Survival, Physiological Response, and Recovery of Enteric Bacteria Exposed to a Polar Marine Environment

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Survival, sublethal injury, and recoverability of Escherichia coli, Enterococcus faecalis, Salmonella typhimurium, and Yersinia enterocolitica were investigated by using diffusion chambers over 54 to 56 days of in situ exposure to a polar marine environment (−1.8°C; salinity, 34.5 ppt) at McMurdo Station, Antarctica. Plate counts were used to assess recoverability and injury, whereas direct viable counts (DVCs) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction were utilized to determine substrate responsiveness and respiratory activity, respectively. Tₘ values (times for 10-fold decreases in numbers of recoverable cells) on nonselective medium were ca. 216 to 259 h for E. coli, S. typhimurium, and Y. enterocolitica and 432 h for E. faecalis. Sublethal injury was greater in populations of indicator bacteria than in pathogens. DVCs, CTC reduction, and plate counts indicated progressive increases in viable but nonculturable cells in E. coli, S. typhimurium, and Y. enterocolitica cultures throughout the 54-day exposure. Forty-eight-day exposure of E. coli, S. typhimurium, and Y. enterocolitica by the DVC and CTC methods remained within 1% of inoculum values during 54 days of exposure, indicating some long-term persistence in the viable-butanineutralizable state. Percentages of respiring E. coli and S. typhimurium increased significantly upon addition of nutrients at all temperatures tested, indicating that nutrient availability rather than temperature limited enteric bacterial activity in this very cold environment. Large nutrient inputs to low-temperature marine environments may thus allow for the long-term persistence of enteric bacteria in a nonrecoverable state.

In the last few decades, the persistence of human enteric pathogens and indicator organisms has assumed increasing environmental and public health significance, particularly in environments considered pristine (11, 20, 33, 49). Sewage is often discharged untreated into low-temperature marine environments. Approximately 90% of marine environments are 5°C or less, with polar regions constituting roughly 14% of the earth’s surface (34). In polar environments, release of untreated sewage is primarily dictated by the logistical difficulties and expenses associated with treatment in isolated, relatively small communities (6, 19, 20). Polar regions (>66°33' latitude) also represent the low-temperature extremes for coastal marine environments, with seawater frequently poised at, or near, its freezing point (ca. −1.8°C). Particularly in areas where fast ice forms, water column temperature is stable, with solar radiation being highly attenuated and seasonal (38). In addition, community primary and secondary production is relatively large and highly seasonal (3, 17, 48). Survival of enteric bacteria in these environments is generally enhanced by reduced grazing rates of heterotrophic nanoflagellates and larvae, in addition to the effects of lower seawater temperatures (2, 39, 40, 42).

Factors affecting the survival of allochthonous bacteria in marine environments include predation, osmotic stress, solar radiation, nutrient availability, bacteriophage, algae, autochthonous microbial toxins, hydrostatic pressure, growth phase, and temperature (8, 11, 14, 25). Lower temperatures have been shown to extend survival of enteric bacteria, and it has been frequently reported that temperature is the most important factor in predicting fecal coliform survival in marine environments (2, 5, 12, 28, 51).

Techniques for the enumeration of fecal indicator organisms and specific pathogens from marine environments are widely accepted (1). Recent studies describing the persistence of human enteric bacteria in aquatic environments have demonstrated that many of these organisms enter an altered physiological state termed viable but nonculturable (VBNC) (11, 24, 44). This is commonly defined as the inability to form colonies on a given solid medium while remaining active by direct-viable-count (DVC) methods. Stressors within aquatic environments also induce sublethal physiological and structural changes, termed injury, in enteric bacteria. In this injured state, bacteria are unable to reproduce under conditions which allow for growth of uninjured cells, including restrictive temperatures and the presence of selective agents (30). As an example, a VBNC bacterium may appear viable by DVC methods yet not form colonies on a nonselective growth medium which normally supports its growth. If this bacterium can form colonies on nonselective medium but not on medium which contains selective agents to which the organism is normally resistant, it is termed injured. Since the vast majority of enumeration techniques for fecal indicator and pathogenic bacteria require cultivating with selective media, the quantitative detection of viable organisms in the environment may be seriously compromised. Previous marine studies have demonstrated that cold shock, starvation, and possibly osmotic shock result in sublethal injury and the VBNC response (25, 29, 35, 44). Most of these experiments have used laboratory microcosms, which are subject to "bottle effects," including the termination of solute exchange with the environment (13, 32). In addition, the relationship between environmental stress, sublethal injury, and the VBNC state remains largely unresolved.

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Considering that the minimum temperature at which growth of *Escherichia coli* has been reported is ca. 7.5°C, the question of whether most sewage bacteria can actively grow in situ but whether they can adapt and persist through the formation of dormant, or metabolically quiescent, cells which can respond to subsequent increases in temperature and/or substrate concentrations (16, 24, 47, 52). It is also of interest whether, and to what degree, these cells display sublethal injury or VBN responses.

In order to determine the long-term physiological responses and recoverability of enteric bacteria exposed to extremely low-temperature marine environments, we assessed survival, sublethal injury, recoverability, and the VBN state of enteric bacteria under polar marine conditions. Experiments were performed by using diffusion chamber exposure of two indicators (*E. coli* and *Enterococcus faecalis*) and two pathogenic (*Salmonella typhimurium* and *Yersinia enterocolitica*) bacterial species in situ for periods of 54 to 56 days at McMurdo Station, Antarctica. The *E. coli* strain used was enterotoxigenic, representing both an indicator and a pathogen. Recoverable and injured cells were enumerated by plate counts on selective and nonselective media. Viable cells numbers and substrate responsiveness were determined by use of the DVC method (27).

In addition, tetrazolium reduction with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which indicates active respiration, was used to identify respiring cells (43).

### MATERIALS AND METHODS

#### Organisms, growth conditions, and exposure

By using 0.1 ml of overnight starter culture inocula, 100-ml cultures of enterotoxigenic *E. coli* Tsx432, *S. typhimurium* SL320, *Y. enterocolitica* O:8, and *E. faecalis* were grown in tryptone-lactose-yeast extract (TLY) in 250-ml Erlenmeyer flasks at 37°C, with shaking at 150 rpm. Cultures were grown to early stationary phase (20 h) as monitored by *A*₅₄₀. Cells were pelleted at 3,000 × g for 10 min and washed twice at ca. 20°C in filter-sterilized (1- and 0.22-μm-pore-size filters), aged seawater (FSASW) collected weekly from Winter Quarters Bay, Antarctica. Cells were then resuspended at a concentration of ca. 10⁷ cells per ml (10¹⁰ CFU/ml for *E. faecalis*) and injected slowly into duplicate 30-ml diffusion chambers (31). Time zero samples were taken, and chambers were then simultaneously suspended at a depth of 1 m in a constant-flow, 1,240-liter circulating aquarium (D [dilution rate] = 3.92 h⁻¹) at McMurdo Station, Antarctica (166°40′E, 77°51′S) during the austral summer of 1992 (October through December) for periods of up to 64 days. The aquarium was fed with seawater directly from Winter Quarters Bay, and the tank contained no net metal fittings. Temperature and salinity were –1.8°C and 34.5 ppt, respectively, and remained constant throughout the experiment. Subsamples for all assays (total, 0.5 ml) were removed with a sterile 1- or 5-ml syringe, kept at the in situ temperature, and transported to the laboratory immediately. Cells were diluted in –1.8°C FSASW, allowed to reach ca. 25°C over a period of 1 h, and plated for all assays described below.

#### Plate counts and injury

Dilutions of all cultures were membrane filtered or spot- or spread-plated onto various media. *E. coli, S. typhimurium,* and *Y. enterocolitica* were plated on TLY agar with 0.1% deoxycholate (TLYD; selective) and on TLY agar without deoxycholate (nonselective). *E. faecalis* was plated on brain heart infusion agar (BHI agar; nonselective) and mE agar (selective). Standard incubation was at 25°C, except for *E. faecalis*, which was incubated at 37°C. All colonies were counted at 24, 48, and 144 h, or until CFU no longer increased. Atypical or slowly growing colonies which formed were periodically picked from plates after various periods of exposure and streaked onto either tryptone-7 agar (*E. coli*), salmonella-shigella (SS) agar (*S. typhimurium*), Yersinia-selective agar (*Y. enterocolitica*), or mE agar (*E. faecalis*) to check for contamination. Atypical colonies of *E. coli* and *S. typhimurium* were also inoculated on triple sugar iron (TSI) agar. No contamination was indicated in any of the chambers by this method with incubations at 37°C. *E. coli*, *S. typhimurium*, and *Y. enterocolitica* cells exposed for 54 days were incubated at –1.8, 8, 20, and 37°C for up to 15 days to determine optimal plating recovery temperatures. Sublethal injury was calculated as (CFU_nonselective – CFU_early/CFU_nonselective) × 100, as described previously (45). T₀₉₀ and T₀₉₀ decay times (rates required for 1- and 2-order-of-magnitude reductions in recoverable cell numbers, respectively) and diffusion chamber volume turnover times were calculated as described previously and are included here for comparative purposes (28, 51).

**DVCs.** Aliquots of *E. coli, S. typhimurium,* and *Y. enterocolitica* (0.1 ml) were diluted into 0.9 ml of FSASW at –1.8°C and assayed for substrate responsivity by the method of Kogure et al. (27), as modified by Singh et al. (46). Final concentrations of components were as follows: 0.025% yeast extract, 0.3% Casamino Acids, and 0.002% nalidixic acid. After a 2-h period of acclimation at 25°C, incubation was continued at 37°C for 8 h at 100 rpm. Incubations were stopped by addition of 0.1 ml of 37% formaldehyde. Cells were then filtered and stained with 0.01% acridine orange (AO) in 2 mM Tris buffer (pH 7.0) for 3 min. Elongated (DVC-positive) [DVC(+)] as well as total cells (DVCDC) were enumerated by epifluorescence microscopy.

Aliquots of all organisms (0.1 ml) were assayed for electron transport (respiration) by the method of Rodriguez et al. (43) with a final concentration of 5 mM CTC in FSASW. CTC incubations were at 37°C and 100 rpm for 4 h after a 2-h period of acclimation at 25°C. *E. coli, S. typhimurium,* and *Y. enterocolitica* cells exposed for 48 days were incubated at –1.8, 8, 20, and 37°C with and without addition of 0.1× TLY (final concentration, ca. 3.5 g of organic carbon per liter) to determine whether temperature or nutrient availability limited in situ cellular respiration. Incubations were terminated with formaldehyde as described above. Bacterial suspensions were filtered and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml) for 10 min, and cells containing fluorescent CTC-formazan deposits (CTC-positive cells) [CTC(+) as well as total cells, were enumerated by epifluorescence microscopy.

All epifluorescence counts were performed with Nuclepore 0.22-μm-pore-size, preblackened polycarbonate filtration membranes (no. 110656) and a Zeiss standard 16 microscope equipped with a 100-W U.V. mercury lamp (Opti-quip 1500). Zeiss filter set no. 48-77-09 (BP 490, FT 510, and LP 520) for AO was used for DVCs, while set no. 48-77-01 (BP 365/11, FT 395, and LP 397) was used for CTC and DAPI counts. During CTC counts, total cells were first counted with the DAPI filter set and then CTC(+) cells were enumerated by switching to the DVC filter set. Formaldehyde (3.7%)-killed CTC and DVC assay controls were used for all organisms. Control experiments also indicated no observable increases in total cell numbers with 8-h (DVC) and 4-h (CTC) incubations as described above. A minimum of 800 cells in at least eight fields were counted for each sample, and cell size was noted by using a calibrated ocular grid. Error was calculated as described previously (22).

In order to determine diffusion rates of metabolites across chamber membranes in situ, rhodamine diffusion across the chamber membranes was assayed by using a sterile control.
chamber. Also, in order to determine decreases in diffusion rates due to membrane fouling, diffusion chambers in which E. coli, S. typhimurium, and E. faecalis had been exposed for 64 days were emptied, but not flushed, and 30 ml of 1 mM rhodamine 6G in FSASW (molecular weight, 479.0) was injected. Chambers were placed in situ, and diffusion was monitored by measuring Asat of subsamples. Readings were corrected for dilution due to sampling and plotted against time in situ. A Beckman DU 74000 spectrophotometer was used for all absorbance measurements.

Chemicals, media, and equipment. Rhodamine 6G, α-lactose, Tris base, Tris-HCl, DAPI, AO, 37% formaldehyde, nalidixic acid, sodium hydroxide, and deoxycholate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Yeast extract, Casamino Acids, agar, tryptic soy broth without dextrose, BHI broth, and mE agar were from Difco Laboratories (Detroit, Mich.). CTC was obtained from Polysciences, Inc. (Warrington, Pa.). All cell manipulations for low-temperature incubations (8 and −1.8°C) were performed in a refrigerated environmental room.

RESULTS

Recoverability and injury. Upon exposure to the polar marine environment, all bacteria examined demonstrated declining recoverability, determined by colony formation, in addition to increases in sublethal injury as assessed by the TLY/TLYD CFU ratio (Fig. 1 and 2). The fraction of slowly growing colonies that required >24 h for colony formation also generally increased with time of exposure for all organisms on all media used (data not shown). Sublethal injury on TLYD decreased rapidly throughout the 54-day exposure, with a T50 of approximately 336 h (Fig. 1A). Recoverability on TLY indicated a T50 of approximately 480 h. Sublethal injury rapidly increased to 96% after 4 days of exposure and then remained between 92 and 98.5% throughout the remainder of the 54-day exposure (Fig. 2).

S. typhimurium recoverability on TLYD and TLY decreased by 1 log unit after 4 and 11 days of exposure, respectively. This was followed by continuing decreases in recoverability on both media (Fig. 1B). T50 values for TLYD and TLY recoveries were 408 and 528 h, respectively. Sublethal injury rapidly increased to a maximum of 87% at 4 days, after which a general decreasing trend was observed. After 11 days, injury appeared lower for the S. typhimurium population than for the other test organisms throughout the 54-day exposure (Fig. 2). After 54 days of exposure, plate counts on both TLY and TLYD were approximately 5 × 10⁵ CFU/ml. It was also observed that S. typhimurium colonies changed from convex and round to flat and spreading after 7 days of exposure. These colonies showed reactions typical of S. typhimurium when grown on TSI and SS agar.

Y. enterocolitica plate counts on TLY showed little decrease in the first 4 days, while counts on TLYD decreased by approximately 1 order of magnitude (Fig. 1C). After 4 days, counts on both TLY and TLYD continued to decrease. After 15 days of exposure, plate counts on TLYD and TLY decreased steadily, with T50 values of 360 and 504 h, respectively. As with E. coli and S. typhimurium, injury reached its highest initial value of 80% at 4 days and then increased to 99% because of a sharp 2-log-unit decrease in TLYD counts by 15 days, while TLY counts remained stable (Fig. 2). After 54 days of exposure, plate counts on TLYD and TLY were 10³ and 10⁴ CFU/ml, respectively.

E. faecalis recoverability on BHI agar decreased 1 log unit over the first 27 days of exposure and decreased somewhat more rapidly to a total decrease of 3 orders of magnitude after 56 days (Fig. 1D). In contrast, mE agar plate counts decreased 0.4 order of magnitude until 4 days, after which CFU on this medium rapidly decreased to <0.2 CFU/ml by 27 days. Injury increased gradually to >90% after 1 week of exposure and remained effectively 100% after approximately 27 days (Fig. 2). T50 values averaged 192 and 840 h on mE agar and BHI agar, respectively.

After 54 days of environmental exposure, E. coli, S. typhimurium, and Y. enterocolitica cells failed to form colonies on TLY or TLYD at 37°C (Fig. 3). Optimal colony-forming temperatures on TLY and TLYD were −1.8 and 8°C (E. coli), 20 and 20°C (S. typhimurium), and 8 and −1.8°C (Y. enterocolitica), respectively. S. typhimurium would not form colonies at 8 and −1.8°C on TLYD (Fig. 3B), while Y. enterocolitica showed greater colony-forming ability on this medium at these temperatures (Fig. 3C). Growth at 8°C required an average of 6 days for colony formation and an additional 2 to 3 days for visible colony formation by slow growing S. typhimurium. Sublethal exposure of randomly picked colonies onto media used to check for contamination (see Materials and Methods) indicated that the observed growth was not due to contamination of chambers with autochthonous marine bacteria. In control experiments using freshly grown cells, none of the organisms tested formed colonies at −1.8°C and only Y. enterocolitica had the ability to form colonies at 8°C. Also, differences between TLY and TLYD recoveries at 20 and 37°C for all bacteria tested were noted in this experiment. By contrast, reincubation of 100 ml of TLY prewarmed to 37°C with 0.5 ml of 64-day-exposed E. coli, S. typhimurium, and E. faecalis produced growth curves very similar to those obtained with fresh overnight inocula (i.e., similar growth rates and cell densities), except that E. coli and E. faecalis showed prolonged lag phases (data not shown).

DVCs. E. coli CTC(+) cell numbers were consistently lower than DVC(+) E. coli numbers by 0.2 to 1.5 orders of magnitude following environmental exposure (Fig. 1A). DVC(+) cell numbers gradually declined over the first 15 days of exposure and then stabilized at ca. 10% of initial values. After 25 days of exposure, these numbers steadily increased to within 1 order of magnitude of inoculum values by 54 days. The same general trend was observed for CTC(+) counts, which stabilized at ca. 1% by 25 days and then rose steadily to 90% of initial values by 54 days.

CTC(+) and DVC(+) cell numbers correlated well for the S. typhimurium and Y. enterocolitica cultures throughout the 54 days of exposure (Fig. 1B and C). These numbers decreased steadily between days 4 and 20. CTC(+) and DVC(+) numbers in each culture then stabilized at 1% of initial values. After 40 days, CTC(+) S. typhimurium cell numbers gradually increased to within 79% of total cell numbers by 54 days while Y. enterocolitica CTC(+) cell numbers increased to within 10% of initial values (Fig. 1B and C). The VBNC response was greatest for S. typhimurium, with 2- to 4-order-of-magnitude underestimations of DVC(+) and CTC(+) numbers after 11 days. Y. enterocolitica and E. coli nonselective plate counts underestimated DVC(+) and CTC(+) numbers by 1 to 3 orders of magnitude after 20 days and by 1 to 4.5 orders of magnitude after approximately 25 days, respectively.

E. faecalis is resistant to nalidixic acid and therefore cannot be assayed for substrate responsiveness by the DVC method. After a transient increase in E. faecalis CTC(+) cell numbers to 100% of total cells at 4 days, these cell numbers decreased rapidly (Fig. 1D). This was followed by a sharp decline in the number of recoverable cells on mE agar. After 7 days, no CTC(+) E. faecalis cells were observed, although plate counts on BHI agar remained ca. 10⁵ CFU/ml (see above).
Total cell numbers for all bacteria tested remained relatively constant (only AODC values are presented for clarity [Fig. 1]). Slight increases in total cell numbers and decreases in cell size were observed within the first week of exposure.

Addition of 0.1× TLY to cell populations of *E. coli* and *S. typhimurium* exposed for 48 days significantly increased the percentage of cells displaying respiratory activity at −1.8, 8, 20, and 37°C in comparison with values for controls without nutrient addition (Fig. 4). *E. coli* showed the greatest relative increase, from ca. 8% to greater than 90% at 20°C (Fig. 4A). *S. typhimurium* had the greatest percentages of respiring cells at all temperatures after nutrient addition, with values reaching over 90% for most temperatures. *S. typhimurium* also showed the greatest percentage of respiring cells in the absence of added nutrients, averaging approximately 30% respiring cells at all temperatures examined (Fig. 4B). *Y. enterocolitica* had the least increase, with very slight increases in percentages of CTC(+) cells at 8 and 20°C in the presence of added nutrients. With nutrient addition, *Y. enterocolitica* reached a maximum of only ca. 12% CTC(+) cells at 20°C (Fig. 4C).

**Diffusion rates.** The rate of diffusion of rhodamine 6G from the chamber and times required for 90 and 99% exchange were calculated as described previously (51). Times for 90 and 99% exchange of control chamber volume under in situ conditions were 31.7 and 63.4 h, respectively. Chambers in which bacteria
had been exposed for 64 days showed 90 and 99% exchange times of 41.6 and 83.38 h (E. coli), 43.9 and 87.9 h (S. typhimurium), and 44.2 and 88.4 h (E. faecalis), respectively. Diffusion rates in inoculated chambers decreased by 24.0% (E. coli), 27.9% (S. typhimurium), and 28.4% (E. faecalis) in comparison with control levels after 64 days of exposure.

**DISCUSSION**

Total bacterial counts remained relatively constant and near the level of the original inoculum for all organisms, indicating minimal cell lysis with environmental exposure of 54 to 56 days. Slight increases in total cell numbers and decreases in cell size within the first week of exposure were likely due to reductive division (Fig. 1; cell size data not shown) (25).

Decreases in diffusion rates (ca. 30%) were expected because of fouling of the diffusion chamber membrane by microbial cells and suspended particulates. Control chamber diffusion rates in this study were slightly higher than those reported by Vasconcelos and Swartz for a marine environment (51). From the diffusion rates and total cell counts obtained in this study, we conclude that most metabolites diffused out of the chambers.

All organisms, except E. coli on TLYD, remained recoverable on selective and nonselective media throughout the exposures (Fig. 1). Recoverable cell counts over the 54-day exposure for the three gram-negative organisms (E. coli, S. typhimurium, and Y. enterocolitica) yielded somewhat similar T90 values (216 to 259 h; TLYD T90 values were always lower than those for TLY) for both the selective and nonselective media. This is lower than the value obtained in an early polar marine microcosm study addressing recoverability of E. coli at 0°C (T90 = 395 to 540 h on tertigol-7) (18) but approximately two to six times greater than those obtained in more recent marine survival studies in <5°C marine environments (2, 5, 28). Although direct comparisons between these results are difficult, the previously observed trends of prolonged survival at low temperatures and reduced recoveries on selective media were observed in the present study.

E. faecalis also retained high levels of recoverable cells on nonselective medium (BHI agar) (T90 = 576 h) after 56 days of exposure, although this organism became nonrecoverable on mE agar after only 14 days (T90 = 96 h) (Fig. 1D). This indicates that the use of this selective medium leads to lower levels of detection of injured cells, signified by the dramatic underestimation of viable cell numbers detectable by BHI agar. In previous studies, T90s for E. faecalis of 269 h at 4°C with TSA agar (5) and of 122 h at 0°C with mE agar were found (28). Collectively, these results indicate that while E. faecalis survives at least as well or better than pathogens when recovery is measured on nonselective media, recoveries on mE agar increasingly underestimated numbers of viable E. faecalis cells in <10°C marine environments with time of exposure.

Recoverability of all organisms examined was consistently lower on selective than on nonselective media. This indicates that in this environment, significant proportions of the enteric bacterial populations sustained sublethal injury which was greatest within the first week of exposure (Fig. 2). Organisms in order of maximal levels of injury throughout the study are as follows: E. faecalis > E. coli > Y. enterocolitica > S. typhimurium. However, the system used for assessing E. faecalis injury was different from that used for the gram-negative organisms. Both indicator organisms tested generally demonstrated greater initial and sustained injury than the pathogens.
upon exposure to the polar marine environment (45). These results may explain the relatively poor recoveries of these bacteria on highly selective media in the previous low-temperature marine survival studies described above.

Incubation of 54-day-exposed cells of *E. coli*, *S. typhimurium*, and *Y. enterocolitica* at various temperatures indicated that polar marine exposure shifted optimal and permissive growth temperatures downwards, allowing *E. coli* and *S. typhimurium* cells to form colonies slowly at -1.8 and 8°C (Fig. 3). This suggests that long-term cold exposure may induce physiological changes in these organisms which permit growth below ca. 7.5°C. The observed loss of colony-forming ability of these organisms at 37°C indicates that exposure also induced marked thermosensitivity with respect to colony formation at normal incubation temperatures. Environmental adaptation through changes in membrane lipids at low temperatures and expression of cold shock proteins is known to occur in *E. coli* (16, 21, 23, 41). These adaptations may produce physiological changes which result in increased thermosensitivity as well as allowing long-term survival at low temperatures. Mutational events in culture subpopulations during exposure could also have produced the observed growth temperature responses. Control experiments with freshly grown cells indicated that none of the bacteria tested (with the exception of *Y. enterocolitica*) contained subpopulations capable of growth at <8°C over ca. 15 days of exposure on agar plates. These results indicate that subpopulations capable of growth at <8°C were not present in cultures upon initial exposure but developed only after long-term, in situ exposure. Although the trend of increasing thermosensitivity and lowered optimal growth temperature was observed for all strains tested (*E. coli*, *S. typhimurium*, and *Y. enterocolitica*), mechanisms of adaptation may differ between organisms and within culture subpopulations. A significant proportion of the exposed pathogenic bacteria (enterotoxigenic *E. coli*, *S. typhimurium*, and *Y. enterocolitica*) became nonrecoverable on nonselective medium at 37°C. However, these bacteria retained respiratory activity and the capability for regrowth at the same temperature in liquid medium. This indicates the possibility of regrowth and pathogenesis in an appropriate host. The increasing fraction of slowly growing colonies is consistent with previous observations of delayed regrowth of *E. coli* upon recovery from exposure to reduced temperatures (21). Relatively high-level recoveries of *Y. enterocolitica* observed at lower temperatures in the control and exposed populations are thought to be due to the psychrotrophic nature of this organism (50).

Good general agreement between DVC(+) and CTC(+) values indicated that these assays were similar in their ability to assess physiological activity, although they measure different cellular processes. The stability of CTC(+) and DVC(+) cell numbers (at ca. 1% of the inoculum) for the enteric bacilli indicates that these organisms persisted in an active and substrate-responsive state at ca. 0.1% of inoculum numbers for at least 54 days. When these results are compared with nonselective plate count recoveries, it is clear that there was a progressive shift to a VBNC state upon polar marine exposure in suspensions of *E. coli*, *S. typhimurium*, and *Y. enterocolitica* (Fig. 4A through C). This general trend has been previously observed with many enteric bacteria on exposure to aquatic environments (4, 26, 44). These observations are also consistent with those of Dawe and Penrose, who found no decrease in ATP levels but decreasing plate counts in coliform suspensions exposed to an 8- to 12°C marine environment (10). The present results indicate that plate count enumerations, particularly on selective media, may significantly and progressively underestimate numbers of viable enteric bacteria in cold (ca. <10°C) marine environments. Results observed with *E. faecalis* indicated that CTC reduction assays significantly underestimated viable cell numbers on nonselective medium following environmental exposure (Fig. 1D). Thus, CTC reduction is a poor indicator of *E. faecalis* viability in this environment.

Addition of relatively high nutrient concentrations to cells exposed for 48 days significantly increased percentages of respiring *E. coli* and *S. typhimurium* cells over levels observed for controls with no nutrient addition at all temperatures examined (Fig. 4A and B). Slight increases were also noted for *Y. enterocolitica* cells (Fig. 4C). This indicates that large nutrient inputs increase respiratory activities of dormant (i.e., showing no respiratory activity by CTC reduction) *E. coli*, and *S. typhimurium*, at the low in situ temperature, revealing that the majority of these cells maintained the capacity for uptake and metabolism of added nutrients. Coastal polar marine environments are characterized by seasonally high productivity and some of the highest reported particulate and dissolved organic carbon concentrations in marine surface waters (38, 54). It was noted that increases in respiring numbers of *E. coli*, *S. typhimurium*, and *Y. enterocolitica* after 25 days of exposure corresponded with the annual phytoplankton bloom observed during concurrent studies (20, 39). Thus, large nutrient inputs may allow *E. coli*, *S. typhimurium*, and possibly *Y. enterocolitica* to persist in a nongrowing, metabolically active, VBNC state by providing substrates for cellular maintenance energy require-
ments. Increased levels of nutrients have been found to increase survival rates of enteric bacteria in seawater (by using plate counts as a measure of viability) (8, 35). In addition, Wibberg et al. recently demonstrated that increased salinity concentrations are required to maintain growth rates of both autochthonous mesophilic marine heterotrophic bacteria and E. coli at minimal growth temperatures (10°C for E. coli) (53).

However, several reports have challenged that this observation holds true at temperatures below 12°C (9, 28). Previous short-term cold exposure experiments have also shown that translation initiation, as well as binding and transport of amino acids, is inhibited in E. coli below 8°C (7, 15). However, transport, incorporation, and mineralization of added amino acids were not completely inhibited. Cold survival in the VBNC state with reduced rates of respiratory activity has also been reported for the enteric pathogen Vibrio vulnificus (37). Low temperature, rather than minimal nutrients, appears to be an important determining factor limiting respiratory activity and promoting the VBNC state in this organism (36). In the present study, respiratory activity of E. coli and S. typhimurium appears to be limited by nutrient availability rather than by temperature in this cold environment (Fig. 4A and B).

Incubation of 54-day-exposed E. coli, S. typhimurium, and Y. enterocolitica at 37°C in liquid medium (DVC, CTC, and regrowth experiments) revealed large numbers of cells capable of displaying or regaining metabolic activity, while no cells could be recovered as colonies on the solid media used at that temperature (Fig. 3). Evidently, there was some response in 54-day-exposed cells which precluded colony formation on solid media, but not respiration or growth in liquid medium at 37°C. This may be due to the different osmotic environment of a liquid medium in comparison with the surface of an agar plate.

In summary, exposure of enteric bacteria to a very cold, polar marine environment increased thermosensitivity, as demonstrated by a subsequent reduction in optimal temperature for colony formation from 37 to ≤20°C. Respiratory activity in E. coli, S. typhimurium, and Y. enterocolitica was limited by nutrient availability rather than by temperature in this environment. Decay rates of E. coli, S. typhimurium, and Y. enterocolitica were two to four times greater in the present study than in previous reports in situ marine studies of E. coli survival at <10°C. Exposure also resulted in a progressive increase in the number of injured, VBNC, and inactive cells in these populations with time, resulting in significant underestimated numbers of viable organisms in the environment by the plate count method. While E. faecalis decay rates were the lowest of all organisms tested on nonselective medium, this organism rapidly became nonrecoverable on the selective medium commonly used for its detection and therefore is an unsuitable indicator of the presence of enteropathogenic bacteria in this environment. While the decay rates of recoverable cells appeared similar for E. coli, S. typhimurium, and Y. enterocolitica on all media tested, it is suggested that incubation of samples for coliform enumeration from <10°C marine environments be reduced to ≤20°C if plating techniques are used. Most-probable-number enumerations with liquid media may also give better recoveries than agar plate assays. However, data thus obtained should be considered minimum estimates of viable organism numbers in this extreme environment because of the considerable number of VBNC and injured coliforms expected on the basis of the present study.

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