APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1996, p. 1287–1294 Vol. 62, No. 4
0099-2240/96/$04.00+0

Responses to Stress and Nutrient Availability by the Marine Ultramicrobacterium *Sphingomonas* sp. Strain RB2256

MITSURU EGUCHI,[1] TAKANORI NISHIKAWA,[1] KAREN MACDONALD,[2]† RICARDO CAVICCHIOLI,[2] JAN C. GOTTSCHAL,[3] AND STAFFAN KJELLEBERG[2]*

Department of Fisheries, Kinki University, Nara 631, Japan[1]; Department of Microbiology, University of Groningen, 9751 NN Haren, The Netherlands[3]; and School of Microbiology and Immunology, The University of New South Wales, Sydney 2052, Australia[2]

Received 7 November 1995/Accepted 31 January 1996

***Sphingomonas* sp. strain RB2256 was isolated from Resurrection Bay in Alaska and possibly represents the dominant bacterial species in some oligotrophic marine environments. Strain RB2256 has a high-affinity nutrient uptake system when growing under nutrient-limiting conditions, and growing cells are very small (<0.08 μm^3). These characteristics indicate that RB2256 is highly evolved for withstanding nutrient limitations and grazing pressure by heterotrophic nanoflagellates. In this study, strain RB2256 was subjected to nutrient starvation and other stresses (high temperature, ethanol, and hydrogen peroxide). It was found that growing cells were remarkably resistant, being able to survive at a temperature of 56°C, in 25 mM hydrogen peroxide, or in 20% ethanol. In addition, growing cells were generally as resistant as starved cells. The fact that vegetative cells of this strain are inherently resistant to such high levels of stress-inducing agents indicates that they possess stress resistance mechanisms which are different from those of other nondifferentiating bacteria. Only minor changes in cell volume (0.03 to 0.07 μm^3) and maximum specific growth rate (0.13 to 0.16 h^{-1}) were obtained for cells growing in media with different organic carbon concentrations (0.8 to 800 mg of C per liter). Furthermore, when glucose-limited, chemostat-grown cultures or multiple-nutrient-starved batch cultures were suddenly subjected to excess glucose, maximum growth rates were reached immediately. This immediate response to nutrient upshift suggests that the protein-synthesizing machinery is constitutively regulated. In total, these results are strong evidence that strain RB2256 possesses novel physiological and molecular strategies that allow it to predominant in natural seawater.**

Natural seawater normally contains 10^5 to 10^6 bacterial cells per ml (44). The bacterial composition of natural seawater is regulated by such factors as grazing pressure (40, 47), nutrient limitation or starvation (19, 21), and various other physicochemical stresses (e.g., temperature variation, oxidative stress, etc.). In response to starvation in the normally oligotrophic seawater environment, some marine bacteria form nongrowing ultramicrocells which are highly resistant to secondary stresses (20, 31). Although starved heterotrophic ultramicrobacteria exhibit enhanced survival, they cannot become the predominant biomass in natural bacterial assemblages as long as they remain in the nongrowing phase. In contrast, marine bacteria that can survive oligotrophic conditions while maintaining cell growth have the opportunity to become the predominant species in natural seawater.

Sphingomonas sp. strain RB2256 was isolated from natural seawater in Resurrection Bay, Seward, Alaska, by an extinction dilution method (2, 3, 38). For further information on this organism, see reference 37. Strain RB2256 was isolated from 10^5-times-diluted natural seawater, indicating that 1 ml of the original seawater contained at least 10^5 cells of strain RB2256, making this organism a numerically important member of the total population in Resurrection Bay (approximately 1.0×10^6 cells per ml [38]). Moreover, subsequent 16S rRNA probing work suggested the presence of similar species in the North Sea (37).

Strain RB2256 is a gram-negative, yellow-pigmented, rod-shaped bacterium (37, 38). It is catalase and oxidase positive and is resistant to kanamycin (85 μg/ml) and streptomycin (200 μg/ml) (38). Growing cells of strain RB2256 were shown to have high-affinity uptake systems under nutrient-limiting conditions (38, 39), similar to that of starved cells of a marine bacterium such as *Vibrio* sp. strain S14 (1, 25). This characteristic may help strain RB2256 to scavenge the very small amounts of bioavailable substances in the marine oligotrophic environment. As the cell size of strain RB2256 is <0.08 μm^3 (see Table 1 and reference 38), this organism belongs to the ultramicrobacteria as proposed by Torrella and Morita (42) and more recently reviewed by Gottschal (9). Grazing of bacterial cells by heterotrophic nanoflagellates has been found to be size selective in that ultramicrobacteria, such as strain RB2256 and starved microcells, may more easily escape grazing by heterotrophic nanoflagellates than do larger bacteria (7, 40). It therefore appears that strain RB2256 is highly evolved for surviving both grazing pressures and nutrient limitation in oligotrophic marine environments.

The growth behavior so far identified for strain RB2256 raises important questions concerning its physiotype. It has been suggested that more detailed studies of strain RB2256, which apparently retains its low growth rate and small size under different growth conditions (37), may provide novel information on the characteristics of typical marine pelagic bacteria. While in laboratory cultures strain RB2256 grows heterotrophically like *Escherichia coli* or a marine *Vibrio* sp., it is essential to address whether the physiology of RB2256 is fundamentally different from that of the well-characterized ma-

* Corresponding author. Mailing address: School of Microbiology and Immunology and Centre of Marine Biofouling and Bio-Innovation (CMBB), The University of New South Wales, Sydney 2052, Australia. Phone: 61-2-385 2102. Fax: 61-2-313 6528. Electronic mail address: s.kjelleberg@unsw.edu.au.

† Present address: AMRAD Operations Pty. Ltd., Boronia, Victoria 3155, Australia.

rine species isolated so far. As the apparent DNA content of RB2256 is one-third that of the *E. coli* chromosome (38), it is of particular interest to elucidate how strain RB2256 responds to nutrient starvation and other environmental stresses and whether nutrient-limited and starved cells develop a general cross protection from stress, as is found for most heterotrophic nondifferentiating bacteria (20). Such responses appear to aid in the successful adaptation to, and survival in, nutrient-limiting and starvation conditions.

In this study, we initiated an examination of the responses of RB2256 to shifts in growth and environmental conditions. Such studies provide essential information on the physiological characteristics of a main representative of the marine bacterial community and address earlier hypotheses on distinctly different physiotypes of oligotrophic bacteria (12, 34). The present study examined the effect of starvation on the resistance of batch- and chemostat-grown RB2256 cells to the physiochemical stresses of high temperature, oxidation (hydrogen peroxide), and ethanol. In addition, we studied the effect that starvation and medium richness have on cell morphology and assessed cell growth characteristics in response to the sudden availability of nutrients.

MATERIALS AND METHODS

Organism, growth conditions, and media. The strain used in this study is the heterotrophic marine ultramicrobacterium *Sphingomonas* sp. strain RB2256 (37, 38). The organism was maintained as freeze-dried cultures and as frozen stock cultures at −80°C. In batch culture, RB2256 was grown in a range of different media (VNSS broth [32, 43] without soluble starch, V/10, V/100, V/1000, and nine-salts solution [NSS]) at room temperature (23 to 25°C) on a rotary shaker. VNSS contained the following components per 1,000 ml of NSS: peptone, 1.0 g; yeast extract, 0.5 g; glucose, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; Na_2HPO_4, 0.01 g. NSS contained the following (per 1,000 ml of deionized water): NaCl, 17.6 g; Na_2SO_4, 1.47 g; $NaHCO_3$, 0.08 g; KCl, 0.25 g; KBr, 0.04 g; $MgCl_2 \cdot 6H_2O$, 1.87 g; $CaCl_2 \cdot 2H_2O$, 0.041 g; $SrCl_2 \cdot 6H_2O$, 0.01 g; H_3BO_3, 0.01 g. To prepare the V/10, V/100, and V/1000 media, VNSS broth without soluble starch was diluted 10, 100, and 1,000 times, respectively, with sterilized NSS (32). The pH of all media was adjusted to 7.8. Concentrations of dissolved organic carbon in these media were 800 (VNSS), 80 (V/10), 8 (V/100), and 0.8 (V/1000) mg/liter.

For chemostat cultivation and some batch cultures (see Results), a completely defined artificial seawater (ASW) basal medium was used. This medium had the following composition (grams per liter of deionized water): NaCl, 24; Na_2SO_4, 4; KCl, 0.68; KBr, 0.1; H_3BO_3, 0.025; NaF, 0.002; $MgCl_2 \cdot 6H_2O$, 10.8; $CaCl_2 \cdot 2H_2O$, 1.5; $SrCl_2 \cdot 6H_2O$, 0.024; $NaHCO_3$, 0.2; $NaHPO_4$, 0.04; NH_4Cl, 0.5; trace element solution, 1.0 ml/liter; vitamin solution, 1.0 ml/liter. The bicarbonate, phosphate, ammonium chloride, and trace element solutions were autoclaved separately and added as small, concentrated volumes to the autoclaved basal salts after cooling to room temperature. The vitamin solution was filter sterilized and added separately. The trace element solution contained the following (milligrams per liter of Milli-Q-purified water): $CuCl_2 \cdot 2H_2O$, 15; $NiCl_2 \cdot H_2O$, 25; $Na_2MoO_4 \cdot 2H_2O$, 25; $ZnCl_2$, 70; $MnCl_2 \cdot 4H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 120. It also contained $FeCl_3$ at 4.0 g/liter, EDTA at 2.0 g/liter, and 6.5 ml of 25% HCl. The final solution was neutralized to pH 7 to 8 with concentrated NaOH. The vitamin solution contained the following (milligrams per liter of Milli-Q-purified water): *p*-aminobenzoic acid, 50; pyridoxine-HCl, 100; thiamine-HCl, 50; riboflavin, 50; nicotinic acid, 50; D-Ca-pantothenate, 50; lipoic acid, 50; nicotinamide, 50; vitamin B_{12}, 50; biotin, 20; folic acid, 20. For growth in the chemostat, glucose was used as the sole carbon and energy source (0.54 g/liter; 3 mM) and was also added as a separately autoclaved, concentrated solution.

In batch cultures grown on ASW medium, alanine, acetate, or glucose (3 to 15 mM) from an autoclaved stock solution was added as a growth substrate. In such cultures, morpholinepropanesulfonic acid (MOPS) buffer (4 g/liter) was included in the basal medium to maintain the pH between 6.9 and 7.6. For plate counting of chemostat-grown cells, ASW-glucose (ASWG) medium was used, which consisted of ASW medium with 3 mM glucose and 1.5% agar. For growth under glucose limitation, the bacteria were grown at 30°C in a chemostat with a working volume of 1,050 ml with constant automatic adjustment of the pH with a sterile 0.25 M NaOH solution.

For determination of the optimum growth temperature, RB2256 was grown in ASWG and ASWG broth plus 8 g of yeast extract per liter and maximum specific growth rates were determined at various temperatures.

Starvation conditions. Cells grown in VNSS and V/10 were harvested in the log phase (4×10^8 cells per ml at an optical density at 610 nm [OD_{610}] of 0.1 in VNSS or 0.07 in V/10) by centrifugation (8,000 × *g*, 10 min), washed once, and resuspended in sterilized NSS buffered with MOPS (pH 7.8). Starvation of chemostat-grown cells was initiated by transferring samples from the glucose-limited culture into sterile conical flasks without centrifugation or resuspension. Multiple-nutrient starvation refers to the use of media devoid of carbon, nitrogen, and phosphorus. All starvation experiments were carried out at 25°C.

Viability measurements. Viable counts in batch-grown cultures were estimated from the number of CFU on VNSS agar, both with and without streptomycin (200 μg/ml) and kanamycin (85 μg/ml), by the drop plate method (13). There were no significant differences between CFU counts with and without antibiotics. Growth and final cell densities were monitored by determining OD_{610}. Viable counts of chemostat-grown cells were obtained as CFU counts on ASWG agar.

Stress exposure protocols. Stress resistance was assessed by recording the number of CFU of RB2256 challenged with the following stresses: (i) heat stress, as experienced by a shift from 25°C (or 30°C for chemostat-grown cells) to 56°C; (ii) ethanol (20%) exposure; (iii) exposure to hydrogen peroxide (25 mM; freshly prepared from a 30% commercial stock solution). A range of high temperatures and ethanol and hydrogen peroxide concentrations were tested on RB2256 to determine a satisfactory level for stress experiments. Exposure to ethanol or hydrogen peroxide was done at 25°C. Stress resistance was determined on samples from log-phase batch-grown cells in VNSS and V/10, on samples taken within 3 min following withdrawal from the chemostat, and on cells taken at appropriate times during subsequent multiple-nutrient starvation. As cells grown in VNSS or V/10 showed equivalent stress responses, V/10 was adopted for cell growth. The number of repeats for each stress experiment is indicated in each figure legend.

Morphological observations. Cell size and shape were determined by 4′,6-diamidino-2-phenylindole (DAPI) epifluorescence photomicrography and scanning electron microscopy. At appropriate times, samples were withdrawn from growing or starving cultures and fixed in 0.5% (wt/vol) glutaraldehyde. Fixed cells were pretreated with 0.1% Triton X-100 to improve stain penetration (38), stained with the fluorochrome DAPI, and observed as described by Porter and Feig (35), with an epifluorescence microscope (Olympus). For scanning electron microscopy, growing cells were sampled from various media and filtered onto 0.2 μm-pore-size Nucleopore membrane filters (1-cm diameter). Cells on filters were transferred to 75, 50, and 25% NSS; dehydrated once in 50, 70, 80, and 90% ethanol and twice in 100% ethanol; treated with 100% isoamyl acetate; and dried at the critical point. Finally cells were sputter coated with 15-nm Au particles, and viewed on a JSM-T200 (JEOL) at a magnification of ×5,000 to ×10,000.

RESULTS

Growth rate, size, and viability of batch- and chemostat-grown cells. *Sphingomonas* sp. strain RB2256 was grown in chemostat and batch cultures to determine what effect medium richness has on growth rate, cell size, and viability. Only minor changes in the maximum specific growth rate were observed in batch-grown cells in response to different concentrations of organic compounds in complex media. These growth rates were 0.16, 0.15, 0.15, 0.15, and 0.13 h^{-1} at 25°C (Table 1) in VNSS, V/10, V/100, V/1000, and NSS, respectively. While growth rates remained constant, the final cell numbers varied with medium richness, cell number being directly proportional to the concentrations of organics in the different media (Table 1). Consistent with the observed small differences in growth rates, only relatively minor changes in cell volume (Table 1) and shape (data not shown) were noted during growth in these media. Growing cells always appeared as short, nonmotile rods. A similar cellular morphology was also observed when cells were grown in defined ASW medium in a glucose-limited chemostat at a dilution rate of 0.027 h^{-1} (data not shown). These results show that RB2256 grows slowly irrespective of medium richness and that growing cells maintain a fixed cell volume and shape.

Multiple-nutrient (carbon, nitrogen, and phosphorus) starvation (NSS medium) had a minimal effect on growth rate and cell size in comparison with growth in VNSS (Table 1). Furthermore, while cells were slightly smaller, they remained short rods (data not shown). When batch-grown cells were starved, during the first 24 h of starvation, CFU increased approximately 1.5-fold (data not shown); probably because of the completion of ongoing rounds of DNA replication followed by cell division and partitioning. In addition, the OD_{610} decreased by approximately 20% (data not shown), which is consistent

TABLE 1. Characteristics of RB2256 cells batch grown in VNSS, V/10, V/100, V/1000, and NSS media

Medium	Final density (cells/ml)	Growth rate (h^{-1})	Mean cell vol (μm^3) ± SD (no. of cells examined)		Cell shape
			DAPI	SEM[a]	
VNSS	9.0×10^9	0.16	0.07 ± 0.02 (61)	0.04 ± 0.01 (30)	Shortrods
V/10	7.0×10^8	0.15	0.04 ± 0.02 (60)	0.04 ± 0.01 (25)	Shortrods
V/100	2.3×10^7	0.15	0.03 ± 0.01 (60)	0.02 ± 0.01 (30)	Shortrods
V/1000	3.4×10^6	0.15	0.03 ± 0.01 (62)	0.02 ± 0.01 (25)	Shortrods
NSS	6.7×10^5	0.13	0.03 ± 0.01 (61)	ND[b]	Shortrods

[a] SEM, scanning electron microscopy.
[b] ND, not determined.

with starved cells being slightly smaller than growing cells (Table 1). After 24 h, both CFU and OD_{610} remained constant throughout the starvation period (168 h) (data not shown). Viability in samples taken from the glucose-limited chemostat changed very little during the first 168 h, and CFU dropped thereafter to stabilize at a level of 1 to 3% between 10 and 30 days after the onset of starvation (data not shown).

Stress responses of batch-grown cells. Growing and starved cells were examined for sensitivity to temperature, hydrogen peroxide, and ethanol stress. To evaluate the effect of starvation, log-phase cells were harvested and resuspended in nutrient-free NSS medium and starved for up to 168 h prior to being stressed (see Materials and Methods). To examine heat stress sensitivity, cells were subjected to heat stress by rapid transfer from 25 to 56°C for 15, 30, and 60 min (Fig. 1). All cells were remarkably heat tolerant, surviving, albeit at 10^{-3}%, for up to 60 min at 56°C. Starvation did not confer additional heat tolerance, with starved cells being essentially as tolerant as actively growing cells.

The ability of RB2256 to withstand high temperatures is also reflected in its growth temperature profile (Fig. 2). While RB2256 was able to grow at 5°C at a very low rate of approximately 0.01 h^{-1} (results not shown), it was also able to grow at temperatures above 45°C (Fig. 2).

Cells were remarkably resistant to hydrogen peroxide, being able to withstand 25 mM for up to 60 min (Fig. 3). Qualitatively, the survival patterns were similar to those observed in heat stress experiments, with growing and starved cells being able to survive the stress equally well. In a fashion similar to the survival of cells exposed to high levels of heat and hydrogen peroxide, RB2256 cells were intrinisically resistant to a high level of ethanol (20%) (Fig. 4). However, while growing cells were resistant to ethanol (<1% at 60 min), starved cells were even more resistant (about 100-fold). Evidently, 60 min of exposure to 20% ethanol poses little harm to RB2256 cells, especially those that had been starved for up to 168 h. Interestingly, cells that have just been starved ($t = 0$) appear to be extremely sensitive to ethanol, with survivors being undetectable after 30 min of exposure to ethanol (Fig. 4). An explanation for this phenomenon is unknown; however, Eguchi et al. are currently examining the accumulation of excreted metab-

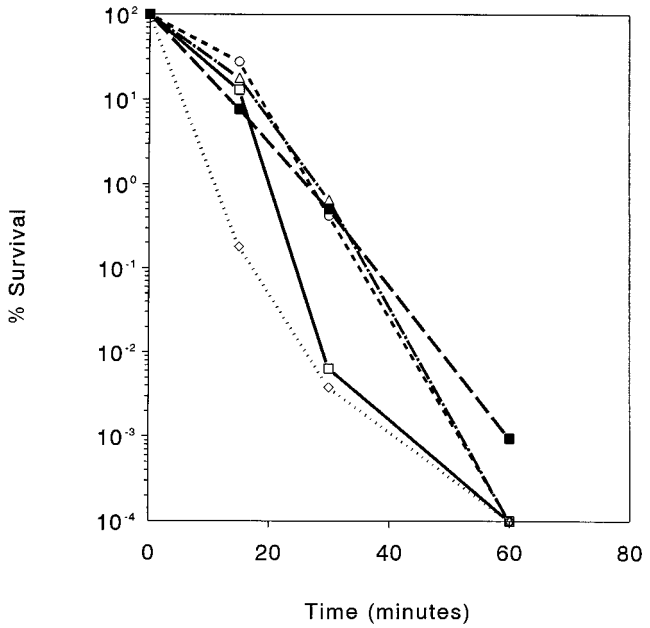

FIG. 1. Percent survival of *Sphingomonas* sp. strain RB2256 CFU following a heat shock in the form of a shift from 25 to 56°C with continuous exposure at 56°C for up to 60 min. Samples were taken directly from exponentially growth batch culture cells (□) or washed cells starved for various periods of time, i.e., 0 (◇), 24 (○), 72 (△), or 168 (■) h. Experiments were performed at least five times, and the results of a typical experiment are shown.

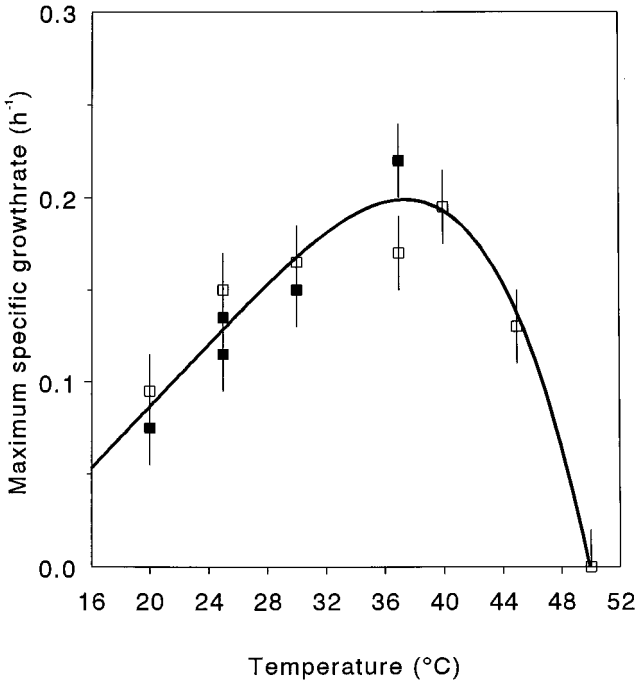

FIG. 2. Maximum specific growth rates of *Sphingomonas* sp. strain RB2256 in batch cultures at different temperatures in ASWG medium plus 8 g of yeast extract per liter (■) and in ASWG medium (□). Experiments were performed at least twice, and standard error bars are shown.

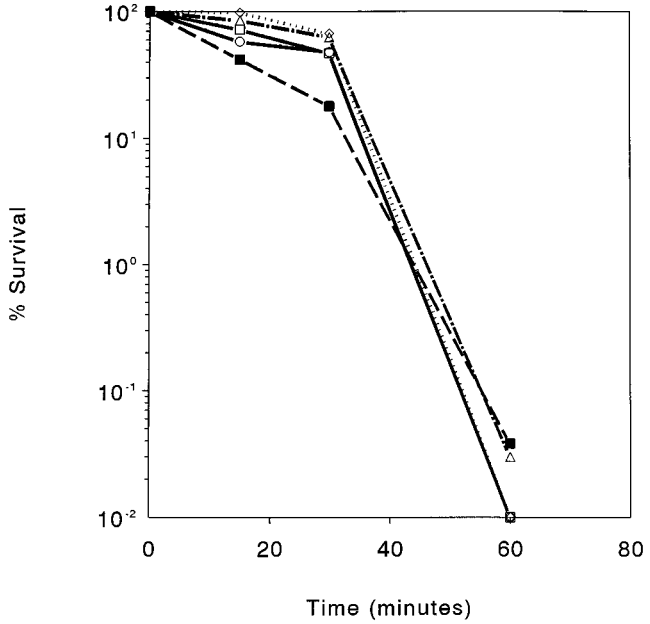

FIG. 3. Percent survival of *Sphingomonas* sp. strain RB2256 CFU following exposure to 25 mM hydrogen peroxide for up to 60 min. Samples were taken directly from exponentially growing batch culture cells (□) or washed cells starved for various periods of time, i.e., 0 (◇), 24 (○), 72 (△), or 168 (■) h. Experiments were performed at least five times, and the results of a typical experiment are shown.

olites in the culture medium that may contribute to cell viability (5).

Stress responses of chemostat-grown cells. The sensitivity of strain RB2256 to hydrogen peroxide, ethanol, and heat stress (30 to 56°C) was examined with glucose-limited, chemostat-grown cells grown at a steady-state dilution rate (i.e., specific growth rate) of 0.027 h^{-1}. As shown in Fig. 5, cells taken from the chemostat and exposed directly (without a washing procedure) to the various stress conditions were extremely stress resistant. Viability never decreased below 50% for any treatment up to a 60- to 75-min exposure time. Furthermore, stress resistance was independent of growth phase, with actively growing and starved cells remaining largely unaffected by the stress applied. In addition, these results also show that while batch-grown cells are highly stress resistant, chemostat-grown cells are even more resilient.

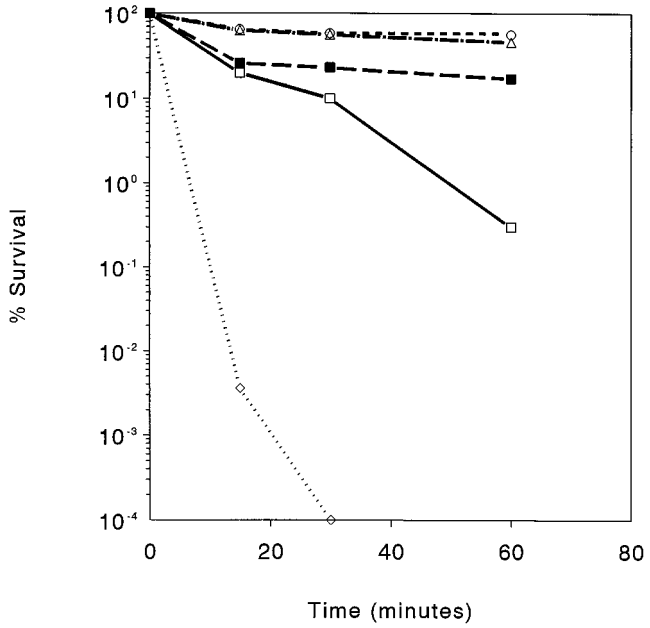

FIG. 4. Percent survival of *Sphingomonas* sp. strain RB2256 CFU following exposure to 20% (vol/vol) ethanol for up to 60 min. Samples were taken directly from exponentially growing batch culture cells (□) or washed cells starved for various periods of time, i.e., 0 (◇), 24 (○), 72 (△), or 168 (■) h. Experiments were performed at least five times, and the results of a typical experiment are shown.

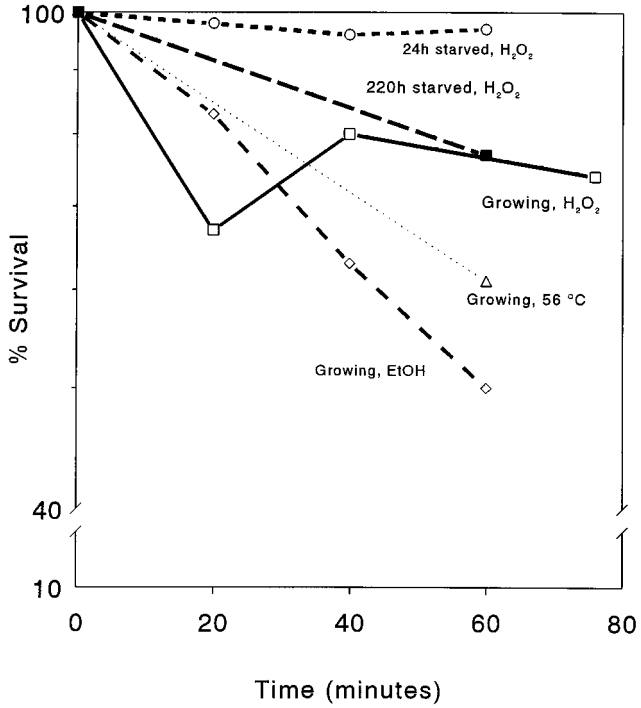

FIG. 5. Percent survival of *Sphingomonas* sp. strain RB2256 CFU grown in glucose-limited continuous culture at a dilution rate of 0.027 h^{-1}. Cells were transferred immediately from the chemostat into test tubes and exposed to hydrogen peroxide (25 mM, □), heat (56°C, △), or ethanol (EtOH; 20%, ◇) for 0 to 60 (or 76) min. In the case of hydrogen peroxide exposure, cells were also subjected to 24 h (○) or 220 (■) h of starvation prior to the stress exposure. Chemostat experiments were performed twice, and CFU were counted five times for each time point. A standard deviation of 15 to 18% was observed for each time point.

Transitions to growth in the presence of excess glucose. Experiments were performed to examine the response of strain RB2256 to the sudden availability of a nutrient source. Samples were taken from glucose-limited chemostat cultures grown at a dilution rate of 0.027 h^{-1}, supplied with 3 mM glucose, and incubated further at 30°C as a batch culture. Growth was recorded in such cultures by measuring OD_{660} from the moment of glucose addition. Despite the low rate of growth in the chemostat, cells grew at a rate of 0.185 h^{-1} immediately following transfer to conditions of glucose excess (Fig. 6). When the same experiment was repeated with OD_{660} readings taken at much shorter intervals of 6 to 10 min (Fig. 6, insert), it was evident that the OD_{660} increase occurred immediately within the first 6 min.

The effect of a sudden addition of glucose was also examined in the following way. Glucose-limited, chemostat-grown cultures were allowed to grow for approximately 1 generation

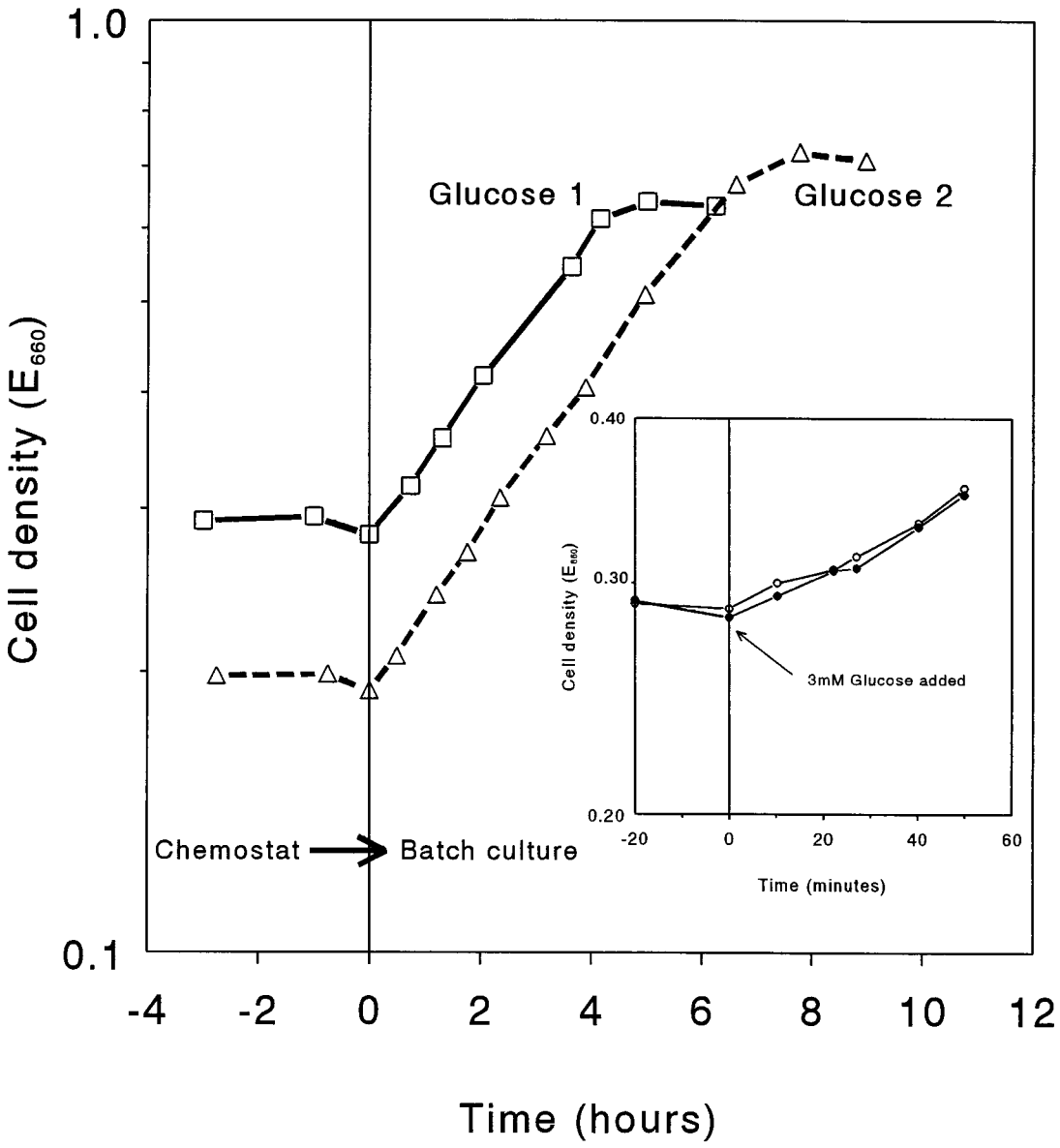

FIG. 6. Growth of *Sphingomonas* sp. strain RB2256 following addition of 3 mM glucose to 20-ml chemostat samples in conical flasks placed on a shaking incubator at 30°C. The experiment was performed 4 days after startup of the first chemostat run (Glucose 1, □) and 6 days after startup of the second chemostat run (Glucose 2, △). The insert shows the growth response (in duplicate) 12 days after the startup of the first chemostat run recorded at much shorter time intervals. Chemostat experiments were performed twice, and CFU were counted five times for each time point. A standard deviation of 15 to 18% was observed for each time point.

time period on alanine in a batch culture, and the 3 mM glucose was added. Cells exposed to this regimen began to grow instantaneously at a maximum rate (0.18 to 0.20 h^{-1}; Fig. 7). During the first 30 to 40 min after glucose addition, cell density increased at an even higher rate (0.28 to 0.29 h^{-1}), probably because of rapid initial intracellular polyglucose accumulation (36). In a fashion similar to sudden glucose supplementation, when cells were transferred from the glucose-limited chemostat into acetate- or alanine-containing ASW batch cultures, growth was immediately established at rates of 0.02 and 0.04 h^{-1}, respectively (data not shown). These results indicated that regardless of the nutrient source encountered (glucose, acetate, or alanine), RB2256 is able to resume its maximum growth rate immediately upon supplementation to nonlimiting concentrations, without any lag phase, as defined by the nutrient source and the growth temperature.

DISCUSSION

Heterotrophic bacteria usually adapt their growth rate and cell size to both the nature and concentration of organic nutrients in the environment. Recent studies have also demonstrated that most nondifferentiating bacteria display differentiation-like alterations during adaptation to stress, starvation, and growth arrest (20). A large number of genes are involved in such non-growth-related responses in nondifferentiating heterotrophic bacteria (20). The marine low-nutrient-adapted organism studied in this report, *Sphingomonas* sp. strain RB2256, exhibits few similarities to the adaptation displayed by heterotrophic bacteria hitherto studied. In fact, this communication provides results that suggest that strain RB2256 has evolved a genotype with few phenotypic adjustments to changes in the

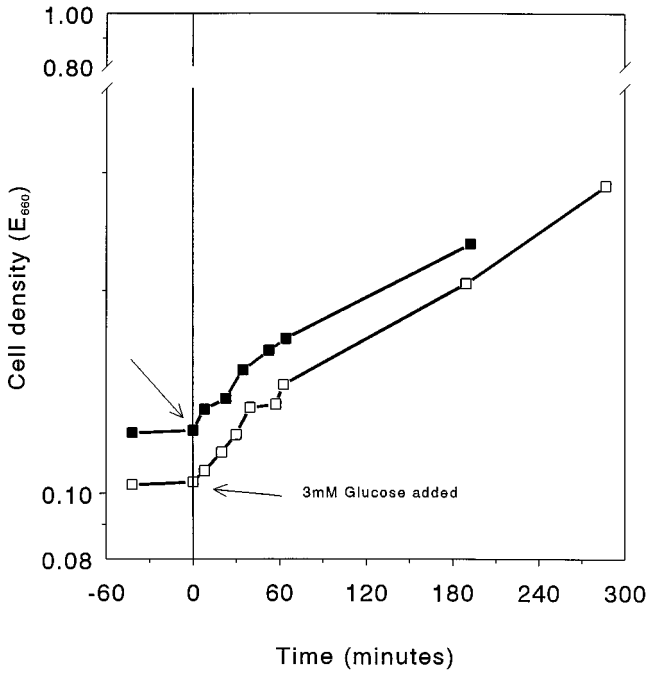

FIG. 7. Growth of *Sphingomonas* sp. strain RB2256 following addition of 3 mM glucose to 20-ml alanine-grown batch cultures in the late log phase (in duplicate). Cells had been transferred from the glucose-limited chemostat directly into the alanine-supplemented batch culture medium. The arrows indicate the time at which 3 mM glucose was added to each batch culture. Chemostat experiments were performed twice, and CFU were counted five times for each time point. A standard deviation of 15 to 18% was observed for each time point.

environment, akin to the presumed characteristics (12, 34) of a model oligotrophic bacterium.

An important way in which bacteria alter their growth rates is by adapting the capacity of protein synthesis to accommodate growth demands (45). Key molecules for such a regulatory mechanism are the protein and RNA molecules (45) of the ribosomes. The number of ribosomes is suggested to reflect variations in the growth rate of the bacterial cell. Interestingly, *Sphingomonas* sp. strain RB2256 showed little variation in its maximum growth rate (0.13 to 0.16 h^{-1}) or cell size in response to a 10-, 100-, or 1,000-fold dilution of complex growth medium (Table 1). It even maintained the same rate of growth in NSS in which a 2,000- to 3,000-times-lower cell number was obtained than in VNSS medium. Although the constancy of the maximum growth rate over such a large range of nutrient concentrations is truly remarkable, it should be noted that the maximum growth rate exhibited by strain RB2256 is relatively low in comparison with those of most bacteria isolated from marine or freshwater communities (2, 10, 24). Other growth studies with strain RB2256 indicated that no other substrates or media could be found supporting higher rates of growth (38). Only much lower rates were observed in defined minimal media with acetate or alanine (this study) or mixtures of several other amino acids (38) and, as would be expected, under substrate limitation in continuous culture. It may be tentatively concluded that strain RB2256 is not genetically geared to high growth rates. Not only does it contain as little as 1.5 fg of DNA per cell (38), which is approximately one-third the size of a single copy of the *E. coli* genome (15), but the organism was also shown to contain only 1 copy of the rRNA operon (4), compared with the 8 to 11 copies in most of the marine organisms tested to date (46).

Experiments with glucose-limited chemostat cultures (D = 0.027 h^{-1}) indicated that when cells of RB2256 were abruptly confronted with excess glucose, maximum growth rates occurred, either in chemostat cultures or after the cells had been transferred to batch cultures. This occurred without a noticeable lag and at rates very similar to those observed in batch cultures grown with excess glucose. Although a rapid increase in growth rate is to be expected, an immediate sevenfold increase, as was observed in this study, is unusual. In earlier studies on the nutrient upshift responses of chemostat-grown *E. coli* (11, 23) and *Klebsiella aerogenes* (41) cultures, it was shown that time is required (from several hours to 2 to 3 doubling times) to increase the rRNA content from the low value at substrate-limited growth to a content typical for growth at the maximum rate. Thus, *E. coli* grown at dilution rates below 0.3 h^{-1} showed an immediate increase in growth rate, but never more than two- to threefold, followed by a gradual further increase to its maximum rate of growth. Similar experiments were performed with *Cytophaga johnsonae* grown under glucose limitation at 0.15 and 0.03 h^{-1} (14). Growth of cells at 0.15 h^{-1} and an upshift to glucose excess resulted in immediate growth at the maximum rate (D = 0.20 h^{-1}), but with cells grown at 0.03 h^{-1}, a 1- to 2-h lag period was observed in which no growth was detected at all, followed by a period of nearly 20 h during which the growth rate gradually increased to 85% of its maximum value. The immediate response by *Sphingomonas* sp. strain RB2256 to nutrient upshift suggests that in this organism an increase in rRNA content does not necessarily take place, perhaps because the organism does not downregulate its protein synthesis machinery during glucose limitation and is possibly not capable of doing so. To substantiate the validity of this suggestion, the rate of protein synthesis has to be studied directly and compared in fast-growing, slowly growing, and starved cells.

Sphingomonas sp. strain RB2256 was the predominate species in Resurrection Bay near Alaska, where the seawater temperature was 4 to 8°C (36). Nevertheless, while RB2256 is psychrotrophic in nature and well adapted to low temperatures, its optimum growth temperature in defined glucose-mineral medium appeared to be between 35 and 40°C and growth was still possible between 44 and 48°C. Moreover, the resistance of cells grown in batch culture at 25°C and subsequently exposed to 56°C is remarkable. After 15 min of exposure to 56°C, 10 to 30% of the initial cell number remained capable of forming colonies on agar plates, with no significant differences between cells taken directly from growing cultures or from cultures starved for up to 7 days. This resistance to temperature extremes exhibited by RB2256 is significantly greater than that observed in other bacteria. Growing cells of *E. coli* K-12 lose viability to less than 1% after 6 min of exposure to 57°C (17). With regard to well-characterized marine isolates, the CFU of cells of *Vibrio* sp. strains S14 and DW1 grown at 26°C decreased by more than 3 log units after 30 min of exposure to 40 and 37°C, respectively (18, 33). In contrast, a much higher temperature of 56°C was required to kill a similar proportion of RB2256 cells.

Batch culture-grown cells of strain RB2256 were also greatly resistant to a high concentration of hydrogen peroxide (25 mM). After 30 min of exposure, at least 50% of the initial population was still capable of forming colonies. The same length of exposure, albeit with 15 mM hydrogen peroxide, resulted in more than 99% loss of viability in *E. coli* cultures (17). Similarly, exponentially growing cells of *Vibrio anguillarum* demonstrated a 10^5-fold reduction in viability after 30 min of exposure to concentrations of hydrogen peroxide as low as 2 mM (30).

The remarkably pronounced stress resistance in strain RB2256 was further highlighted by the results obtained for stress exposure of chemostat-grown cultures. Cells from slowly growing, glucose-limited chemostat cultures retained higher viability following 1 h of exposure to ethanol, hydrogen peroxide, or high temperature than did batch-grown cells: 160, 7,500, and 6×10^5 times more CFU, respectively, than for batch-grown cells. We know of no obvious reason why chemostat-grown cells should be inherently more stress resistant than batch-grown cells. However, the results serve to highlight the remarkable stress resistance of RB2256 during growth under severe nutrient limitation, regardless of the growth constraints placed on them. Moreover, these results complement observations made by others who have shown that bacteria grown at low, nutrient-limited growth rates in chemostats exhibit better starvation survival than the same bacteria grown at higher dilution rates or in batch culture (8).

The marine environment is extremely oligotrophic (26–28), which implies that microorganisms must face significant periods of shortage of carbon and energy (19, 22). Although strain RB2256 seems extremely well adapted to growth at low nutrient concentrations, it is unlikely to escape periods of starvation altogether. No loss in viability was found during the initial 7 days of starvation of strain RB2256 after pregrowth in a batch or 10 days of starvation of chemostat-grown cells. A hallmark of the starvation response in most nondifferentiating heterotrophic bacteria is the development, in a sequential fashion, of cross protection against a series of different stress conditions. In fact, the starved, growth-arrested cell carries a distinct resemblance to the stress-resistant spore stage of differentiating bacteria, such as *Bacillus* species. In this study, the response of starved cells of strain RB2256 to stress conditions differed markedly from that generally observed in starved heterotrophic bacteria (20). There was no development of cross protection against heat or peroxide stress. This is almost invariably observed with other species, such as *E. coli*; *Vibrio* sp. strains S14, DW1, and Ant300; and *Pseudomonas putida* (6, 17, 18, 33, 36). The only instance in which strain RB2256 displayed some degree of protection as a result of a preceding starvation period was during ethanol stress. A possible explanation for this difference may be the nature of the killing and possible repair mechanisms involved. Both heat and hydrogen peroxide are predominantly DNA-damaging agents (29), whereas ethanol most likely affects the cell membrane and thus interferes with regulation of the internal pH, membrane transport, and, indirectly, the translation process (16, 29). Nevertheless, it may be hypothesized that strain RB2256 adds another strategy. That is, that actively growing cells are already substantially resistant to extreme conditions of stress. In fact, this study demonstrates that the stress conditions employed to provoke a loss in the viability of growing cells are significantly more severe than those reported for other strains in studies of stress responses in bacteria.

In conclusion, it is suggested that the marine bacterium *Sphingomonas* sp. strain RB2256 displays a phenotype that apparently lacks or rarely employs inducible systems for growth rate and cell size changes under different nutrient regimens. This is evidenced by the facts that starved cells of this organism generally do not become more resistant to stress conditions than are growing cells and cell size does not alter as a result of starvation-induced growth arrest. Furthermore, growing, as well as nongrowing, cells of strain RB2256 are resistant to conditions of severe stress and appear to display a constitutively resistant phenotype under all growth conditions. We infer that the properties which are considered typical for a "model" oligotrophic bacterium (12, 34) are reflected in the characteristics identified for *Sphingomonas* sp. strain RB2256.

ACKNOWLEDGMENTS

We thank Akira Kawai of Kinki University for continuous interest in this project and Julie Lim for assistance with RB2256 growth and survival experiments.

REFERENCES

1. **Albertson, N. H., T. Nyström, and S. Kjelleberg.** 1990. Starvation-induced modulations in binding protein-dependent glucose transport by the marine *Vibrio* sp. S14. FEMS Microbiol. Lett. **70:**205–210.
2. **Austin, B.** 1988. Methods in aquatic bacteriology. John Wiley & Sons, Chichester, England.
3. **Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson.** 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedure, and initial results. Appl. Environ. Microbiol. **59:**881–891.
4. **Cavicchioli, R., et al.** Unpublished data.
5. **Eguchi, M., et al.** Unpublished data.
6. **Givskov, M., L. Eberl, S. Møller, L. K. Poulsen, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176:**7–14.
7. **Gonzalez, J. M., E. B. Sherr, and B. F. Sherr.** 1993. Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. Mar. Ecol. Prog. Ser. **102:**257–267.
8. **Gottschal, J. C.** 1990. Phenotypic response to environmental changes. FEMS Microbiol. Ecol. **74:**93–102.
9. **Gottschal, J. C.** 1992. Substrate capturing and growth in various ecosystems. J. Appl. Bacteriol. Symp. Suppl. **72:**39S–48S.
10. **Gottschal, J. C., W. Harder, and R. A. Prins.** 1991. Principles of enrichment, isolation, cultivation, and preservation of bacteria, p. 149–196. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.
11. **Harvey, R. J.** 1970. Metabolic regulation in glucose-limited chemostat cultures of *Escherichia coli*. J. Bacteriol. **104:**698–706.
12. **Hirsch, P., M. Bernhard, S. S. Cohen, J. C. Ensign, H. W. Jannasch, A. L. Koch, K. C. Marshall, A. Matin, J. S. Poindexter, S. C. Rittenberg, D. C. Smith, and H. Veldkamp.** 1979. Life under conditions of low nutrient concentrations, p. 261–279. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Berlin Dahlem Conference. Verlag Chemie, Weinheim, Germany.
13. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44:**1246–1247.
14. **Hofle, M. G.** 1984. Transient responses of glucose-limited cultures of *Cytophaga johnsonae* to nutrient excess and starvation. Appl. Environ. Microbiol. **47:**356–362.
15. **Ingraham, J. L., O. Maaloe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Inc., Sunderland, Mass.
16. **Ingram, L. O., and T. M. Buttke.** 1984. Effects of alcohols on micro-organisms. Adv. Microbiol. Physiol. **25:**253–300.
17. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. **170:**3910–3914.
18. **Jouper-Jaan, A., A. E. Goodman, and S. Kjelleberg.** 1992. Bacteria starved for prolonged periods develop increased protection against lethal temperatures. FEMS Microbiol. Ecol. **101:**229–236.
19. **Kirchman, D. L.** 1990. Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. Mar. Ecol. Prog. Ser. **62:**47–54.
20. **Kjelleberg, S., N. Albertson, K. Flärdh, L. Holmquist, A. Jouper-Jaan, R. Marouga, J. Ostling, B. Svenblad, and D. Weichart.** 1993. How do non-differentiating bacteria adapt to starvation? Antonie van Leeuwenhoek **63:** 333–341.
21. **Kjelleberg, S., K. B. G. Flärdh, T. Nyström, and D. J. W. Moriarty.** 1993. Growth limitation and starvation of bacteria, p. 289–320. *In* T. E. Ford (ed.), Aquatic microbiology—an ecological approach. Blackwell Scientific Publications, Oxford.
22. **Kjelleberg, S., M. Hermansson, and P. Marden.** 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Annu. Rev. Microbiol. **41:**25–49.
23. **Koch, A. L.** 1979. Microbial growth in low concentrations of nutrients, p. 261–279. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Berlin Dahlem Conference. Verlag Chemie, Weinheim, Germany.
24. **Mallory, L. M., B. Austin, and R. R. Colwell.** 1977. Numerical taxonomy and ecology of oligotrophic bacteria isolated from the estuarine environments. Can. J. Microbiol. **23:**733–750.
25. **Marden, P., T. Nyström, and S. Kjelleberg.** 1987. Uptake of leucine by a gram-negative heterotrophic bacterium during exposure to starvation conditions. FEMS Microbiol. Ecol. **45:**233–241.

26. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34:**436–441.
27. **Morita, R. Y.** 1990. The starvation-survival state of microorganisms in nature and its relationship to bioavailable energy. Experientia **46:**813–817.
28. **Munster, U.** 1993. Concentrations and fluxes of organic carbon substrates in the aquatic environment. Antonie van Leeuwenhoek **63:**243–274.
29. **Neidhardt, F. C., R. A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18:**295–329.
30. **Nelson, D. et al.** Unpublished data.
31. **Nyström, T., K. Flärdh, and S. Kjelleberg.** 1990. Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **172:**7085–7097.
32. **Nyström, T., P. Marden, and S. Kjelleberg.** 1986. Relative changes in incorporation rates of leucine and methionine during starvation of two bacteria isolated from marine waters. FEMS Microbiol. Ecol. **38:**285–292.
33. **Nyström, T., R. M. Olsson, and S. Kjelleberg.** 1992. Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. Appl. Environ. Microbiol. **58:**55–65.
34. **Poindexter, J. S.** 1987. Bacterial responses to nutrient limitation. Symp. Soc. Gen. Microbiol. **41:**283–317.
35. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
36. **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.
37. **Schut, F.** 1994. Ecophysiology of a marine ultramicrobacterium. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
38. **Schut, F., E. J. de Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59:**2150–2160.
39. **Schut, F., M. Jansen, T. M. Pedro-Gomes, J. C. Gottschal, W. Harder, and R. A. Prins.** 1995. Substrate uptake and utilization by a marine ultramicrobacterium. Microbiology **141:**351–361.
40. **Simek, K., and T. H. Chrzanowski.** 1992. Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. Appl. Environ. Microbiol. **58:**3715–3720.
41. **Tempest, D. W., D. Herbert, and P. J. Phipps.** 1967. Studies on the growth of *Aerobacter aerogenes* at low dilution rates in a chemostat, p. 240–253. *In* E. O. Powell, C. G. T. Evans, R. E. Strange, and D. W. Tempest (ed.), Microbial Physiology and Continuous Culture, proceedings of the 3rd International Symposium. Her Majesty's Stationary Office London.
42. **Torrella, F., and R. Y. Morita.** 1981. Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl. Environ. Microbiol. **41:**518–527.
43. **Vaatraen, P.** 1976. Microbiological studies in coastal waters of the northern Baltic Sea. I. Distribution and abundance of bacteria and yeasts in the Tvarminne area. Walter Andree de Nottbeck Found. Sci. Rep. **1:**1–58.
44. **Van Es, F. B., and L. A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria. Adv. Microb. Ecol. **6:**111–170.
45. **Wagner, R.** 1994. The regulation of ribosomal RNA synthesis and bacterial cell growth. Arch. Microbiol. **161:**100–109.
46. **Wolfe, C. J., and M. G. Haygood.** 1993. Bioluminescent symbionts of the Caribbean flashlight fish (*Kryptophanaron alfredi*) have a single rRNA operon. Mol. Mar. Biol. Biotechnol. **2:**189–197.
47. **Wright, R. T.** 1988. Methods for evaluating the interaction of substrate and grazing as factors controlling planktonic bacteria. Ergeb. Limnol. **31:**229–242.