Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment

by James Joseph Smith

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University

Abstract:

This report describes an assessment of the survival, physiological response and recovery of common human fecal indicator and enteropathogenic bacteria with long-term polar marine exposure. The maintenance and expression of antibiotic-resistance (R) and conjugative (F) plasmids were also examined. Direct (non-culture-based) methods for assessing bacterial viability were employed to determine fractions of viable-but-nonculturable (VBNC) bacteria. A systematic study of the mechanisms behind one of these techniques (5-cyano-2,3-ditolyl tetrazolium chloride [CTC] reduction) was also investigated in a model prokaryote Polar marine exposure experiments were performed using in situ diffusion chambers filled with cultures of 2 indicator, (Escherichia coli, Enterococcus faecalis), and 2 pathogenic bacteria (Salmonella typhimurium, Yersinia enterocolitica). These were placed in situ at Winter Quarters Bay, McMurdo Station, Antarctica for periods of 54-56 days (-1.8°C, 34.5 ppt salinity). E. coli cells harboring R-(pUC19), as well as F-(pFamp), plasmids were exposed for 54 and 21 days, respectively. Exposure significantly extended the survival of all enteric bacteria examined when compared to survival studies in more temperate marine environments. Little cell lysis or decreases in total cell numbers were noted. A progressive increase in amounts of VBNC and injured cells with exposure was noted in E. coli, S. typhimurium, and Y. enterocolitica. These organisms also became markedly thermosensitive after 48 days exposure, and would no longer form colonies on plating media at 37°C. E. coli, S. typhimurium, and Y. enterocolitica respiratory activity was limited by nutrient availability rather than temperature in the environment. R-, and F- plasmids were maintained and expressed for up to 54 days.

Experiments demonstrated that both CTC and INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5 -phenyl tetrazolium chloride) were reduced in response to bacterial respiratory activity. CTC appeared to be reduced by the primary aerobic dehydrogenases (NADH, succinate), while INT was reduced by these as well as ubiquinone and possibly cytochromes b555,556. Both CTC and INT were reduced under all anaerobic conditions examined. However, CTC appears to form a nonfluorescent formazan in the presence of high concentrations of inorganic phosphate.

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A thesis submitted in partial fulfillment
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6-12-95
Date

Chairperson, Graduate Committee

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6-16-95
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7/20/95
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Graduate Dean

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Advisor: Gordon A. McFeters

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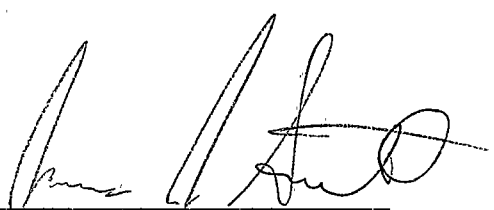
Signature 

Date 5/22/95

This work is dedicated to my late little-brother, Andrew William Smith.

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CHAPTER 1

GENERAL INTRODUCTION

Indicators

In the last few decades the use of marine outfalls for the disposal of sanitary wastes has increased greatly, as have the urban populations inhabiting coastal areas (12, 89). The survival of human enteric pathogens and indicator organisms in marine environments has subsequently assumed increasing public health significance with respect to the presence and enumeration of these types of microorganisms in commercial fish and shellfish, marine mammals, and recreational waters (14, 69). Techniques for the enumeration of indicator organisms (i.e. *E. coli*, coliforms) and specific pathogens from marine environments have been developed and accepted as standard methods (1). However, these procedures, such as the multiple-tube fermentation (MTF or MPN), and membrane filtration (MF), as well as indicator organisms, were largely developed for the examination of relatively warm (>10°C), freshwater systems (most common sources of bathing and drinking water) (14, 69). It has been recognized that the behavior of indicators and pathogens in the marine environment differs greatly from that of freshwater (9, 43, 53). In addition, the survival of the primary indicator of fecal pollution, *E. coli* , in seawater has been shown to be inversely related to temperature,

with colder temperatures extending survival time by a factor of 2 for each 10°C decrease. In fact, numerous studies have determined that water temperature is the most important factor in predicting fecal coliform survival in marine environments (2, 5, 26, 38, 84). This is of particular relevance since sewage is often discharged untreated into low-temperature marine environments and only ca. 10% of marine environments are > 5°C (58).

Current "die-off" studies of human enteric and genetically engineered microorganisms in the environment have determined that for many, if not most bacteria, there appear to be what are termed "injured" and/or "viable-but-nonculturable" (VBNC) states (67, 77). 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). VBNC is commonly defined as the inability to form colonies on a given solid medium while remaining active by direct viable-count (DVC) methods (67, 77). Stressors within aquatic environments also induce sublethal physiological and structural changes, termed injury, in enteric bacteria (51). 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 (79). As an example, a bacterium shown to be viable by DVC methods but which will not form a colony on nonselective medium is termed VBNC. A bacterium which will form a colony on nonselective medium, but not on this same medium containing a selective agent to which it is normally resistant is termed injured. It should be emphasized that both injured and VBNC responses are commonly observed in bacteria exposed to environmental stress. Since the majority of enumeration

techniques for enteric bacteria require culturing, results may seriously underestimate numbers of viable bacteria in the environment. This is especially true in environments where stressors such as cold and starvation may promote entry into the VBNC state. Marine studies using laboratory microcosms or in situ diffusion chambers have shown that cold shock, starvation, and possibly osmotic shock are inducers of this state (2, 18, 38, 63, 66). Most of these experiments have used laboratory, batch-culture microcosms which are subject to "bottle effects" (1, 17). This confinement terminates exchange of nutrients and metabolites with the ambient water, which is important for an evaluation of in situ survival and physiological processes in natural aquatic environments. A study by McFeters and Terzieva found significant differences in *E. coli* and *Yersinia enterocolitica* survival and sublethal injury levels between experiments using bacterial suspensions in diffusion chambers in laboratory containers, in situ in a mesotrophic stream, and in sample bottles (50). The use of diffusion chambers deployed in situ largely alleviates these problems (49). Experiments using in situ diffusion chambers for the study of enteric bacterial survival associated with sewage in the marine environment are few (2, 39), and none used techniques to determine the extent of bacteria in the VBNC state.

Survival of Enteric Bacteria in the Marine Environment

Marine waters present unfavorable conditions for those copiotrophic bacteria found in raw sewage, which commonly persist in temperate freshwater environments. Salinity, pressure, UV-irradiation, competition with, and predation by the autochthonous flora and fauna, temperature

fluctuations, and (in many cases) oligotrophic conditions all exert effects on the survival and physiology of sewage bacteria (4, 9, 10, 23, 39, 77). Reviews of the literature have shown that the primary effectors of pathogenic and indicator bacterial survival and activity in seawater are the relatively oligotrophic nature of most marine environments, as well as temperature (14, 53, 60). Grazing by larvae and heterotrophic zooplankton may also reduce numbers of enteric bacteria from the environment. Some authors feel that protistan grazing may be the primary factor influencing enteric bacterial survival in aqueous environments (23). However, it appears that temperature plays an important role in regulating rates of bacterial removal through grazing, with lower rates at lower temperatures. In an estuarine study of the effect of eukaryotic grazing pressure and temperature on *E. coli* survival in diffusion chambers, Anderson et al. (2) found that grazing exerted little or no decline in cell numbers below 3°C over 6 days. However, at 13 and 24°C grazing did exert an effect. In addition, several studies in the Antarctic marine environment have found that grazing rates are significantly reduced compared to more temperate regions (71, 73).

Studies on the effects of nutrient levels on enteric bacterial survival are conflicting. Lessard et al. (39) observed no correlation between diel cycles, or dissolved organic carbon and *E. coli* survival over a range of temperatures (0-25°C) in a salt marsh. Temperature was found to be the most significant factor in that study. In addition, Chamberlain and Mitchell (10) described little effect of nutrients on enteric bacterial survival below ca. 12°C. However, studies by Carlucci and Pramer (9), as well as Munro et al. (61) found that increased levels of nutrients prolonged enteric bacterial survival in seawater at ca. 24-28°C using plate counts as a measure of viability. Also,

Weibe et al. recently showed that increased substrate concentrations are required to maintain growth rates of *E. coli* at minimal (10°C), compared to higher growth temperatures (86). Thus, it appears that while increased nutrient levels may allow for the increased persistence of enteric bacteria in temperate environments, this effect may be reduced, or nonexistent at temperatures below ca. 10°C.

Starvation, Cold Shock, Adaptation, and the "Viable-but-Nonculturable" State

Considerable research has been done on the starvation of both autochthonous bacteria from aquatic environments and organisms of public health significance (57). However, it appears that the starvation-survival response of enteric bacteria is not identical to that of many autochthonous marine bacteria (16, 35, 77). A question arises as to whether some sewage bacteria, such as *Vibrio cholerae* and *Salmonella* spp. persist indefinitely in the marine environment (85). Reviews by Stevenson (81), Morita (59, 60), Kjelleberg et al. (35), and Matin et al. (44) indicate that the persistence of marine autochthonous microrganisms in seawater is primarily a function of their ability to adapt to the environment, and/or through the ability to form "dormant" cells. Several terms have been proposed for this state including "microcysts", "somnicells", "dormant", and "viable-but-nonculturable" (VBNC). The latter term refers to the inability of cells to divide and form recognizable colonies or turbidity in plating and MPN assays using conventional selective and/or nonselective media. The other terms refer largely to a spore-like state of nonsporeforming bacteria, largely as a result of starvation and/or cold shock. In this state bacteria are seen to reduce their size considerably

("dwarfing"), and switch from specific, low-affinity membrane uptake systems to non-specific, relatively high-affinity uptake systems (35, 59, 60). Other physiological changes include increased intracellular levels of ppGpp and induction of the stringent response (35, 64), increased adhesion to surfaces (33), aggregation (40), sublethal injury (79), non-culturability (67), as well as increased resistance to gastric pH, cell wall synthesis inhibitors, and chaotrophic agents (21, 45). It is also apparent that synthesis of specific starvation and cold-shock proteins takes place as analyzed by 2-D gel electrophoresis (35, 45). Alterations in membrane fatty acids have been noted in *Vibrio vulnificus* (41), and *Vibrio cholerae* (25, 28) in response to nutrient deprivation in marine microcosms. It has long been recognized that *Escherichia coli* markedly increases the proportion of unsaturated fatty acids in the cell membrane in response to low temperatures (< ca. 10-20°C) (13, 30, 65). This is an apparent adaptation to allow cells to retain a fluid ("non-ordered" lipid phase) membrane at lower temperatures (30, 31). Loss of membrane fluidity leads to segregation of protein/lipid in the membrane and appears to lead to a loss of certain enzymatic functions (83).

It has been hypothesized that virtually all bacteria, including copiotrophs, can possibly use both K- and r strategies during periods of their life cycle (3). The r-strategists rely on high reproductive rates for survival while K-strategists depend on adaptation to the carrying capacity of the environment. This would help explain the persistence of enteric, allochthonous, copiotrophic bacteria when exposed to oligotrophic conditions. It is emphasized that under conditions of marine starvation and/or cold shock, subpopulations of enteric bacteria are seen to become nonculturable (11, 76, 78). This phenomenon has been observed in: *E. coli* (57), *Klebsiella aerogenes*,

Vibrio cholerae (6, 11, 78, 81, 88),*Vibrio vulnificus* (63, 66), *Shigella sonnei, Shigella flexneri* (11), *Camphylobacter jejuni, Campylobacter pylori* (72), *Salmonella enteriditis* (78), *Legionella pneumophila* , *Aeromonas* spp., *Aeromonas salmonicida* (56), *Alteromonas esperjiana* , *Enterococcus* spp., *Micrococcus* spp., and *Nitrobacter* spp. (77). Environmental stressors, particularly cold-shock, have also been found to induce sublethal physiological and structural changes, termed injury, in enteric bacteria (51, 79). This is defined as the inability of an organism to reproduce under conditions which allow for growth of uninjured cells (restrictive temperatures, selective agents, etc.). The public health implications are obvious as enumeration of the pathogenic and indicator bacteria listed above are based largely upon culture methods, and VBNC bacteria have been shown to retain their pathogenicity and plasmid-encoded antibiotic resistance (7, 79). The usefulness of the coliform and fecal coliform indices for public health water quality monitoring may thus be seriously compromised especially with respect to colder marine environments (1, 69, 76, 84).

Effects of Culture Physiology on Survival and Culturability

A question remains as to the effects of injury (i.e. sublethal stress) on formation of the VBNC state in the marine environment. Gauthier et al. (20), Oliver et al. (66), Meynell (52), and Gauthier et al. (19) respectively, have shown that survival and the formation of VBNC cells are functions of growth phase, prior starvation, prior exposure to cold, and increased salinity. Unstarved, non-salinity or cold exposed, log-phase cells seem to be most susceptible to cell death without formation of VBNC cells (46). Stationary

phase, pre-starved, -cold shocked, and -saline exposed cells have an increased ability to form VBNC cells. Recent work by Oliver et al. has shown that there may be an antagonistic effect of cold shock and starvation on the formation of VBNC cells, with time of prestarvation as well as culture O.D. directly related to formation of VBNC *Vibrio vulnificus* in 5°C seawater (66). In addition, Nilsson et al. have demonstrated that formation of the VBNC state is independent of nutrient levels for this organism (63). A rise in temperature to 25°C was also demonstrated to increase culturability to initial levels.

Specific proteins are synthesized in response to cold shock and starvation. However, a question remains as to whether and to what degree these proteins and their functions overlap in the induction of the VBNC state (22, 34, 44). Individual protein functions are difficult to assess, but transcriptional, and metabolic regulators have been implicated. Studies by Matin (44, 46) describe the current knowledge of the genetic basis of starvation responses. McCann et al. (48) have recently discovered the KatF (*rpoS*) gene in *E. coli* is a major switch controlling expression of some 32 starvation-mediated *pex* genes. Induction of *pex* genes is independent of cyclic AMP, and is cross-protective for heat, osmotic and oxidative stresses. KatF has also been identified as a putative sigma factor (σ^S) controlling expression of stress-induced genes (42). KatF homologues have also been found in *Salmonella typhimurium, Klebsiella pneumoniae, Shigella flexneri* and *Pseudomonas aeruginosa*. This coordinate control of multiple unlinked genes and operons by a common regulatory element appears to be a form of "global control system" responding to environmental stress (35, 64). Sets of coordinately regulated genes induced under a given environmental stimulus are termed stimulons (64). However, while responses to heat shock and

oxidative stress have been studied extensively, the physiologic and genetic regulation of bacterial adaptation to survival under cold marine conditions remains largely unexplored.

Enumeration and Activity Measurements of Bacteria in Aqueous Environments

Various methods have been employed with respect to the enumeration of environmentally stressed bacteria. The most widely used techniques for enumeration of total, active, or viable cells in a population are; acridine orange direct counts (AODC, total cells), spread-, or drop-plating (plate count, recoverable or culturable cells), direct viable counts by the method of Kogure et al. (36, 80), p-iodonitrotetrazolium chloride (INT) or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction (32, 74, 90) (active cellular respiration), and plating on non-selective and selective media (injury) (79). A somewhat typical assay of a population of cells would involve total AODC (DAPI can also be used) combined with INT reduction to determine total count and the fraction of actively respiring cells (82). CTC reduction in particular is beginning to be widely used as an indicator of actively respiring, and hence viable cells. However, the use of CTC and INT for assessment of bacterial activity is hampered by a lack of knowledge regarding the specific cellular processes they measure. This information is essential for accurate interpretation of physiological and viability data obtained using these indicators. A systematic study of reduction of these indicators in prokaryotes is needed.

A comparison of DVC and radiolabeled substrate uptake (microautoradiography) methods using VBNC *Salmonella enteriditis* and *E. coli* in marine microcosms showed that ca. 90% of cells responsive to the DVC

method were metabolically active, and no significant difference was observed between these methods (76). Radioisotopic measurements discussed by Hobbie (27) have been used to determine more precisely the uptake kinetics and metabolism of bacterial populations. Several authors have also used ATP assays as well as tritiated thymidine and/or ^{14}C-substrate uptake to measure population activities, DNA synthesis and endogenous metabolism (33, 60, 62). The use of tritiated uridine/leucine to measure RNA/protein synthesis rates can give an estimation of biosynthesis in an exposed bacterial population, while uptake of ^{14}C-labeled α-methyl-glucose allows for estimation of membrane transport of exogenously provided carbohydrate. Elucidation of whether enteric bacteria exposed to extreme low-temperature marine environments continue to synthesize macromolecules and retain the ability to transport exogenously supplied nutrients may help to define the metabolic state of these organisms while trying to survive.

Plasmid Maintenance and Expression

Prolonged survival of plasmid-harboring enteric bacteria in sewage released into low-temperature marine environments presents the possibility of horizontal transfer virulence and/or antibiotic resistance (R) genes to autochthonous bacteria (89). Virulence-associated genes in *E. coli, Y. enterocolitica, S. typhumurium, and S. flexneri* are also under temperature regulation, and are most commonly repressed at low temperatures (47). Natural genetic exchange through transformation, conjugation, and transduction has been described in marine environments, as well as transfer of R plasmids from enteric to marine bacteria and fish pathogens (24, 37, 70,

89). Since cold-shock as well as starvation have been shown to induce sublethal injury and the VBNC response, the possibility of underestimation of plasmid harboring enteric bacterial numbers also exists. Plasmids have been demonstrated to be stably maintained and expressed in VBNC *E. coli*, as well as *E. coli* exposed to well, lake, and marine waters at temperatures of 15-25°C (7, 8). Whether enteric bacteria will maintain and express R-, and F-plasmids, which code for congugative functions and certain bacteriophage receptors, in situ under low-temperature stress and marine exposure is unknown. In addition, it remains to be seen whether plasmid-containing and wild-type enteric bacteria have similar survival responses upon environmental exposure.

Enteric Bacteria in Polar Marine Environments

Recent interest in the biological and chemical pollution problems associated with the U.S. Antarctic bases has provided the impetus for research into the fate of pollutants in the marine environment, and in marine animal life (39). Nowhere has this drawn as much focus as the largest Antarctic base, McMurdo station (29, 54). Untreated raw sewage mixed with ≈ 40 ppt salinity, ≈ 37°C desalinization brine is discharged (ca. 20-40,000 gal/day) after comminutation (grinding with an impeller blade) through a heated, submerged outfall located ≈ 15 m below the water surface. This outfall is located almost directly seaward from the station. Ambient seawater conditions in the general area are a nearly constant -1.8°C (seawater is poised at its freezing point), 34.5 ppt salinity, with an average current (mean tidal speed) of 180 m/day, and a mean net speed of 26 m/d (close to the lower limit of

detection). Effluent conditions have been described as 33-38°C, 30-36.5 ppt salinity, and 0.0060-0.0081 m^3/sec (72). Enteric bacteria in the effluent are challenged by very cold temperatures, osmotic shock, and gradually increasing oligotrophic conditions as the sewage plume disperses. In addition, solar radiation is highly attenuated by 2-3 m of fast ice (and snow) cover for 10 months of the year, and is effectively absent for up to 4 months during polar winters. Yearly inputs of organic nutrients through primary and secondary productivity are some of the highest reported for marine surface waters due to under-ice phytoplankton and diatom blooms, but are highly seasonal (68). The blooms regularly last for ca. 1 month, and have been found to produce amino acid and sugar concentrations in the water column of up to 490 and 25 μM, respectively (87).

Thus, the Antarctic marine environment represents a unique setting in which to study the survival patterns and physiological consequences of sublethal stress in enteric sewage bacteria exposed to cold, osmotic stress, and a "feast and famine" existence.

Research Goals and Objectives

Validation of Tetrazolium Reduction Methods

1) Elucidate the site(s) and mechanism(s) by which 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) are reduced to intracellular formazan crystals in a model prokaryote.
2) Determine whether and to what extent CTC and INT are biologically reduced under anaerobic conditions with different substrates in a model prokaryote.

3) Define the substrates which give optimal CTC and INT reduction under aerobic conditions with different substrates in a model prokaryote.
4) See how different substrates effect CTC (+)/INT (+) cell numbers in relation to plate counts.

Exposure of Enteric Bacteria to a Polar Marine Environment

1) Determine the survival kinetics, injury, and loss of culturability of representitive enteric organisms (indicators and pathogens) when exposed to cold (-1.8°C) seawater.
2) Determine whether indicators and pathogens share similar survival responses, and which indicators are optimal for this environment.
3) Use the above data to determine if exposure to this type of environment enhances survival/persistence of enteric organisms.
4) Elucidate the specific cellular responses of bacteria, in terms of substrate uptake and macromolecular synthesis to cold marine environmental exposure, focusing on cold shock and starvation responses.
5) Determine whether nonculturability of viable enteric bacteria found in sewage is promoted under cold marine conditions, and, if so, the degree to which these bacteria persist over time and could jeopardize public health.

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