann.** 1995. Modelling diffuse hydrothermal flow in black smoker vent fields, p. 159–173. *In* L. M. Parson, C. L. Walker, and D. R. Dixon (ed.), Hydrothermal vents and processes. Geological Society, London.

464. **Patching, J. W., and D. Eardly.** 1997. Bacterial biomass and activity in the deep waters of the eastern Atlantic — evidence of a barophilic community. Deep-Sea Res. I **44:**1655–1670.

465. **Pautot, G., P. Guennoc, A. Coutelle, and N. Lyberis.** 1984. Discovery of a large brine deep in the northern Red Sea. Nature **310:**133–136.

466. **Pichereau, V., A. Hartke, and Y. Auffray.** 2000. Starvation and osmotic stress induced multiresistances: influence of extracellular compounds. Int. J. Food Microbiol. **55:**19–25.

467. **Pledger, R. J., and J. A. Baross.** 1991. Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures of up to 110°C isolated from a submarine hydrothermal vent environment. J. Gen. Microbiol. **137:**203–211.

468. **Pledger, R. J., B. C. Crump, and J. A. Baross.** 1994. A barophilic response by two hyperthermophilic, hydrothermal vent *Archaea*: an upward shift in the optimal temperature and acceleration of growth rate at supra-optimal temperatures by elevated pressure. FEMS Microbiol. Ecol. **14:**233–241.

469. **Polz, M. F., and C. M. Cavanaugh.** 1995. Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. Proc. Natl. Acad. Sci. USA **92:**7232–7236.

470. **Polz, M. F., and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:**3724–3730.

471. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting microflora. Limnol. Oceanogr. **25:**943–948.

472. **Preyer, J. M., and J. D. Oliver.** 1993. Starvation-induced thermal tolerance as a survival mechanism in a psychrophilic marine bacterium. Appl. Environ. Microbiol. **59:**2653–2656.

473. **Prieur, D.** 1988. Étude préliminaire de communautés bactériennes hétérotrophes associées à des invertébrés des sources hydrothermales profondes. Oceanol. Acta **SP8:**139–145.

474. **Prieur, D., and C. Jeanthon.** 1987. Preliminary study of heterotrophic bacteria isolated from two deep sea hydrothermal vent invertebrates: *Alvinella*

pompejana (polychaete) and *Bathymodiolus thermophilus* (bivalve). Symbiosis **4:**87–98.

475. **Quesada, E., V. Bejar, M. J. Valderrama, and A. Ramos-Cormenzana.** 1987. Growth characteristics and salt requirements of *Deleya halophila* in a defined medium. Curr. Microbiol. **16:**21–25.

476. **Quesada, E., M. J. Valderrama, V. Béjar, A. Ventosa, M. C. Gutierrez, F. Ruíz-Berraquero, and A. Ramos-Cormenzana.** 1990. *Volcaniella eurihalina* gen. nov., sp. nov., a moderately halophilic nonmotile Gram-negative rod. Int. J. Syst. Bacteriol. **40:**261–267.

477. **Quesada, E., A. Ventosa, F. Ruíz-Berraquero, and A. Ramos-Cormenzana.** 1984. *Deleya halophila*, a new species of moderately halophilic bacteria. Int. J. Syst. Bacteriol. **34:**287–292.

478. **Radax, C., C. Gruber, and H. Stan-Lotter.** 2001. Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt. Extremophiles **5:**221–228.

479. **Ramboz, C., E. Oudin, and Y. Thisse.** 1988. Geyser-type discharge in Atlantis II Deep, Red Sea: evidence of boiling from fluid inclusions in epigenetic anhydrite. Can. Mineral. **26:**765–786.

480. **Ranjard, L., E. Brothier, and S. Nazaret.** 2000. Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. Appl. Environ. Microbiol. **66:**5334–5339.

481. **Ranjard, L., S. Nazaret, F. Gourbière, J. Thioulouse, P. Linet, and A. Richaume.** 2000. A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints. FEMS Microbiol. Ecol. **31:**107–115.

482. **Rappé, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni.** 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418:**630–633.

483. **Rappé, M. S., P. F. Kemp, and S. J. Giovannoni.** 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. Limnol. Oceanogr. **42:**811–826.

484. **Rathgeber, C., N. Yurkova, E. Stackebrandt, J. T. Beatty, and V. Yurkov.** 2002. Isolation of tellurite- and selenite-resistant bacteria from hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean. Appl. Environ. Microbiol. **68:**4613–4622.

485. **Reddy, G. S. N., P. U. M. Raghavan, N. B. Sarita, J. S. S. Prakash, N. Nagesh, D. Delille, and S. Shivaji.** 2003. *Halomonas glaciei* sp. nov. isolated from fast ice of Adelie Land, Antarctica. Extremophiles **7:**55–61.

486. **Rengpipat, S., S. E. Lowe, and J. G. Zeikus.** 1988. Effect of extreme salt concentrations on the physiology and biochemistry of *Halobacteroides acetoethylicus*. J. Bacteriol. **170:**3065–3071.

487. **Rensing, C., and G. Grass.** 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. **27:**197–213.

488. **Reynolds, T. J., and R. E. Beane.** 1985. Evolution of hydrothermal fluid characteristics at the Santa Rita, New Mexico, porphyry copper deposit. Econ. Geol. **80:**1328–1347.

489. **Reysenbach, A.-L., and J. W. Deming.** 1991. Effects of hydrostatic pressure on growth of hyperthermophilic archaebacteria from the Juan de Fuca Ridge. Appl. Environ. Microbiol. **57:**1271–1274.

490. **Reysenbach, A.-L., K. Longnecker, and J. Kirshtein.** 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. Appl. Environ. Microbiol. **66:**3798–3806.

491. **Robb, F. T., and D. S. Clark.** 1999. Adaptation of proteins from hyperthermophiles to high pressure and high temperature. J. Mol. Microbiol. Biotechnol. **1:**101–105.

492. **Robertson, D. E., M.-C. Lai, R. P. Gunsalus, and M. F. Roberts.** 1992. Composition, variation, and dynamics of major osmotic solutes in *Methanohalophilus* strain FDF1. Appl. Environ. Microbiol. **58:**2438–2443.

493. **Robey, M., A. Benito, R. H. Hutson, C. Pascual, S. F. Park, and B. M. Mackey.** 2001. Variation in resistance to high hydrostatic pressure and *rpoS* heterogeneity in natural isolates of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **67:**4901–4907.

494. **Robigou, V., J. R. Delaney, and D. S. Stakes.** 1993. Large massive sulfide deposits in a newly discovered active hydrothermal system, the High-Rise field, Endeavour Segment, Juan de Fuca Ridge. Geophys. Res. Lett. **20:**1887–1890.

495. **Robinson, C. R., and S. G. Sligar.** 1994. Hydrostatic pressure reverses osmotic pressure effects on the specificity of *Eco*RI-DNA interactions. Biochemistry **33:**3787–3793.

496. **Robinson, C. R., and S. G. Sligar.** 1995. Heterogeneity in molecular recognition by restriction endonucleases: osmotic and hydrostatic pressure

effects on *Bam*HI, *Pvu* II, and *Eco*RV specificity. Proc. Natl. Acad. Sci. USA **92:**3444–3448.

497. **Robinson, C. R., and S. G. Sligar.** 1995. Hydrostatic and osmotic pressure as tools to study macromolecular recognition. Methods Enzymol. **259:**395–427.

498. **Robison, B. H., and T. M. Lancraft.** 1984. An upward transport mechanism from the benthos. Naturwissenschaften **71:**322–324.

499. **Rodríguez-Valera, F., F. Ruíz-Berraquero, and A. Ramos-Cormenzana.** 1981. Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentrations. Microb. Ecol. **7:**235–243.

500. **Rodríguez-Valera, F., A. Ventosa, E. Quesada, and F. Ruíz-Berraquero.** 1982. Some physiological features of a *Halococcus* sp. at low salt concentrations. FEMS Microbiol. Lett. **15:**249–252.

501. **Roesijadi, G., and E. A. Crecelius.** 1984. Elemental composition of the hydrothermal vent clam *Calyptogena magnifica* from the East Pacific Rise. Mar. Biol. **83:**155–161.

502. **Roesijadi, G., J. S. Young, E. A. Crecelius, and L. E. Thomas.** 1985. Distribution of trace metals in the hydrothermal vent clam, *Calyptogena magnifica*. Bull. Biol. Soc. Wash. **6:**311–324.

503. **Rogers, D. R., and K. J. Edwards.** 2003. Personal communication.

504. **Romanenko, L. A., P. Schumann, M. Rohde, V. V. Mikhailov, and E. Stackebrandt.** 2002. *Halomonas halocynthiae* sp. nov., isolated from the marine ascidian *Halocynthia aurantium*. Int. J. Syst. Evol. Microbiol. **52:**1767–1772.

505. **Ron, E. Z., D. Minz, N. P. Finkelstein, and E. Rosenberg.** 1992. Interactions of bacteria with cadmium. Biodegradation **3:**161–170.

506. **Rona, P. A.** 2003. Resources of the sea floor. Science **299:**673–674.

507. **Rosenbauer, J. L., and R. J. Bischoff.** 1983. Uptake and transport of heavy metals by heated seawater: a summary of the experimental results, p. 177–197. *In* P. A. Rona, K. Boström, L. Laubier, and K. L. Smith, Jr. (ed.), Hydrothermal processes at seafloor spreading centers. Plenum Press, New York.

508. **Rosenberg, A.** 1983. *Pseudomonas halodurans* sp. nov., a halotolerant bacterium. Arch. Microbiol. **136:**117–123.

509. **Rouch, D. A., B. T. O. Lee, and A. P. Morby.** 1995. Understanding cellular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance. J. Ind. Microbiol. **14:**132–141.

510. **Ruelas-Inzunza, J., L. A. Soto, and F. Páez-Osuna.** 2003. Heavy-metal accumulation in the hydrothermal vent clam *Vesicomya gigas* from Guaymas basin, Gulf of California. Deep-Sea Res. I **50:**757–761.

511. **Russell, N. J., and R. L. Adams.** 1991. Effects of salinity on membrane lipids and membrane-derived oligosaccharides, p. 225–231. *In* F. Rodríguez-Valera (ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York.

512. **Ryan, W. B. F., E. M. Thorndike, M. Ewing, and D. A. Ross.** 1969. Suspended matter in the Red Sea brines and its detection by light scattering, p. 153–157. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

513. **Sabry, S. A., H. A. Ghozlan, and D.-M. Abou-Zeid.** 1997. Metal tolerance and antibiotic resistance patters of a bacterial population isolated from sea water. J. Appl. Microbiol. **82:**245–252.

514. **Sako, Y., S. Nakagawa, K. Takai, and K. Horikoshi.** 2003. *Marinithermus hydrothermalis* gen. nov., sp. nov., a strictly aerobic, thermophilic bacterium from a deep-sea hydrothermal vent chimney. Int. J. Syst. Evol. Microbiol. **53:**59–65.

515. **Sako, Y., K. Takai, Y. Ishida, A. Uchida, and Y. Katayama.** 1996. *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. Int. J. Syst. Bacteriol. **46:**1099–1104.

516. **Salmassi, T. M., K. Venkateswaran, M. Satomi, K. H. Nealson, D. K. Newman, and J. G. Hering.** 2002. Oxidation of arsenite by *Agrobacterium albertimagni*, AOL15, sp. nov., isolated from Hot Creek, California. Geomicrobiol. J. **19:**53–66.

517. **Sandaa, R.-A., Ø. Enger, and V. Torsvik.** 1999. Abundance and diversity of *Archaea* in heavy-metal-contaminated soils. Appl. Environ. Microbiol. **65:**3293–3297.

518. **Sandaa, R.-A., V. Torsvik, Ø. Enger, F. L. Daae, T. Castberg, and D. Hahn.** 1999. Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol. Ecol. **30:**237–251.

519. **Santini, J. M., L. I. Sly, A. Wen, D. Comrie, P. de Wulf-Durand, and J. M. Macy.** 2002. New arsenite-oxidizing bacteria isolated from Australian gold mining environments — phylogenetic relationships. Geomicrobiol. J. **19:**67–76.

520. **Santini, J. M., J. F. Stolz, and J. M. Macy.** 2002. Isolation of a new arsenate-respiring bacterium — physiological and phylogenetic studies. Geomicrobiol. J. **19:**41–52.

521. **Santoro, M. M., Y. Liu, S. M. A. Khan, L.-X. Hou, and D. W. Bolen.** 1992. Increased thermal stability of proteins in the presence of naturally occurring osmolytes. Biochemistry **31:**5278–5283.

522. **Santos, H., and M. S. da Costa.** 2001. Organic solutes from thermophiles and hyperthermophiles. Methods Enzymol. **334:**302–315.

523. **Santos, H., and M. S. da Costa.** 2002. Compatible solutes of organisms that live in hot saline environments. Environ. Microbiol. **4:**501–509.

524. **Sass, A. M., H. Sass, M. J. L. Coolen, H. Cypionka, and J. Overmann.** 2001. Microbial communities in the chemocline of a hypersaline deep-sea basin (Urania Basin, Mediterranean Sea). Appl. Environ. Microbiol. **67:**5392–5402.

525. **Satomi, M., B. Kimura, G. Takahashi, and T. Fujii.** 1997. Microbial diversity in kusaya gravy. Fish. Sci. **63:**1019–1023.

526. **Schaefer, J. K., J. Letowski, and T. Barkay.** 2002. *mer*-mediated resistance and volatilization of Hg(II) under anaerobic conditions. Geomicrobiol. J. **19:**87–102.

527. **Schleifer, K. H., and R. Kilpper-Bälz.** 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int. J. Syst. Bacteriol. **34:**31–34.

528. **Schmid, A. K., and M. E. Lidstrom.** 2002. Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. **184:**6182–6189.

529. **Schmidt, H. A., K. Strimmer, M. Vingron, and A. von Haeseler.** 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics **18:**502–504.

530. **Scholten, J. C., P. Stoffers, D. Garbe-Schönberg, and M. Moammar.** 2000. Hydrothermal mineralization in the Red Sea, p. 369–395. *In* D. S. Cronan (ed.), Handbook of marine mineral deposits. CRC Press, Boca Raton, Fla.

531. **Scholz, S., J. Sonnenbichler, W. Schäfer, and R. Hensel.** 1992. Di-myo-inositol-1,1'-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. FEBS Lett. **306:**239–242.

532. **Schrenk, M. O., D. S. Kelley, J. R. Delaney, and J. A. Baross.** 2003. Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. Appl. Environ. Microbiol. **69:**3580–3592.

533. **Schröder, I., E. Johnson, and S. de Vries.** 2003. Microbial ferric iron reductases. FEMS Microbiol. Rev. **27:**427–447.

534. **Schuliger, J. W., S. H. Brown, J. A. Baross, and R. M. Kelly.** 1993. Purification and characterization of a novel amylolytic enzyme from ES4, a marine hyperthermophilic archaeum. Mol. Mar. Biol. Biotechnol. **2:**76–87.

535. **Scientific Staff of Cruise *Bannock* 1984-12.** 1985. Gypsum precipitation from cold brines in an anoxic basin in the eastern Mediterranean. Nature **314:**152–154.

536. **Scott, J. A., and S. J. Palmer.** 1988. Cadmium bio-sorption by bacterial exopolysaccharide. Biotechnol. Lett. **10:**21–24.

537. **Severin, J., A. Wohlfarth, and E. A. Galinski.** 1992. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. **138:**1629–1638.

538. **Seyfried, W. E., Jr., and K. Ding.** 1995. Phase equilibria in subseafloor hydrothermal systems: a review of the role of redox, temperature, pH and dissolved Cl on the chemistry of hot spring fluids at mid-ocean ridges, p. 248–272. *In* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thompson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union, Washington, D.C.

539. **Shanks, W. C., III, and J. L. Bischoff.** 1977. Ore transport and deposition in the Red Sea geothermal system: a geochemical model. Geochim. Cosmochim. Acta **41:**1507–1519.

540. **Sheu, D. D.** 1990. The anoxic Orca Basin (Gulf of Mexico): geochemistry of brines and sediments. Rev. Aquat. Sci. **2:**491–507.

541. **Shokes, R. F., P. K. Trabant, B. J. Presley, and D. F. Reid.** 1977. Anoxic, hypersaline basin in the northern Gulf of Mexico. Science **196:**1443–1446.

542. **Sievert, S. M., T. Heidorn, and J. Kuever.** 2000. *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and

emended description of the genus *Halothiobacillus*. Int. J. Syst. Evol. Microbiol. **50:**1229–1237.

543. **Silver, S.** 1994. Exploiting heavy metal resistance systems in bioremediation. Res. Microbiol. **145:**61–67.

544. **Silver, S.** 1996. Bacterial resistances to toxic metal ions — a review. Gene **179:**9–19.

545. **Silver, S., and L. T. Phung.** 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. **50:**753–789.

546. **Skerratt, J. H., P. D. Nichols, C. A. Mancuso, S. R. James, S. J. Dobson, T. A. McMeekin, and H. Burton.** 1991. The phospholipid ester-linked fatty acid composition of members of the family *Halomonadaceae* and genus *Flavobacterium:* a chemotaxonomic guide. Syst. Appl. Microbiol. **14:**8–13.

547. **Sleator, R. D., C. G. M. Gahan, and C. Hill.** 2003. A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*. Appl. Environ. Microbiol. **69:**1–9.

548. **Sleator, R. D., and C. Hill.** 2001. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. **26:**49–71.

549. **Smets, B. F., J. B. Morrow, and C. A. Pinedo.** 2003. Plasmid introduction in metal-stressed, subsurface-derived microcosms: plasmid fate and community response. Appl. Environ. Microbiol. **69:**4087–4097.

550. **Smibert, R. M., and N. R. Krieg.** 1994. Systematics: phenotypic characterization, p. 607–654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.

551. **Smirnova, G. V., T. A. Krasnykh, and O. N. Oktyabrsky.** 2001. Role of glutathione in the response of *Escherichia coli* to osmotic stress. Biochemistry (Moscow) **66:**973–978.

552. **Smirnova, G. V., N. G. Muzyka, M. N. Glukhovchenko, and O. N. Oktyabrsky.** 2000. Effects of menadione and hydrogen peroxide on glutathione status in growing *Escherichia coli*. Free Radic. Biol. Med. **28:**1009–1016.

553. **Smith, D. C., M. Simon, A. L. Alldredge, and F. Azam.** 1992. Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature **359:**139–142.

554. **Smith, D. H.** 1967. R factors mediate resistance to mercury, nickel, and cobalt. Science **156:**1114–1116.

555. **Smith, K. L., Jr., P. M. Williams, and E. R. M. Druffel.** 1989. Upward fluxes of particulate organic matter in the deep North Pacific. Nature **337:**724–726.

556. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.

557. **Solioz, M., and J. V. Stoyanov.** 2003. Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol. Rev. **27:**183–195.

558. **Solovieva, I. M., and K.-D. Entian.** 2002. Investigation of the *yvgW Bacillus subtilis* chromosomal gene involved in Cd^{2+} ion resistance. FEMS Microbiol. Lett. **208:**105–109.

559. **Spröer, C., E. Lang, P. Hobeck, J. Burghardt, E. Stackebrandt, and B. J. Tindall.** 1998. Transfer of *Pseudomonas nautica* to *Marinobacter hydrocarbonoclasticus*. Int. J. Syst. Bacteriol. **48:**1445–1448.

560. **Stackebrandt, E., C. Koch, O. Gvozdiak, and P. Schumann.** 1995. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int. J. Syst. Bacteriol. **45:**682–692.

561. **Staley, J. T., and J. J. Gosink.** 1999. Poles apart: biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol. **53:**189–215.

562. **Stanley, S. O., and C. M. Brown.** 1974. Influence of temperature and salinity on the amino-acid pools of some marine pseudomonads, p. 92–103. *In* R. R. Colwell and R. Y. Morita (ed.), Effect of the ocean environment on microbial activities. University Park Press, Baltimore, Md.

563. **Stanley, S. O., and R. Y. Morita.** 1968. Salinity effect on the maximal growth temperature of some bacteria isolated from marine environments. J. Bacteriol. **95:**169–173.

564. **Starinsky, A., and A. Katz.** 2003. The formation of natural cryogenic brines. Geochim. Cosmochim. Acta **67:**1475–1484.

565. **Steil, L., T. Hoffmann, I. Budde, U. Völker, and E. Bremer.** 2003. Genome-wide transcriptional profiling analysis of adaptation of *Bacillus subtilis* to high salinity. J. Bacteriol. **185:**6358–6370.

566. **Steinsbu, B. O., and T. Torsvik.** Unpublished data from the National Center for Biotechnology Information website.

567. **Straube, W. L., M. O'Brien, K. Davis, and R. R. Colwell.** 1990. Enzymatic profiles of 11 barophilic bacteria under in situ conditions: evidence for pressure modulation of phenotype. Appl. Environ. Microbiol. **56:**812–814.

568. **Strimmer, K., and A. von Haeseler.** 1996. Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. Mol. Biol. Evol. **13:**964–969.

569. **Summit, M., and J. A. Baross.** 1998. Thermophilic subseafloor microorganisms from the 1996 North Gorda Ridge eruption. Deep-Sea Res. II **45:**2751–2766.

570. **Summit, M., and J. A. Baross.** 2001. A novel microbial habitat in the mid-ocean ridge subseafloor. Proc. Natl. Acad. Sci. USA **98:**2158–2163.

571. **Summit, M., B. Scott, K. Nielson, E. Mathur, and J. Baross.** 1998. Pressure enhances thermal stability of DNA polymerase from three thermophilic organisms. Extremophiles **2:**339–345.

572. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.

573. **Suzuki, M. T., M. S. Rappé, Z. W. Haimberger, H. Winfield, N. Adair, J. Ströbel, and S. J. Giovannoni.** 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. **63:**983–989.

574. **Swinnerton, J. W., and V. J. Linnenbom.** 1969. Low molecular weight hydrocarbon analyses of Atlantis II waters, p. 251–253. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

575. **Takai, K., F. Inagaki, S. Nakagawa, H. Hirayama, T. Nunoura, Y. Sako, K. H. Nealson, and K. Horikoshi.** 2003. Isolation and phylogenetic diversity of members of previously uncultivated ε-Proteobacteria in deep-sea hydrothermal fields. FEMS Microbiol. Lett. **218:**167–174.

576. **Takai, K., H. Kobayashi, K. H. Nealson, and K. Horikoshi.** 2003. *Deferribacter desulfuricans* sp. nov., a novel sulfur-, nitrate- and arsenate-reducing thermophile isolated from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. **53:**839–846.

577. **Takai, K., T. Komatsu, F. Inagaki, and K. Horikoshi.** 2001. Distribution of Archaea in a black smoker chimney structure. Appl. Environ. Microbiol. **67:**3618–3629.

578. **Takami, H., K. Kobata, T. Nagahama, H. Kobayashi, A. Inoue, and K. Horikoshi.** 1999. Biodiversity in deep-sea sites located near the south part of Japan. Extremophiles **3:**97–102.

579. **Tamura, K., T. Shimizu, and H. Kourai.** 1992. Effects of ethanol on the growth and elongation of *Escherichia coli* under high pressures up to 40 MPa. FEMS Microbiol. Lett. **99:**321–324.

580. **Tanaka, T., J. G. Burgess, and P. C. Wright.** 2001. High-pressure adaptation by salt stress in a moderately halophilic bacterium obtained from open seawater. Appl. Microbiol. Biotechnol. **57:**200–204.

581. **Tay, P. L., L. Lonergan, M. Warner, K. A. Jones, and the IMERSE Working Group.** 2002. Seismic investigation of thick evaporite deposits on the central and inner unit of the Mediterranean Ridge accretionary complex. Mar. Geol. **186:**167–194.

582. **Tebo, B. M.** 1995. Metal precipitation by marine bacteria: potential for biotechnological applications, p. 231–263. *In* J. K. Setlow (ed.), Genetic engineering, vol. 17. Plenum Press, New York.

583. **Tehei, M., B. Franzetti, M.-C. Maurel, J. Vergne, C. Hountondji, and G. Zaccai.** 2002. The search for traces of life: the protective effect of salt on biological macromolecules. Extremophiles **6:**427–430.

584. **Templeton, A. S., T. P. Trainor, A. M. Spormann, and G. E. Brown, Jr.** 2003. Selenium speciation and partitioning within *Burkholderia cepacia* biofilms formed on α-Al_2O_3 surfaces. Geochim. Cosmochim. Acta **67:**3547–3557.

585. **Teske, A., T. Brinkhoff, G. Muyzer, D. P. Moser, J. Rethmeier, and H. W. Jannasch.** 2000. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. Appl. Environ. Microbiol. **66:**3125–3133.

586. **Tesone, S., A. Hughes, and A. Hurst.** 1981. Salt extends the upper temperature limit for growth of food-poisoning bacteria. Can. J. Microbiol. **27:**970–972.

587. **Thackray, P. D., and A. Moir.** 2003. SigM, an extracytoplasmic function sigma factor of *Bacillus subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. J. Bacteriol. **185:**3491–3498.

588. **Thomas, D. N., and G. S. Dieckmann.** 2002. Antarctic sea ice — a habitat for extremophiles. Science **295:**641–644.

589. **Thompson, G. A., and R. J. Watling.** 1987. Bioaccumulation potential of heterotrophic bacteria for lead, selenium, and arsenic. Bull. Environ. Contam. Toxicol. **38:**1049–1054.

590. **Thompson, J. R., L. A. Marcelino, and M. F. Polz.** 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR.' Nucleic Acids Res. **30:**2083–2088.

591. **Timoney, J. F., J. Port, J. Giles, and J. Spanier.** 1978. Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. Appl. Environ. Microbiol. **36:**465–472.

592. **Tivey, M. K.** 1995. The influence of hydrothermal fluid composition and advection rates on black smoker chimney mineralogy: insights from modeling transport and reaction. Geochim. Cosmochim. Acta **59:**1933–1949.

593. **Tivey, M. K., and J. R. Delaney.** 1986. Growth of large sulfide structures on the Endeavour Segment of the Juan de Fuca Ridge. Earth Planet. Sci. Lett. **77:**303–317.

594. **Tivey, M. K., and R. E. McDuff.** 1990. Mineral precipitation in the walls of black smoker chimneys: a quantitative model of transport and chemical reaction. J. Geophys. Res. **95:**12,617–12,637.

595. **Tohoyama, H., M. Inouhe, M. Joho, and T. Murayama.** 1995. Production of metallothionein in copper- and cadmium-resistant strains of *Saccharomyces cerevisiae*. J. Ind. Microbiol. **14:**126–131.

596. **Touati, D.** 2000. Iron and oxidative stress in bacteria. Arch. Biochem. Biophys. **373:**1–6.

597. **Trefry, J. H.** 1977. Transition metal chemistry in the Orca Basin. Eos, Trans. AGU, Suppl. **58:**1175.

598. **Trefry, J. H., D. B. Butterfield, S. Metz, G. J. Massoth, R. P. Trocine, and R. A. Feely.** 1994. Trace metals in hydrothermal solutions from Cleft segment on the southern Juan de Fuca Ridge. J. Geophys. Res. **99:**4925–4935.

599. **Trüper, H. G.** 1969. Bacterial sulfate reduction in the Red Sea hot brines, p. 263–271. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

600. **Trüper, H. G., and E. A. Galinski.** 1990. Biosynthesis and fate of compatible solutes in extremely halophilic phototrophic eubacteria. FEMS Microbiol. Rev. **75:**247–254.

601. **Tuovila, B. J., F. C. Dobbs, P. A. LaRock, and B. Z. Siegel.** 1987. Preservation of ATP in hypersaline environments. Appl. Environ. Microbiol. **53:**2749–2753.

602. **Turner, J. S., and N. J. Robinson.** 1995. Cyanobacterial metallothioneins: biochemistry and molecular genetics. J. Ind. Microbiol. **14:**119–125.

603. **Turner, J. S., N. J. Robinson, and A. Gupta.** 1995. Construction of Zn^{2+}/Cd^{2+}-tolerant cyanobacteria with a modified metallothionein divergon: further analysis of the function and regulation of *smt*. J. Ind. Microbiol. **14:**259–264.

604. **Urakawa, H., K. Kita-Tsukamoto, S. E. Steven, K. Ohwada, and R. R. Colwell.** 1998. A proposal to transfer *Vibrio marinus* (Russell 1891) to a new genus *Moritella* gen. nov. as *Moritella marina* comb. nov. FEMS Microbiol. Lett. **165:**373–378.

605. **Valderrama, M. J., M. Monteoliva-Sanchez, E. Quesada, and A. Ramos-Cormenzana.** 1998. Influence of salt concentration on the cellular fatty acid composition of the moderately halophilic bacterium *Halomonas salina*. Res. Microbiol. **149:**675–679.

606. **Valderrama, M. J., E. Quesada, V. Béjar, A. Ventosa, M. C. Gutierrez, F. Ruíz-Berraquero, and A. Ramos-Cormenzana.** 1991. *Deleya salina* sp. nov., a moderately halophilic Gram-negative bacterium. Int. J. Syst. Bacteriol. **41:**377–384.

607. **Valette-Silver, J. N., A. Bates, D. Anderson, J. Deming, and W. H. Zoller.** 1985. Uranium enrichments in smoker material collected from the East Pacific Rise (abstract). Eos, Trans. AGU, Suppl. Abstract omitted from supplement.

608. **Valls, M., and V. de Lorenzo.** 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol. Rev. **26:**327–338.

609. **van der Heide, T., and B. Poolman.** 2000. Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane. Proc. Natl. Acad. Sci. USA **97:**7102–7106.

610. **van der Sloot, H. A., D. Hoede, G. Hamburg, J. R. W. Woittiez, and C. H. van der Weijden.** 1990. Trace elements in suspended matter from the anoxic hypersaline Tyro and Bannock Basins (eastern Mediterranean). Mar. Chem. **31:**187–203.

611. **Vargas, C., R. Fernández-Castillo, D. Cánovas, A. Ventosa, and J. J. Nieto.** 1995. Isolation of cryptic plasmids from moderately halophilic eubacteria of the

genus *Halomonas*. Characterization of a small plasmid from *H. elongata* and its use for shuttle vector construction. Mol. Gen. Genet. **246:**411–418.

612. **Vengosh, A., G. J. de Lange, and A. Starinsky.** 1998. Boron isotope and geochemical evidence for the origin of Urania and Bannock brines at the eastern Mediterranean: effect of water-rock interactions. Geochim. Cosmochim. Acta **62:**3221–3228.

613. **Ventosa, A., J. J. Nieto, and A. Oren.** 1998. Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev. **62:**504–544.

614. **Ventosa, A., E. Quesada, F. Rodríguez-Valera, F. Ruíz-Berraquero, and A. Ramos-Cormenzana.** 1982. Numerical taxonomy of moderately halophilic Gram-negative rods. J. Gen. Microbiol. **128:**1959–1968.

615. **Ventosa, A., F. Rodríguez-Valera, J. S. Poindexter, and W. S. Reznikoff.** 1984. Selection for moderately halophilic bacteria by gradual salinity increases. Can. J. Microbiol. **30:**1279–1282.

616. **Viollier, P. H., G. H. Kelemen, G. E. Dale, K. T. Nguyen, M. J. Buttner, and C. J. Thompson.** 2003. Specialized osmotic stress response systems involve multiple SigB-like sigma factors in *Streptomyces coelicolor*. Mol. Microbiol. **47:**699–714.

617. **Volesky, B., H. May, and Z. R. Holan.** 1993. Cadmium biosorption by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **41:**826–829.

618. **Völker, U., H. Mach, R. Schmid, and M. Hecker.** 1992. Stress proteins and cross-protection by heat shock and salt stress in *Bacillus subtilis*. J. Gen. Microbiol. **138:**2125–2135.

619. **Von Damm, K. L.** 1995. Controls on the chemistry and temporal variability of seafloor hydrothermal fluids, p. 222–247. *In* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union, Washington, D.C.

620. **Von Damm, K. L.** 2002. Personal communication.

621. **Von Damm, K. L., and J. L. Bischoff.** 1987. Chemistry of hydrothermal solutions from the southern Juan de Fuca Ridge. J. Geophys. Res. **92:**11,334–11,346.

622. **Von Damm, K. L., A. M. Bray, L. G. Buttermore, and S. E. Oosting.** 1998. The geochemical controls on vent fluids from the Lucky Strike vent field, Mid-Atlantic Ridge. Earth Planet. Sci. Lett. **160:**521–536.

623. **Von Damm, K. L., L. G. Buttermore, S. E. Oosting, A. M. Bray, D. J. Fornari, M. D. Lilley, and W. C. Shanks, III.** 1997. Direct observation of the evolution of a seafloor 'black smoker' from vapor to brine. Earth Planet. Sci. Lett. **149:**101–111.

624. **Von Damm, K. L., J. M. Edmond, B. Grant, C. I. Measures, B. Walden, and R. F. Weiss.** 1985. Chemistry of submarine hydrothermal solutions at 21°N, East Pacific Rise. Geochim. Cosmochim. Acta **49:**2197–2220.

625. **Von Damm, K. L., S. E. Oosting, R. Kozlowski, L. G. Buttermore, D. C. Colodner, H. N. Edmonds, J. M. Edmond, and J. M. Grebmeier.** 1995. Evolution of East Pacific Rise hydrothermal vent fluids following a volcanic eruption. Nature **375:**47–50.

626. **Vreeland, R. H., R. Anderson, and R. G. E. Murray.** 1984. Cell wall and phospholipid composition and their contribution to the salt tolerance of *Halomonas elongata*. J. Bacteriol. **160:**879–883.

627. **Vreeland, R. H., S. L. Daigle, S. T. Fields, D. J. Hart, and E. L. Martin.** 1991. Physiology of *Halomonas elongata* in different NaCl concentrations, p. 233–241. *In* F. Rodríguez-Valera (ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York.

628. **Vreeland, R. H., and J. H. Huval.** 1991. Phenotypic characterization of halophilic bacteria from ground water sources in the United States, p. 53–60. *In* F. Rodríguez-Valera (ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York.

629. **Vreeland, R. H., C. D. Litchfield, E. L. Martin, and E. Elliot.** 1980. *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. Int. J. Syst. Bacteriol. **30:**485–495.

630. **Vreeland, R. H., and E. L. Martin.** 1980. Growth characteristics, effects of temperature, and ion specificity of the halotolerant bacterium *Halomonas elongata*. Can. J. Microbiol. **26:**746–752.

631. **Vreeland, R. H., and W. D. Rosenzweig.** 2002. The question of uniqueness of ancient bacteria. J. Ind. Microbiol. Biotechnol. **28:**32–41.

632. **Vreeland, R. H., W. D. Rosenzweig, and D. W. Powers.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature **407:**897–900.

633. **Wakeham, S. G., J. P. Cowen, B. J. Burd, and R. E. Thomson.** 2001. Lipid-rich ascending particles from the hydrothermal plume at Endeavour Segment, Juan de Fuca Ridge. Geochim. Cosmochim. Acta **65:**923–939.

634. **Wallmann, K., F. S. Aghib, D. Castradori, M. B. Cita, E. Suess, J. Greinert, and D. Rickert.** 2002. Sedimentation and formation of secondary minerals in the hypersaline Discovery Basin, eastern Mediterranean. Mar. Geol. **186:**9–28.

635. **Walthier, T. N., and C. E. Schatz.** 1969. Economic significance of minerals deposited in the Red Sea deeps, p. 542–549. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

636. **Wang, C. L., A. M. Lum, S. C. Ozuna, D. S. Clark, and J. D. Keasling.** 2001. Aerobic sulfide production and cadmium precipitation by *Escherichia coli* expressing the *Treponema denticola* cysteine desulfhydrase gene. Appl. Microbiol. Biotechnol. **56:**425–430.

637. **Wang, C. L., P. C. Michels, S. C. Dawson, S. Kitisakkul, J. A. Baross, J. D. Keasling, and D. S. Clark.** 1997. Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. Appl. Environ. Microbiol. **63:**4075–4078.

638. **Wang, W.-X., N. S. Fisher, and S. N. Luoma.** 1996. Kinetic determinations of trace element bioaccumulation in the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. **140:**91–113.

639. **Watson, S. W., and J. B. Waterbury.** 1969. The sterile hot brines of the Red Sea, p. 272–281. *In* E. T. Degens and D. A. Ross (ed.), Hot brines and recent heavy metal deposits in the Red Sea. Springer-Verlag, New York.

640. **Weber, A., and K. Jung.** 2002. Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. J. Bacteriol. **184:**5502–5507.

641. **Welch, T. J., and D. H. Bartlett.** 1997. Cloning, sequencing and overexpression of the gene encoding malate dehydrogenase from the deep-sea bacterium *Photobacterium profundum* species strain SS9. Biochim. Biophys. Acta **1350:**41–46.

642. **Welch, T. J., A. Farewell, F. C. Neidhardt, and D. H. Bartlett.** 1993. Stress response of *Escherichia coli* to elevated hydrostatic pressure. J. Bacteriol. **175:**7170–7177.

643. **Wemekamp-Kamphuis, H. H., A. K. Karatzas, J. A. Wouters, and T. Abee.** 2002. Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. Appl. Environ. Microbiol. **68:**456–463.

644. **Wery, N., M.-A. Cambon-Bonavita, F. Lesongeur, and G. Barbier.** 2002. Diversity of anaerobic heterotrophic thermophiles isolated from deep-sea hydrothermal vents of the Mid-Atlantic Ridge. FEMS Microbiol. Ecol. **41:**105–114.

645. **Westbrook, G. K., and T. J. Reston.** 2002. The accretionary complex of the Mediterranean Ridge: tectonics, fluid flow and the formation of brine lakes — an introduction to the special issue of Marine Geology. Mar. Geol. **186:**1–8.

646. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia **40:**51–62.

647. **Whitman, W. B.** 2001. Genus II. *Methanotorris* gen. nov., p. 245–246. *In* D. R. Boone, R. W. Castenholz, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1 (The *Archaea* and the deeply branching and phototrophic bacteria). Springer-Verlag, New York.

648. **Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor.** 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. **180:**3650–3656.

649. **Wiesenburg, D. A., J. M. Brooks, and B. B. Bernard.** 1985. Biogenic hydrocarbon gases and sulfate reduction in the Orca Basin brine. Geochim. Cosmochim. Acta **49:**2069–2080.

650. **Wohlfarth, A., J. Severin, and E. A. Galinski.** 1990. The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*. J. Gen. Microbiol. **136:**705–712.

651. **Wombacher, F., M. Rehkämper, K. Mezger, and C. Münker.** 2003. Stable isotope compositions of cadmium in geological materials and meteorites determined by multiple-collector ICPMS. Geochim. Cosmochim. Acta **67:**4639–4654.

652. **Wood, J. M.** 1999. Osmosensing by bacteria: signals and membrane-based sensors. Microbiol. Mol. Biol. Rev. **63:**230–262.

653. **Xu, Y., Y. Nogi, C. Kato, Z. Liang, H.-J. Rüger, D. De Kegel, and N. Glansdorff.** 2003. *Psychromonas profunda* sp. nov., a psychropiezophilic bacterium from deep Atlantic sediments. Int. J. Syst. Evol. Microbiol. **53:**527–532.

654. **Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi.** 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb.

nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. **39:**897–904.

655. **Yanagibayashi, M., Y. Nogi, L. Li, and C. Kato.** 1999. Changes in the microbial community in Japan Trench sediment from a depth of 6292 m during cultivation without decompression. FEMS Microbiol. Lett. **170:**271–279.

656. **Yancey, P. H., W. R. Blake, and J. Conley.** 2002. Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. Comp. Biochem. Physiol. A **133:**667–676.

657. **Yayanos, A. A.** 1986. Evolution and ecological implications of the properties of deep-sea barophilic bacteria. Proc. Natl. Acad. Sci. USA **83:**9542–9546.

658. **Yayanos, A. A.** 1995. Microbiology to 10,500 meters in the deep sea. Annu. Rev. Microbiol. **49:**777–805.

659. **Yayanos, A. A., and E. F. DeLong.** 1987. Deep-sea bacterial fitness to environmental temperatures and pressures, p. 17–32. *In* H. W. Jannasch, R. E. Marquis, and A. M. Zimmerman (ed.), Current perspectives in high pressure biology. Academic Press, London.

660. **Yayanos, A. A., A. S. Dietz, and R. Van Boxtel.** 1981. Obligately barophilic bacterium from the Mariana Trench. Proc. Natl. Acad. Sci. USA **78:**5212–5215.

661. **Yayanos, A. A., A. S. Dietz, and R. Van Boxtel.** 1982. Dependence of reproduction rate on pressure as a hallmark of deep-sea bacteria. Appl. Environ. Microbiol. **44:**1356–1361.

662. **Yayanos, A. A., and J. C. Nevenzel.** 1978. Rising-particle hypothesis: rapid ascent of matter from the deep ocean. Naturwissenschaften **65:**255–256.

663. **Yayanos, A. A., and R. Van Boxtel.** 1982. Coupling device for quick high-pressure connections to 100 MPa. Rev. Sci. Instrum. **53:**704–705.

664. **Yilmaz, E. I.** 2003. Metal tolerance and biosorption capacity of *Bacillus circulans* strain EB1. Res. Microbiol. **154:**409–415.

665. **Yoon, J.-H., S. H. Choi, K.-C. Lee, Y. H. Kho, K. H. Kang, and Y.-H. Park.** 2001. *Halomonas marisflavae* sp. nov., a halophilic bacterium isolated from the Yellow Sea in Korea. Int. J. Syst. Evol. Microbiol. **51:**1171–1177.

666. **Yoon, J.-H., K.-C. Lee, Y. H. Kho, K. H. Kang, C.-J. Kim, and Y.-H. Park.** 2002. *Halomonas alimentaria* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. **52:**123–130.

667. **Zbinden, M., N. Le Bris, P. Compère, I. Martinez, F. Guyot, and F. Gaill.** 2003. Mineralogical gradients associated with alvinellids at deep-sea hydrothermal vents. Deep-Sea Res. I **50:**269–280.

668. **Zierenberg, R. A., Y. Fouquet, D. J. Miller, J. M. Bahr, P. A. Baker, T. Bjerkgård, C. A. Brunner, R. C. Duckworth, R. Gable, J. Gieskes, W. D. Goodfellow, H. M. Gröschel-Becker, G. Guèrin, J. Ishibashi, G. Iturrino, R. H. James, K. S. Lackschewitz, L. L. Marquez, P. Nehlig, J. M. Peter, C. A. Rigsby, P. Schultheiss, W. C. Shanks, III, B. R. T. Simoneit, M. Summit, D. A. H. Teagle, M. Urbat, and G. G. Zuffa.** 1998. The deep structure of a sea-floor hydrothermal deposit. Nature **392:**485–488.

669. **Zierenberg, R. A., and P. Schiffman.** 1990. Microbial control of silver mineralization at a sea-floor hydrothermal site on the northern Gorda Ridge. Nature **348:**155–157.

670. **Zierenberg, R. A., and W. C. Shanks, III.** 1986. Isotopic constraints on the origin of the Atlantis II, Suakin and Valdivia brines, Red Sea. Geochim. Cosmochim. Acta **50:**2205–2214.

671. **Zierenberg, R. A., and W. C. Shanks, III.** 1988. Isotopic studies of epigenetic features in metalliferous sediment, Atlantis II Deep, Red Sea. Can. Mineral. **26:**737–753.

672. **ZoBell, C. E.** 1970. Pressure effects on morphology and life processes of bacteria, p. 85–130. *In* A. M. Zimmerman (ed.), High pressure effects on cellular processes. Academic Press, New York.

673. **ZoBell, C. E., and A. B. Cobet.** 1962. Growth, reproduction, and death rates of *Escherichia coli* at increased hydrostatic pressures. J. Bacteriol. **84:**1228–1236.

674. **ZoBell, C. E., and A. B. Cobet.** 1964. Filament formation by *Escherichia coli* at increased hydrostatic pressures. J. Bacteriol. **87:**710–719.

675. **ZoBell, C. E., and F. H. Johnson.** 1949. The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J. Bacteriol. **57:**179–189.

676. **ZoBell, C. E., and C. H. Oppenheimer.** 1950. Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. J. Bacteriol. **60:**771–781.

677. **ZoBell, C. E., and H. C. Upham.** 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. **5:**239–292.

Vita

JONATHAN Z. KAYE

Education

2003	PhD, Oceanography, University of Washington
1998	MS, Oceanography, University of Washington
1995	BS, Geology-Biology, Brown University

Positions Held

1995–2003	Research Assistant, School of Oceanography, University of Washington
2000, 2002	Science Consultant, American Museum of Natural History, New York
1999	Teaching Assistant, Interpretation of Oceanic Data (two-quarter research and field course), School of Oceanography, University of Washington
1997–1998	Science Consultant, NOVA (PBS), WGBH, Boston
1995	Field Chemist and Field Biologist, United States Geological Survey, Water Resources Division, National Water-Quality Assessment Program, Towson, MD
1993–1995	Undergraduate Research Assistant, Department of Geological Sciences, Brown University

Research Interests

Microbe-metal interactions, including Fe and Mn oxidation in deep-sea hydrothermal plumes and heavy-metal resistance in vent mesophiles and hyperthermophiles. Distribution of heavy-metal-tolerant bacteria in marine environments. Metal bioremediation. Ecology and characterization of moderately halophilic and euryhaline microorganisms from hydrothermal-vent, subseafloor and pelagic environments. Effects of multiple stresses: hydrostatic pressure, salt and temperature; salt and heavy metals. Astrobiology. Possible brine habitats in hydrothermal-vent and subseafloor environments. Phylogenetic and proteomic studies to understand physiological stress and microbe-metal interactions in an environmentally relevant context.

Field Experience

2000	R/V *Atlantis*, Juan de Fuca Ridge, Tidal Perturbations
1999	R/V *Thompson*, Puget Sound, TA for Interpretation of Oceanic Data course

1999	Troodos Ophiolite field course, Cyprus, RIDGE
1998	R/V *Atlantis*, Southern East Pacific Rise, SouEPR
1998	R/V *Brown*, Axial Seamount, NeMO
1998	R/V *Thompson* and R/V *Tully*, Juan de Fuca Ridge, Sulfide Recovery
1998	Samail Ophiolite field course, Oman, University of Washington and Oregon State University
1997	R/V *Atlantis*, Juan de Fuca Ridge, Edifice Rex
1996	R/V *Thompson*, Middle Valley, TN065
1996	R/V *Thompson*, Juan de Fuca Ridge, REVEL
1996	R/V *Sproul*, Columbia River estuary, CRETM
1994	Geology field methods course, Rocky Mountains, Albion College

Awards and Honors

2002	Outstanding Student Presentation, American Geophysical Union Fall Meeting
1999	Totem Award (Puget Sound Chapter of the Public Relations Society of America) and Communicator of Excellence Award (Washington Press Association) for "Life Down Deep: Scientists explore undersea volcanic vents"
1995	National Science Foundation Graduate Research Fellowship Honorable Mention
1995	National Association of Geology Teachers Internship Award to work with the United States Geological Survey
1995	Magna Cum Laude, Brown University
1995	Undergraduate Thesis with Honors, Brown University
1995	Department of Geological Sciences Senior Prize, Brown University
1995	Sigma Xi
1994	Undergraduate Teaching and Research Assistantship

Publications

Kaye, J. Z., and J. Lynch. 1995. Holocene water-level fluctuations at Crooked Pond, Plymouth, Massachusetts. Undergraduate thesis, Brown University.

Kaye, J. Z., and J. A. Baross. 2000. High incidence of halotolerant bacteria in Pacific hydrothermal-vent and pelagic environments. *FEMS Microbiol. Ecol.* 32: 249–260.

Kargel, J. S., J. Z. Kaye, J. W. Head III, G. M. Marion, R. Sassen, J. K. Crowley, O. P. Ballesteros, S. A. Grant and D. L. Hogenboom. 2000. Europa's crust and ocean: Origin, composition, and the prospects for life. *Icarus* 148: 226–265.

Delaney, J. R., D. S. Kelley, E. A. Mathez, D. R. Yoerger, J. Baross, M. O. Schrenk, J. Kaye and V. Robigou. 2001. "Edifice Rex" sulfide recovery project: Analysis of submarine hydrothermal, microbial habitat. *EOS Trans. AGU* 82(6): 67–73.

Shuman, B., J. Bravo, J. A. Lynch, J. Kaye, P. Newby and T. Webb III. 2001. Late Quaternary water-level variations and vegetation history at Crooked Pond, southeastern Massachusetts. *Quat. Res.* 56: 401–410.

Kaye, J. Z., M. C. Márquez, A. Ventosa and J. A. Baross. In press. *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov., and *Halomonas hydrothermalis* sp. nov.: halophilic bacteria isolated from deep-sea hydrothermal-vent environments. *Int. J. Syst. Evol. Microbiol.*

Abstracts

Kaye, J. Z., and J. A. Baross. 2002. Salinity, pressure and heavy-metal stress response of moderately halophilic bacteria isolated from hydrothermal-vent environments. *Eos Trans. AGU* 83(47) Fall Meet. Suppl.: V72A-1283. San Francisco, CA.

Kaye, J. Z., and J. A. Baross. 2002. The combined effect of salt, pressure and temperature on microbial processes and its application to extraterrestrial waters of unknown salinity. NATO Advanced Studies Institute Astrobiology Conference, Chania, Crete, Greece.

Kargel, J. S., O. Prieto, J. Z. Kaye, S. Vance, G. Marion and D. L. Hogenboom. 2002. Chemical, physical, and biological environment of Europa's floating shell, ocean, and subocean crust. NASA Astrobiology Science Conference, NASA Ames Research Center, Moffet Field, CA.

Kaye, J. Z., A. Ventosa and J. A. Baross. 2002. Moderately halophilic bacterial populations from deep-sea hydrothermal vents: The effect of pressure and community time-series analysis. AGU/ASLO Ocean Sciences Meet. Suppl.: OS31F-92. Honolulu, HI.

Kaye, J. Z., and J. A. Baross. 2001. The combined effect of salt, pressure and temperature on microbial processes and its application to extraterrestrial waters of unknown salinity. University of Washington Astrobiology Conference, Crystal Mountain, WA.

Kaye, J. Z., and J. A. Baross. 2001. High abundance and physiological versatility of *Halomonas* spp. in surface ocean and deep-sea, hydrothermal-vent environments. Halophiles 2001 Conference, Seville, Spain.

Márquez, M. C., D. R. Arahal, J. Z. Kaye, J. A. Baross and A. Ventosa. 2000. Caracterización de *Marinobacter* sp. y *Halomonas* sp. aisladas del Océano Pacífico. III Reunión Científica de Microbiología del Medio Acuatico, Santiago de Compostela, Spain.

Kaye, J. Z., and J. A. Baross. 2000. Microbial adaptation to subseafloor brines in mid-ocean-ridge environments. 5th RIDGE Theoretical Institute: The Subsurface Biosphere at Mid-Ocean Ridges, Big Sky, MT.

Kaye, J. Z., and J. A. Baross. 2000. Hyperthermohalophily? 7th Annual NSF Hyperthermophile Symposium, North Carolina State University, Raleigh, NC.

Kaye, J. Z., and J. A. Baross. 1999. A link between halotolerant microbes and subsurface brines in the deep-sea, mid-ocean-ridge system. 6th Annual NSF Hyperthermophile Symposium, University of Georgia, Athens, GA.

Kaye, J. Z., and J. A. Baross. 1999. A link between halotolerant microbes and subsurface brines in the deep-sea, mid-ocean-ridge system. RIDGE Troodos Field School, Larnaca, Cyprus.

Kaye, J. Z., and J. A. Baross. 1999. Implications of halotolerant microorganisms for subseafloor brines along the Juan de Fuca Ridge. RIDGE Juan de Fuca Results Symposium: A Retrospective and Planning Workshop, Seattle, WA.

Baross, J. A., M. Summit, J. Huber, J. Z. Kaye and S. Roadruck. 1999. Physiological and phylogenetic diversity of subseafloor microbial communities. Geological Society of America Meeting, Denver, CO.

Kaye, J. Z., and J. A. Baross. 1998. Salt-tolerant microbes isolated from hydrothermal-vent environments. *Eos Trans. AGU* 79(45) Fall Meet. Suppl.: 59. San Francisco, CA.

Schrenk, M. O., J. Z. Kaye, J. A. Baross, D. S. Kelley and J. R. Delaney. 1998. Microbe-mineral associations in intact hydrothermal vent chimneys recovered from the Juan de Fuca Ridge, July 1998. *Eos Trans. AGU* 79(45) Fall Meet. Suppl.: 59. San Francisco, CA.

Embley, R. W., D. Butterfield, K. Roe, M. Stapp, W. W. Chadwick, L. Evans, S. Merle, J. Baross, J. Kaye, J. Huber, G. Massoth, J. Gendron, S. Maenner, V. Tunnicliffe, M. Tsurumi, J. Marcus, K. Juniper, D. Grelon, C. Levesque, C. Moyer, K. Pelletreau, G. Wheat, S. Scott, J. Chadwick, M. Perfit, E. Guenther, B. McLaughlin-West, J. Getsiv and E. Williamson. 1998. Time series investigations of an active volcano: The NeMO project. *Eos Trans. AGU* 79(45) Fall Meet. Suppl.: 921. San Francisco, CA.

Kaye, J. Z., and J. A. Baross. 1998. Ecology and characterization of halotolerant and halophilic mesophiles from hydrothermal-vent environments: Implications for a warm, briny subsurface habitat. 5th Annual NSF Hyperthermophile Symposium, University of Washington, Seattle, WA.

Kaye, J. Z., and J. A. Baross. 1997. In search of toxic-metal-tolerant high-temperature microbes from hydrothermal vent environments. 4th Annual NSF Hyperthermophile Symposium, Recombinant BioCatalysis Inc., Del Mar, CA.

Kaye, J. Z., and J. Baross. 1997. Characterization of a novel group of chemolithoautotrophic bacteria isolated from hydrothermal-vent environments. First International Symposium on Deep-Sea Hydrothermal Vent Biology (InterRidge), Funchal, Madeira, Portugal.

Kaye, J. Z., and J. A. Baross. 1997. Ecology and characterization of nine hydrothermal-vent bacterial strains isolated from the Endeavour Segment, Juan de Fuca Ridge. *Eos Trans. AGU* 78(46) Fall Meet. Suppl.: 740. San Francisco, CA.

Kaye, J. Z., and J. A. Baross. 1996. Characterization of a novel group of metal-oxidizing chemolithoautotrophic bacteria isolated from hydrothermal vent environments. 3rd Annual NSF Hyperthermophile Symposium, North Carolina State University, Raleigh, NC.

Newby, P., J. Kaye and J. Lynch. 1995. Holocene water-level fluctuations in southeastern New England. Northeast Regional Conference of the Geological Society of America, Cromwell, CT.

Other

Huber, J., J. Kaye and D. G. Gordon. 1998. "Life down deep: Scientists explore undersea volcanic vents." ABCNews.go.com/sections/science/DailyNews/exped_seamount_intro980821.html