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

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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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of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY, BOZEMAN
Bozeman, Montana

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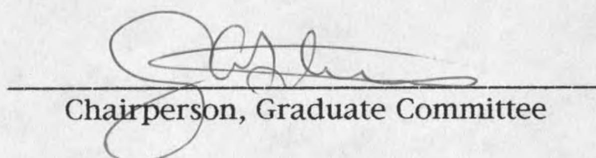
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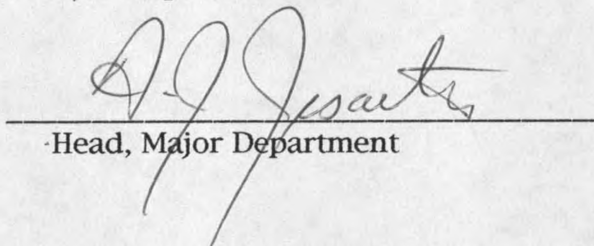
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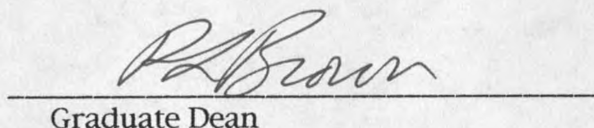
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SURVIVAL, PHYSIOLOGICAL RESPONSE AND RECOVERY OF ENTERIC BACTERIA EXPOSED TO A POLAR MARINE ENVIRONMENT

James Joseph Smith

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Montana State University
1994

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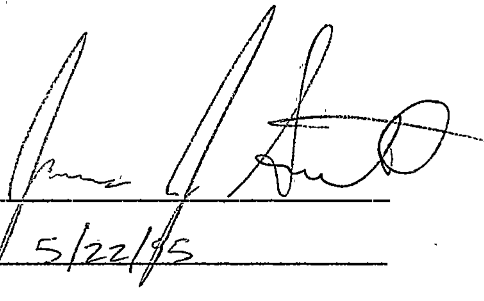

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This work is dedicated to my late little-brother, Andrew William Smith.

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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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†For a broad review of the principal concepts I would refer readers to references 23, 30, 42, and 49.

CHAPTER 2

MECHANISMS OF INT (2-(4-IODOPHENYL)-3-(4-NITROPHENYL)-5-PHENYL TETRAZOLIUM CHLORIDE), AND CTC (5-CYANO-2,3-DITOLYL TETRAZOLIUM CHLORIDE) REDUCTION IN *ESCHERICHIA COLI* K-12

Introduction

Tetrazolium salts are often used as indicators of microbial respiratory activity, as well as viability, particularly in physiological and ecological studies. They are also utilized as differential agents in bacteriological media (TTC [2,3,5-triphenyl-2H-tetrazolium chloride] in Tergitol-7, and mE) (2). INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and more recently CTC (5-cyano-2,3-ditolyl tetrazolium chloride), have been used to indicate the fraction of respiring bacteria in populations. This is done through reduction of the soluble tetrazolium salts to their corresponding colored (INT), or fluorescent (CTC), insoluble, intracellular formazan crystals which can be viewed using epifluorescence microscopy (Fig. 1) (14, 15, 33, 45, 52). INT reduction has also been used to quantify respiratory potential in mixed populations by organic extraction, and colorometric determination of formazan production (6, 17, 26). The INT technique produces dark red, intracellular INT-formazan (INF) crystals which can be difficult to visualize, particularly against an opaque background (12, 44, 45, 46). Unfortunately, this is often the type of background encountered in studies of microbial adhesion, biofilms, and sediments, as well as bacterial enumerations on membrane filters

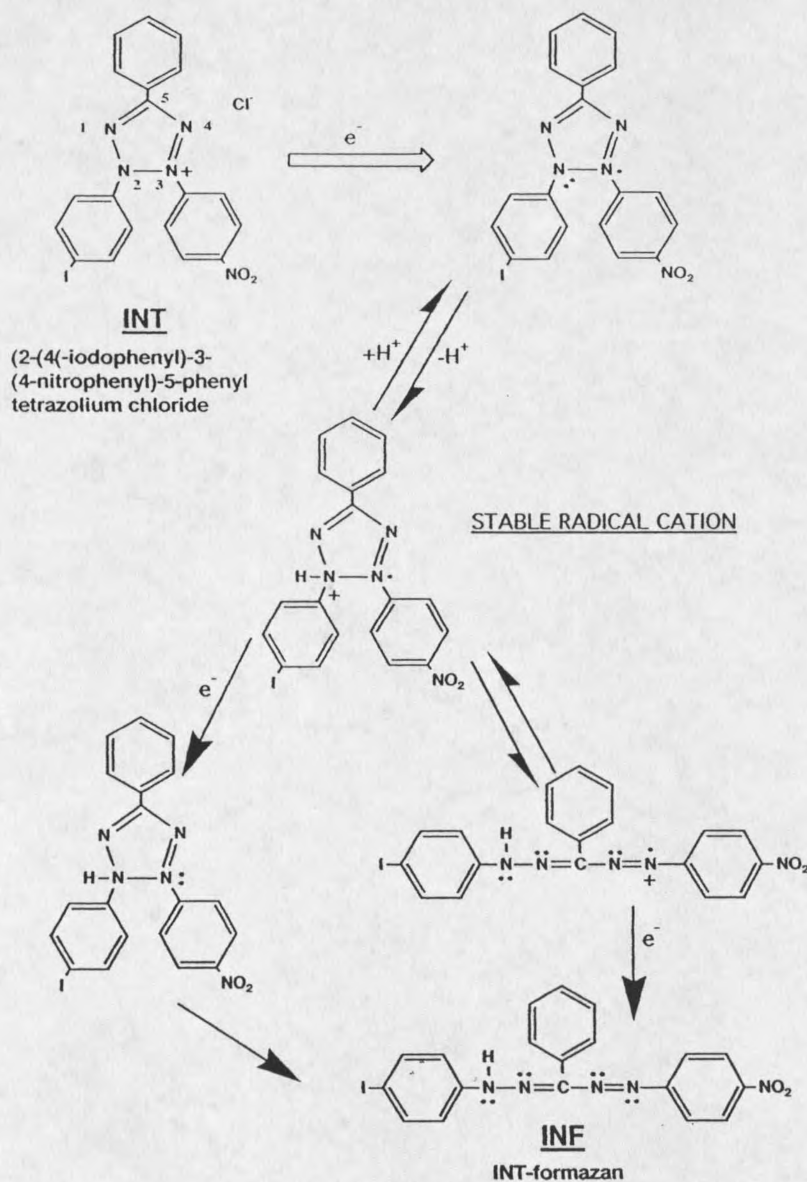

Figure 1(A). Reduction reaction of INT to INF, showing intermediate species between tetrazolium and formazan.

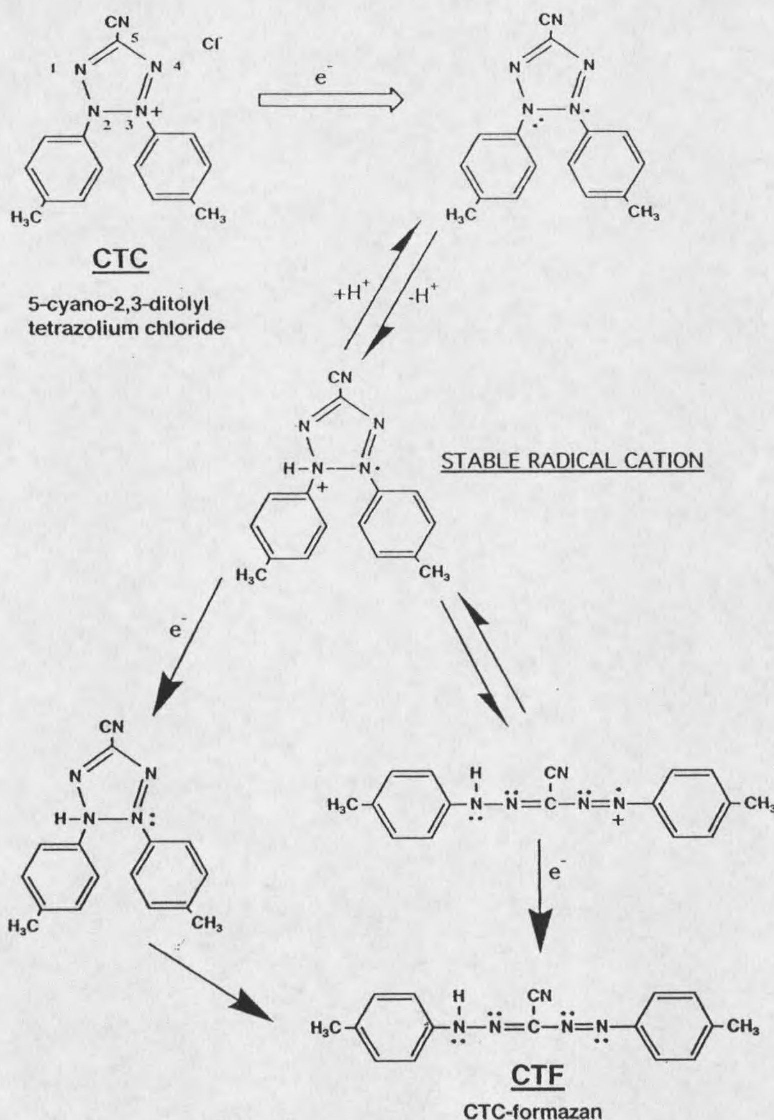

Figure 1(B). Reduction reaction of CTC to CTF, showing intermediate species between tetrazolium and formazan.

using epifluorescence microscopy. INF crystals also appear to be soluble in immersion oil (45, 46). However, the recently developed cyano-substituted, 2,3-diaryl tetrazolium salt CTC forms a fluorescent formazan (CTF) which is relatively easy to visualize against an opaque background by epifluorescence microscopy (33). In addition, CTF is insoluble in immersion oil, and retains its fluorescence upon storage (41). Direct counting of actively respiring bacteria is simplified considerably, and CTF production can be monitored by fluorescence-activated cell sorting (FACS) analysis (14, 33, 38, 52).

With the increasing use of tetrazolium salts for studies of prokaryotic physiology and ecology, questions arise concerning the specific cellular process(es) their reduction measures. Although sites of reduction for INT and CTC in eukaryotic electron transport systems have been fairly well defined, those in prokaryotic cells remain largely unresolved (25, 26, 27, 37). In eukaryotic mitochondria, INT has been found to be reduced by the succinate:ubiquinone oxidoreductase (SDH) complex, or cytochrome c_1 (21,26, 27). Stellmach and Severin found that CTC is reduced primarily by membrane-bound NAD(P)H-dehydrogenase, and possibly superoxide anion (NADH-D) in Ascites tumor cells (43). Kaprelyants and Kell found that CTC was reduced directly by respiratory chain dehydrogenases in *Micrococcus luteus* (15). Although not demonstrated experimentally, SDH has been suggested as the primary site of CTC and INT reduction in prokaryotes (14, 26, 33). It is well known that different tetrazolium salts have different reduction potentials, and the redox potential of CTC is unreported (16, 25, 37). Since the use of different tetrazolium salts and assay conditions for the determination of respiratory potential and activity may yield different findings, results would be easier to

interpret if their redox potentials and site(s) of reduction in the prokaryotic electron transport chain were known.

In order to determine the mechanisms of INT and CTC reduction in a prokaryotic respiratory chain we examined the effects of various inhibitors of electron transport and uncouplers of oxidative phosphorylation with well described sites of action. In general, blockage of the electron transport chain at a point before that at which the tetrazolium salt is reduced will result in decreased reduction to formazan, while blockage after the site of reduction will increase, or produce no change in tetrazolium reduction (25, 27, 38, 43). In order to determine the extent of coupling between tetrazolium reduction and respiration, formazan formation was correlated with oxygen consumption measurements. These experiments were carried out in whole cells and inverted membrane vesicles of *Escherichia coli* K-12, a prokaryote with relatively well-described aerobic and anaerobic electron transport chains (4, 18, 19, 20, 23, 30, 50, 51) (Fig. 2). To determine whether, and to what extent CTC and INT are reduced under anaerobic conditions, whole cells were assayed under conditions designed to stimulate several different anaerobic respiratory chains, as well as glucose fermentation. In order to further characterize the chemical properties of INT and CTC, their mid-point redox potentials ($E_{1/2}$), solubilities, and molar extinction coefficients (ε) in 95% ethanol of CTC and INT were also determined.

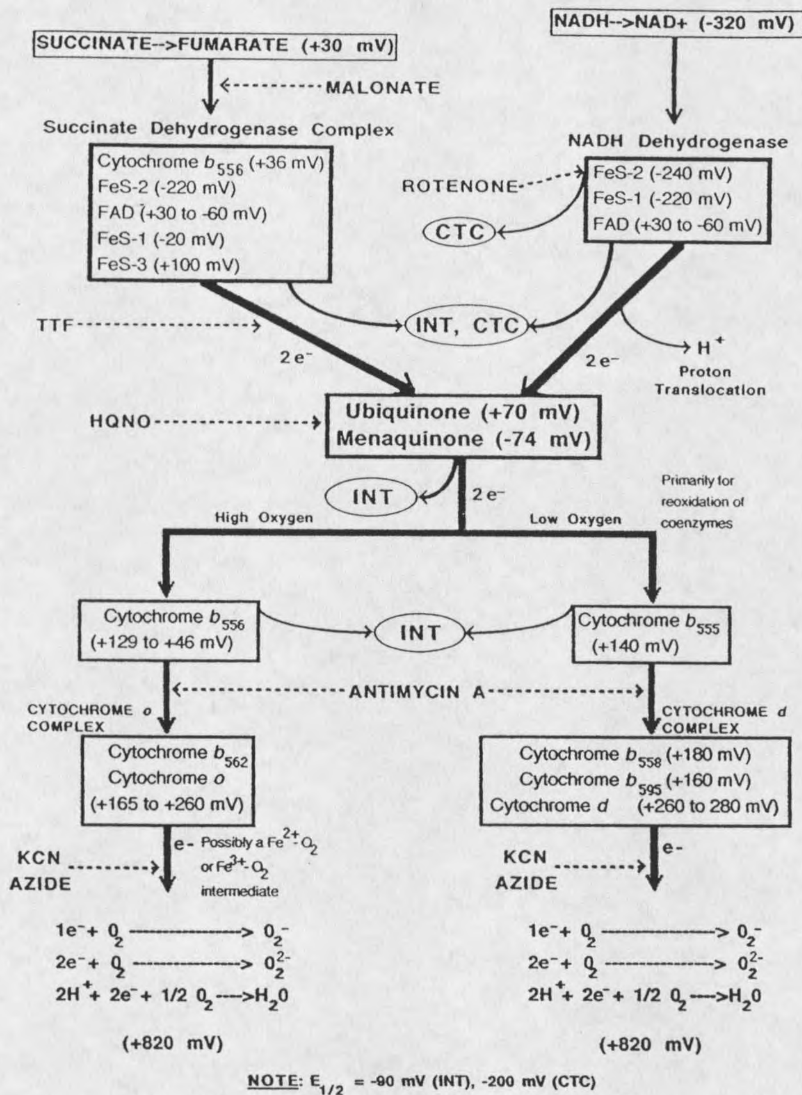

Figure 2. Schematic of the *E. coli* aerobic respiratory chain showing the sites of action of the various inhibitors used in this study. Mid-point redox potentials for the various cytochromes, iron-sulfur centers (Fe-S), and dehydrogenases are given. Sites of reduction of CTC and INT from this study are encircled. Dashed lines indicate sites of inibition of electron transport processes. See materials and methods for chemical abbreviations. Compiled from (4, 18, 19, 23, 25, 27, 29, 30, 32, 48, 50, 51).

Materials and Methods

Determination of INT and CTC Chemical Properties

All chemicals and substrates used were reagent grade or better. CTC was obtained from Polysciences, Inc. (Warrington, Pa.). INT and INF, as well as all substrates, fluorescent stains, and inhibitors were purchased from Sigma Chemical Co. (St Louis, Mo.).

Duplicate redox titrations were carried out in an anaerobic tent under an $N_2/CO_2/H_2$ atmosphere using sodium dithionite as titrant and an Orion 9678BN combination redox electrode. All directly measured redox potentials were corrected by reference to the normal hydrogen electrode (NHE). INT was titrated as a control, as its redox potential has been reported previously ($E_{1/2}$ = -90 mV) (16, 25, 37). Tetrazolium salts were dissolved at 0.1 mM in 50 ml, 0.1 M phosphate buffer, pH 7.0 as described by Karmarker et al. (16). The ascorbic acid (0.1 M)-pH (pH range 2.5-11.5) method of Seidler was also used to determine the relative reducibilities of INT and CTC (36). Molar extinction coefficients were determined by dissolving INT-(INF), and CTC-formazan (CTF) in 95% ethanol. These values were subsequently used to calculate formazan production from absorbance data. CTF was obtained by reduction of 50 ml, 0.1 mM CTC in double-distilled water (dd-H_2O) using sodium dithionite. The precipitate was washed 3 times with dd-H_2O and lyophilized.

Growth Conditions

Escherichia coli K-12 was grown aerobically with vigorous aeration at 25°C to late-log phase in glycerol (8 g/l)-mineral medium supplemented with 1

μM selenic acid and sodium molybdate (9). *E. coli* was also grown anaerobically, and all subsequent manipulations performed in a $N_2/CO_2/H_2$ atmosphere for all anaerobic experiments. The same growth medium as above was used, except glycerol was replaced by glucose. Cells were washed 3 times and resuspended in 0.85% NaCl (pH 6.5) at ca. 10^{10}/ml. Subsamples for aerobic tetrazolium salt experiments were removed after vigorous aeration for 10 min at 25°C. Plate counts were performed by spot-plating subsamples (exposed for the same period, in the same buffer as respective assays) onto R2A medium and incubation for 48 h at 25°C. Plates for anaerobic assays were incubated in the same $N_2/CO_2/H_2$ atmosphere as above.

Substrates, Inhibitors, and Uncouplers

Inhibitors were added as either 0.1 ml 10X solutions, or 10 microliter 100X solutions to the following final concentrations: KCN (2 mm), sodium azide (50 mM), rotenone (ROT, 1 mM), malonate (MAL, 100 mM), thenolyltrifluroacetone (TTF, 1 mM), carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM), antimycin A (20 μM), and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO, 0.2 mM). Rotenone, TTF, CCCP, HQNO, and antimycin A were made as freshly prepared 100X solutions in absolute ethanol and 10 μl added to assays. Final ethanol concentration did not exceed 1%. Superoxide dismutase (SOD) was used at a final concentration of 175 units/ml. Concentrations of substrates for anaerobic incubations were 10 mM each of glucose, formate, glycerol, and fumarate. Combinations were as shown in Figures 3, and 4. R2A broth without phosphate (R2A-P) was used at 0.1-strength, final concentration. Nitrate was present as 40 mM KNO_3. Assays were performed in 10 mM potassium phosphate buffer containing 0.85% NaCl,

5 mM $MgCl_2$, at pH 6.5. The pH of all whole cell assay solutions was adjusted to 6.5 prior to cell and tetrazolium salt addition. Cells were used at a final concentration of ca. 10^9 cells/ml. Cells for nutrient assays were allowed to react for 1 h prior to tetrazolium salt addition. Freshly prepared CTC and INT solutions were added at a final concentration of 1 mM. Incubations were performed in duplicate at 25°C with vigorous aeration for 2 h in the dark (anaerobic incubations were 4 h). Reactions were stopped by addition of 3.7% formaldehyde (final concentration) and immersion in an ice bath. Samples for R/ETS ratio measurements were stopped by addition of 5% trichloroacetic acid (TCA) final concentration. Formaldehyde killed (15 min exposure) controls were performed for all assays. Subsamples (91 μl) for CTC-reduction assays were diluted in filter-sterilized distilled water, filtered through 0.22 μm pre-blackened Nuclepore polycarbonate membranes (no. 110656), and counterstained with 10 μg/ml DAPI (35). Subsamples for INT assays were diluted as above and air-dried as heat-fixed smears using 20 μl aliquots. These were counterstained with 100 mg/l acridine orange (AO) in 2 mM Tris buffer (pH 7.0) for 3 min. and viewed under both epifluorescence and bright field microscopy. Samples for epifluorescence direct counts were viewed using a Leitz Ortholux II microscope equipped with Leitz filter blocks B2, N2.1, and H3 to visualize DAPI, CTC, and INT/AO, respectively. INT (+) cells contained red INT-formazan deposits when viewed under bright-field microscopy, while CTC (+) cells contained fluorescent-orange CTC-formazan deposits. At least 600 cells in ≥10 fields were counted at 1,250 X for each filter. Error was calculated as described previously (40).

Samples for spectrophotometric determination of formazan (0.9 ml) were centrifuged in a high-speed microcentrifuge (5,000 X g, 10 min) and

supernatants discarded. Pellets were then resuspended in 95% EtOH and disrupted by sonication for 15 s using a sonicator microtip (Fisher Scientific Sonic Dismembrator 50) at 30% power, and stored at 4°C overnight. Suspensions were then centrifuged (9,000 X g, 10 min) and absorbance of supernatants determined at 450 nm for CTC, and 485 nm for INT using an HP 8452A spectrophotometer. Cell pellets retained no visible formazan after the above extraction procedure.

Inverted Membrane Vesicles

Three liter cultures of *E. coli* K-12 grown aerobically as described above were harvested in late log phase and inverted membrane vesicles, as well as vesicles depleted in Mg^{2+}-dependent ATPase, were prepared by the method of Burstein et al. (9). Depleted inverted-vesicles lack Mg^{2+}-dependent ATPase activity, but retain membrane-bound dehydrogenase and electron transport activities (9, 34). Study of inhibition of these membrane processes is facilitated through the use of *inverted* membrane vesicles which avoids problems related to inhibitor permeability through intact cell membranes (11, 29). Duplicate assays were performed in a final volume of 1.0 ml, 10 mM phosphate buffer (pH 6.5), containing 5 mM magnesium acetate. Inhibitor concentrations used were the same as those for intact cells above, and sites of action are shown in Figure 2 and Table 1. Vesicle protein (0.25 mg) was added, and allowed to react with agents for 30 min before tetrazolium salt addition. Freshly prepared INT and CTC were added to a final concentration of 1 mM. Substrates were then added to final concentrations of 10 mM succinate, or 0.6 mM NADH which was generated using 50 μg alcohol dehydrogenase, 1% absolute ethanol, and 0.6 mM NAD^+ (29).

Table 1. Substrates, inhibitors, and sites of inhibition for inverted membrane vesicle, tetrazolium reduction assays.

Substrate	Inhibitor	Site of Inhibition
NADH	Rotenone	NADH dehydrogenase
Succinate	Malonate	Succinate dehydrogenase (competetive inhibition)
Succinate	TTF	Quinone binding subunit of succinate dehydrogenase
NADH	HQNO	Ubiquinone-cytochrome *b*
NADH	Antimycin A	Cytochrome *b*-cytochrome *o/d*
NADH	KCN	Cytochrome *o/d* complexes
NADH	Superoxide dismutase	Superoxide anions
NADH	Depleted membranes	Mg+-dependent ATPase
NADH	CCCP	Transmembrane proton gradient
NADH	[a]ADP	Stimulation of ATPase

[a]ADP is considered a stimulator rather than an inhibitor.

There was no detectable formazan production by these substrates in the absence of vesicles. Oxygen consumption rates remained constant throughout the 1 h incubations with both succinate and NADH. Reactions were performed at 25°C for 1 hr in the dark, and were stopped by the addition of 5% TCA (final concentration). TCA-treated controls were performed using succinate and NADH as reductants. There was no detectable formazan produced by TCA-treated vesicles, or vesicles in the absence of substrate. Membranes were pelleted at 175,000 X g for 1 h, resuspended, and formazans dissolved in 95%

ethanol as described for intact cells above. Debris was pelleted by repeating centrifugation, and the supernatants used for absorbance measurements.

Oxygen Consumption

Oxygen consumption was measured using a Gilson 5\6 oxygraph with a Clark-type electrode (29). All experiments were performed in a 2 ml- water-jacketed cell at 25°C. Oxygen saturation was measured using double-distilled water and corrected for altitude and pressure (2). All assays were carried out in the same solutions used for tetrazolium reduction assays. Protein was determined by the micro-BCA method (39). TCA-(membranes), and formaldehyde-(whole cells) treated controls showed no detectable oxygen consumption or formazan formation.

Calculations

In order to correlate oxygen consumption with formazan production it was necessary to convert values obtained to the number of electron equivalents (eq) required for reduction. The values 2 eq/mol formazan, and 4 eq/mol O_2 were used (17, 26, 37). The degree of coupling between oxygen consumption and tetrazolium reduction (nmol O_2-equivalents/nmol formazan-equivalents) was expressed as the respiration/electron transport system (R/ETS) ratio (26). To determine the relative effects of various agents on tetrazolium reduction, all whole cell- or vesicle-formazan values were compared to no-inhibitor or no-substrate controls. Percent deviations from controls were calculated using the equation: [mol $formazan_{(inhibitor\ or\ substrate)}$-mol $formazan_{(control)}$/mol $formazan_{(control)}$] X 100 = % deviation. Statistical significance of differences

between treatments and controls was determined using a one-tailed student's *t*-test assuming unequal variance ($\alpha = 0.05$) (24).

Results and Discussion

Chemical Properties of CTC/INT, and Their Formazans

The physical and chemical properties of CTC and INT, and their corresponding formazans determined in this study are listed in Table 2.

Table 2. Physical and chemical properties of CTC and INT reduction. Molar extinction coefficients, solubilities, and CTC redox potential were determined in this study.

Chemical Property	Tetrazolium Salt		Tetrazolium Formazan	
	CTC	INT	CTF	INF
$E_{1/2}$ (mV)[a]	-200 (-50 ?)	-90	--	--
Solubility[b]	ca. 50 mM	ca. 5 mM	ca. 200 μM	ca. 250 μM
Color	Colorless to light yellow	Colorless to light yellow	Red-orange	Purple-red
λ_{max}	ND[c]	ND	450 nm	485 nm
ε(90% EtOH)	ND	ND	1.624×10^4 l/mol∘cm	6.781×10^3 l/mol∘cm
Fluorescence	NF[d]	NF	Ex: 380 nm Em: 602 nm	NF

[a] Mid-point reduction potential (pH 7.0, 25°C) with reference to the normal hydrogen electrode. Possible CTF production at ca. -50 mV (see text).
[b] Tetrazolium salt solubilities at 25°C were determined in distilled water, formazan solubilities were in 95% ethanol (formazans are water insoluble). CTC solubility varied with solvent (i.e. seawater, distilled water, etc.)
[c] ND, Not determined.
[d] NF, not fluorescent.

During reduction CTC underwent a distinct three-phase transition, from soluble and dark orange, to a brilliant orange colloidal suspension (ca. -50 mV), and finally to a dark orange-red formazan precipitate. During the colloidal phase, redox potential readings took ca. 10 minutes to increase and stabilize after dithionite addition. No such effects were noted with INT which underwent a two-phase transition, from dark red and soluble, to the insoluble purple-red formazan. In the presence of ascorbic acid as a reducing agent, CTC was reduced at pH $\geq$ 4, while INT was reduced at pH $\geq$ 5, indicating CTC was more easily reduced than INT (37). Formazan formation was greatest between pH 5.5-8.5 for INT, and pH 5-10 for CTC.

Tetrazolium salts are converted to formazans by all reducing systems possessing actual redox potentials more negative than the tetrazolium/formazan system (37). Therefore, use of CTC and INT in reducing environments (sediments, media containing reducing agents [thioglycollate, ascorbate, etc.]) where in situ redox potentials are lower than the $E_{1/2}$ values of the tetrazolium salts will lead to non-biological tetrazolium reduction (33, 37). This could explain observations of INF on non-living detrital material in benthic suspensions, and CTC reduction by some growth media (8, 31). In addition, bacterial exopolymeric material (alginate from *Pseudomonas aeruginosa*) and agar have been reported to reduce CTC and INT, respectively in the absence of viable cells (1, 5). This may be due to non-biological reduction by ionized carboxyl-, or pyruvyl -groups in these polysaccharides, particularly under acidic conditions.

While different tetrazolium salts can be compared on the basis of mid-point reduction potentials ($E_{1/2}$) and reducibilities obtained under identical assay conditions, actual reduction potentials will vary dependent on test

conditions (pH, concentration) (16, 37). In general, the less negative a tetrazolium salt redox potential the more easily it is reduced (36, 37). However, results indicated that CTC was slightly more reducible than INT. This may be due to the formation of a colored, colloidal intermediate during CTC reduction at ca. -50 mV. Tetrazolium salts undergo tetrazolinyl-radical cation intermediate stages during reduction, and it appears that this form is more stable and produces a diffuse, poorly localized formazan at redox potentials higher than ca. -200 mV during CTC reduction (Fig. 1) (27, 37). This would explain the evenly-dispersed, weakly-fluorescent intracellular deposits observed in whole cell CTC reduction assays in the presence of certain inhibitors (antimycin A, KCN, CCCP) (see below). Similar results have been observed using mendola blue ($E_{1/2}$ = -110 mV) as an artificial electron donor in *Listeria monocytogenes* and Erlich ascites tumour cells (8, 28, 42). We have also observed this phenomenon in the presence of phosphate over ca. 10 mM in *E. coli* (41, see following chapter). Ethanol-extractable CTF in the absence of CTC (+) cells may also be due to this phenomenon.

Respiration/Tetrazolium Reduction Ratios

R/ETS ratios indicate the moles of respiratory electron flow determined by oxygen consumption (R) per mole of tetrazolium salt reduced (ETS). Thus, R/ETS ratios reflect the degree of coupling between these two processes (see materials and methods) (26). Although R/ETS ratios varied with time of incubation, in general, tetrazolium reduction reflected a fairly constant fraction of respiratory activity over 1 h incubation (particularly after 20 min). Using inverted membrane vesicles, R/ETS values with NADH as the substrate were ca. 45-60 for CTC, and 8-14 for INT over 1 h incubation (Table 3).

Table 3. Ratios of respiration and ETS activity for whole cell and inverted membrane vesicle tetrazolium reduction assays.

	Respiration/ETS[a]					
	Whole Cells		Inverted Membrane Vesicles			
	Endogenous		NADH		SUCCINATE	
	(Oxidant)		(Oxidant)		(Oxidant)	
Time (min)	CTC	INT	CTC	INT	CTC	INT
5	2.4 ± 0.24	47.7 ± 3.2	42.2 ± 3.5	11.8 ± 3.4	59.2 ± 4.1	10.5 ± 1.0
10	5.2 ± 0.79	74.3 ± 4.5	46.2 ± 2.5	13.7 ± 2.1	57.0 ± 3.7	8.7 ± 0.77
20	5.7 ± 0.54	76.5 ± 5.1	47.8 ± 2.2	13.1 ± 1.3	38.2 ± 2.4	4.9 ± 1.2
40	7.8 ± 0.97	115 ± 7.1	58.0 ± 3.2	7.8 ± 1.1	34.7 ± 3.3	2.9 ± 0.97
60	8.9 ± 0.76	91.3 ± 4.3	57.6 ± 2.8	9.1 ± 1.9	39.2 ± 3.1	3.6 ± 1.4

[a]Respiration/ETS = mol O_2 eq/mol formazan eq.

Using succinate as the substrate R/ETS ratios for CTC and INT were ca. 35-60, and 4-10, respectively. Thus, the degree of coupling was roughly similar for individual tetrazolium salts with either succinate, or NADH as the primary electron donor. These results also indicated INT reduction was more tightly coupled than CTC to respiratory electron flow in inverted membrane vesicles by a factor of ca. 5-10 (Table 3). In contrast, R/ETS ratios for whole cells indicated CTC reduction was more tightly coupled to respiratory electron transport than INT by a factor of ca. 10 (Table 3). R/ETS ratios obtained in these systems were also significantly higher than those reported previously

using bacterial cell homogenates (17, 26). These results suggest CTC and/or INT reduction may be limited by penetration through the intact cell envelope to the site(s) of reduction in whole cells. Alternatively, CTC reduction may be more closely linked than INT to dehydrogenases active during endogenous respiration, or may be inhibited by relatively low $\Delta\mu H+$ in inverted vesicles compared to whole cells (see below). One additional possibility is differential NADH-D and SDH activities due to changes in membrane lipids during vesicle formation (10). Slightly lower R/ETS ratios after 20 min. incubation using succinate as compared with NADH as the respiratory substrate indicated reduction of CTC and INT was more closely coupled to SDH than NADH-D activity in vesicles. In other words, normalization of formazan production values to respiratory electron flow as measured by oxygen consumption indicated that SDH activity reduced more INT and CTC to formazan than NADH-D.

Effects of Inhibitors on Tetrazolium Reduction

The methods of mid-, and terminal-chain blockage were used to determine sites of CTC and INT reduction. In general, blockage of electron transport below (i.e. closer to the terminal oxidase) the site of tetrazolium reduction should either increase, or leave unchanged the amount of formazan produced compared to the control (no inhibitor) (25, 27). Blockage prior to the site of reduction (i.e. closer to the primary dehydrogenase) should decrease formazan production (Fig. 2). Effectors of ATP synthesis (ADP, ATPase depleted membranes), and a proton conductor (CCCP) were also utilized to determine their effects on tetrazolium reduction. Inhibition of respiratory electron transport processes using inverted membrane vesicles indicated site(s) of CTC and INT reduction under defined dehydrogenase (SDH or NADH-D) activities,

while experiments using whole cells indicated site(s) of reduction during endogenous respiration (Figs. 2, 3, 4A, and Table 1) (29).

The production of both INF and CTF in inverted membrane vesicles using succinate or NADH as the reductant indicated both CTC and INT reduction was mediated through membrane-bound SDH-, and NADH-D activities. Rotenone treatment strongly increased CTF, but decreased INF production in whole cells (and vesicles), indicating both CTC and INT were reduced by NADH-D in both vesicles and whole cells (Figs. 3 and 4A). However, a decrease in CTF formation in vesicles using rotenone suggests CTC was reduced prior to the FeS-2 center of NADH-D in this system (Figs. 2 and 4A) (29, 51). Malonate inhibition (nearly 100% for CTC), and TTF stimulation of CTF and INF production in vesicles indicated both CTC and INT were also reduced by SDH, prior to the ubiquinone-binding subunit (Figs. 2 and 3) (19, 32, 48). The nearly 7-fold increase in INF formation with TTF indicated INT reduction was tightly coupled to SDH activity, consistent with R/ETS ratio results (see below) (Fig. 3). Using whole cells, SDH inhibition by malonate or TTF had no significant effect on CTC-, while increasing INT-reduction 10-fold (Fig. 4A). Thus, while CTC and INT were reduced by both SDH and NADH-D in vesicles, CTC reduction did not appear to be mediated primarily by SDH in whole cells. Increased INF formation in malonate-treated whole cells may be due to stimulation of other dehydrogenases active during endogenous respiration with $E_{1/2}$ values higher than that of INT, such as formate dehydrogenase ($E_{1/2}$ = -105 mV) (30). Inhibition at ubiquinone (HQNO) and cytochrome *b* (antimycin A) increased CTC reduction in vesicles, while no significant effect was observed using whole cells (Figs. 3 and 4A). This indicated that CTC was reduced prior to ubiquinone in the respiratory chain as has been reported for

Micrococcus luteus (Fig. 2, Table 2) (14, 29). In both vesicles and whole cells INF formation was decreased by HQNO, but strongly increased by inhibition at points further along the transport chain (antimycin A, cyanide, azide), indicating INT reduction by ubiquinone and possibly cytochrome $b_{555, 556}$

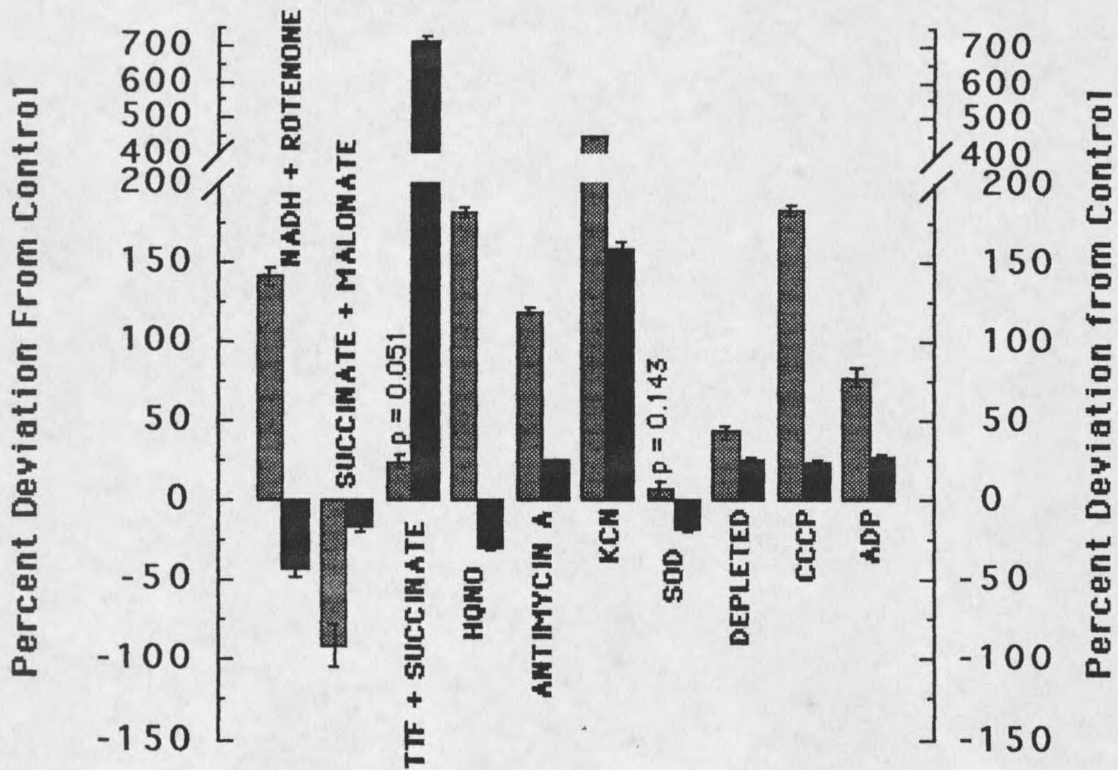

Figure 3. Effects of various inhibitors on CTC (shaded bars), and INT (solid bars) reduction in *E. coli* inverted membrane vesicles. Data are expressed as percent deviations in formazan production from substrates (NADH or succinate) alone. Sites of inhibition are listed in Table 2. P-values are ≤ 0.05 except where noted. Error bars represent standard deviations (n = 2). Calculations and abbreviations are as described in materials and methods.

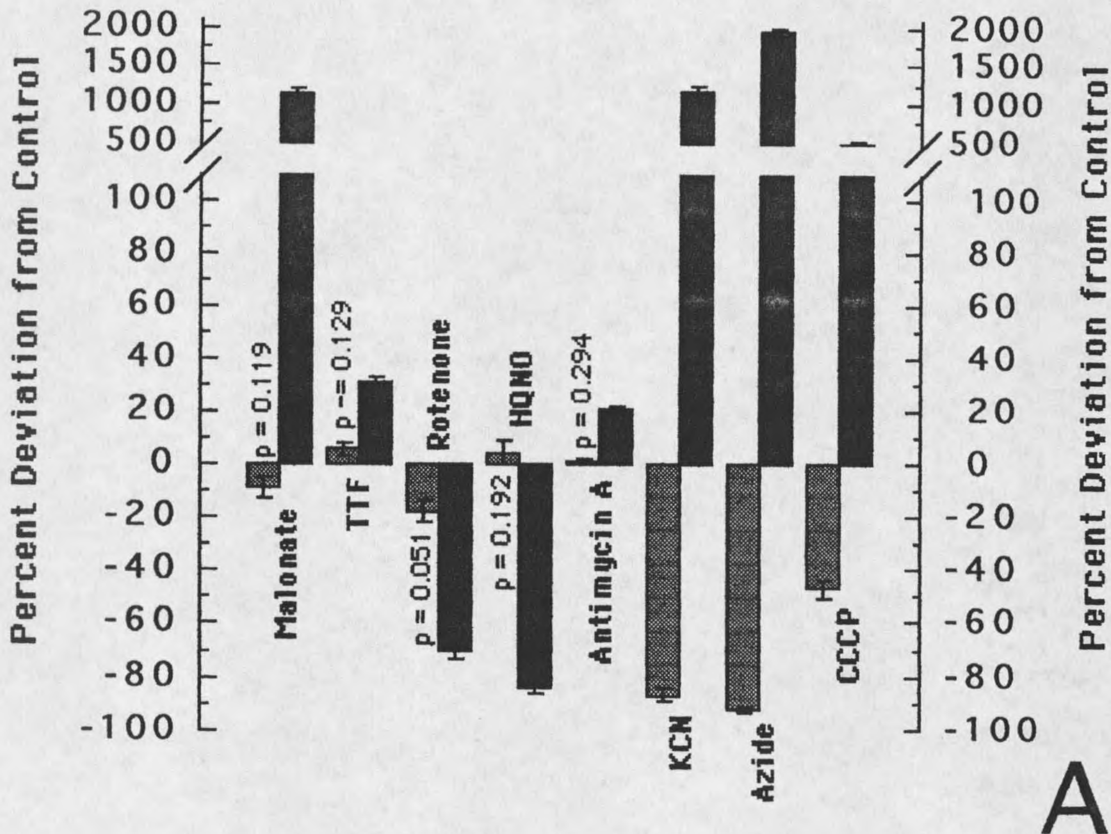

Figure 4(A). Effects of inhibitors on aerobic CTC (shaded bars), and INT (solid bars) reduction in whole cells of *E. coli.* Formazan production values are expressed as a percentage deviations from control without inhibitors (A). P-values are ≤ 0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (B). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent standard deviations (n = 2). See materials and methods for calculations and abbreviations.

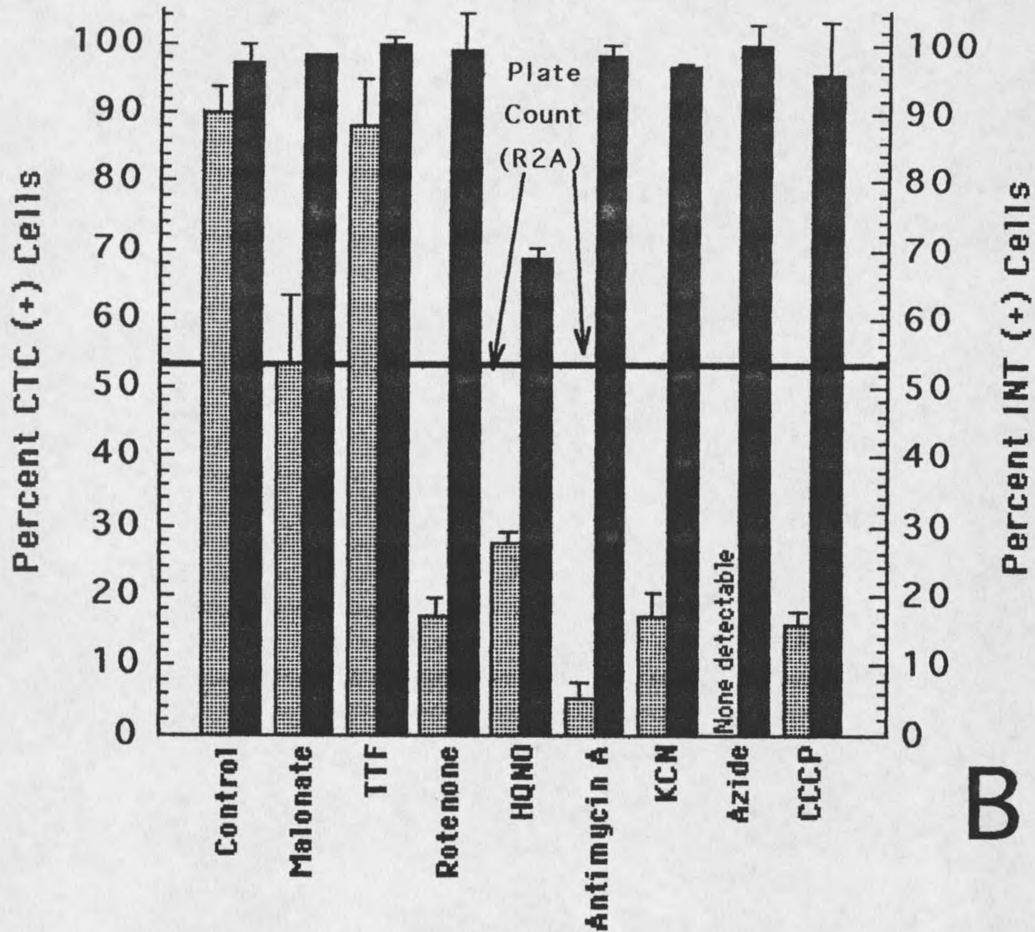

Figure 4(B). Effects of inhibitors on aerobic CTC (shaded bars), and INT (solid bars) reduction in whole cells of *E. coli.* Formazan production values are expressed as a percentage deviations from control without inhibitors (A). P-values are ≤ 0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (B). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent standard deviations (n = 2). See materials and methods for calculations and abbreviations.

(Figs. 2, 3, and 4A) (29, 30). In contrast, KCN and azide inhibition of cytochromes *o* and *d* strongly decreased CTF formation in whole cells, suggesting CTC reduction by the terminal oxidases (Fig. 4A) (4, 30). Similar results have been reported with azide-treated *Pseudomonas diminuta* (33). Considering the $E_{1/2}$ of CTC (-200 mV), it appeared unlikely that the terminal oxidases could directly reduce CTC, as their mid-point potentials are ca. +70 to +280 mV (Fig. 2 and Table 2). SDH and NAD-H are indirectly inhibited by blockage of terminal oxidases (27, 31). In addition, cyanide is known to block FeS-centers in several enzymes directly (22, 35). However, SDH and NADH-D inhibition by KCN are not consistent with increased reduction of both CTC and INT in KCN-treated vesicles, and inhibition by azide (which does not effect FeS-centers) in whole cells (Figs. 3 and 4A). Inhibition of the terminal oxidases also inhibits the major site of proton consumption (in the formation of H_2O by terminal oxidases) and transmembrane electrochemical gradient ($\Delta\mu H+$) generation in *E. coli* cells (Fig. 2). This gradient, formed across the membrane, can be significant (-80 to -180 mV, negative inside) and contributes to the overall redox environment in which tetrazolium reduction takes place (4, 23, 30, 37). CTC reduction is expected to be more sensitive to an increase in redox potential than INT due to CTC's lower $E_{1/2}$, and thus may be selectively inhibited to some degree. The observed decreased CTF-, and increased INF-formation by collapsing $\Delta\mu H+$ in whole cells with CCCP support this hypothesis (Fig. 4A). Although CTC reduction increased in CCCP-treated inverted vesicles, proton consumption is on the outside surface of the membrane in buffered solution, inhibiting normal (i.e. whole cell, or non-inverted vesicle) $\Delta\mu H+$ formation, but still allowing some degree of uncoupling (Fig. 3) (9). However, it should be noted that intracellular CTC reduction by

superoxide anion in whole cells cannot be ruled out. Dismutation of superoxide anions in vesicles using SOD had no effect on CTC reduction, while slightly decreasing INF formation (Figs. 2 and 3, Table 2). INT reduction may thus be mediated to some extent by production of superoxide anions as hypothesized by Seidler (37). Uncoupling (CCCP), depletion of ATPse, and ADP all increased CTF and INF formation in vesicles (Fig. 13). Since these conditions are all known to increase respiratory electron transport activity, it appears CTC and INT reduction was linked to electron transport processes which were, in turn, linked to ATPase activity (9, 29, 34).

Direct Counts

In general, direct counts of CTC and INT (+) aerobic cells reflected formazan production trends compared to controls (Figs. 4A and B). However, cell CTC (+) counts were decreased significantly using HQNO and antimycin A, while CTF production was unaffected (Fig. 4A and B). This suggests that factors other than the total amount of CTF produced affect formation of fluorescent CTF crystals. Size and number of fluorescent CTF crystals per cell was highly variable depending on the particular inhibitor used. Several inhibitors, notably HQNO, antimycin A, cyanide, azide, and CCCP resulted in very faintly fluorescent CTF which was evenly distributed throughout cells. Formation of visible INF crystals appeared less sensitive to the effects of inhibitors than CTF (Table 3). However, visualization of INF crystals was difficult compared to fluorescent CTF, particularly when deposits were small. Using control cells, plate counts on R2A indicated 52.6 ± 1.4% of total cells (AODC) were able to form colonies on this medium while INT and CTC (+) cell numbers were 97.0 ± 2.2, and 89.9 ± 0.9% of total cell numbers, respectively (Fig. 4B). INT (+) cell

numbers were consistently higher than plate counts, even in the presence of inhibitors (Fig. 4B). TTF, rotenone, and azide were the most effective agents for increasing INT (+) cell counts (Fig. 4B). CTC (+) cell numbers were greatest using TTF, and in the no inhibitor control (Fig. 4B). All CTC (+) cell numbers were significantly below plate counts, except for the control, malonate, and TTF samples (Fig. 4B). This may have been due to the use of 1 mM CTC, as maximal formazan production has been found at 2-6 mM in several bacteria (8, 33). The use of cyanide or azide to increase tetrazolium reduction (as has been suggested for INT) will increase INF formation significantly (24, 26, 38, 45, 54) (Fig. 4B). However, this technique may not always be appropriate for CTC reduction assays in intact bacterial cells (42, 43).

The reduction of INT and CTC appeared closely linked to bacterial respiratory activity. However, significant intracellular reduction of both tetrazolium salts was observed in formaldehyde-treated cells if sufficient time (5-15 min) had not passed before CTC or INT addition (data not shown). This effect was much more pronounced for INT than CTC, requiring ca. 15 min contact with formaldehyde to completely eliminate INT-, and 5 min. to eliminate CTC-reduction (CTF produced was diffuse and poorly fluorescent). Similar results were noted using INT in a recent study of tetrazolium reduction in *Campylobacter jejuni* by Boucher et al. (7). This may be related to "nothing dehydrogenase" activity noted in several histochemical studies attributed to residual reduction potential in fixed cells (3, 21, 37). Since INT would continue to be reduced at higher redox potentials than CTC as the potential dissipates (i.e. >100 mV, <90 mV), this may explain more persistent INT reduction after fixation. No detectable tetrazolium reduction was observed in the presence of nutrients or formalin without cells. Therefore, to prevent residual formazan

formation it is important to fix cells with formaldehyde for at least 10-15 min prior to tetrazolium salt addition. This differs from results using R2A as a nutrient source reported by Bovill et al., although the concentration of R2A in the present study was 10-fold lower (8).

Reduction of tetrazolium salts by inverted membrane vesicles suggests their spontanous reduction by "nonviable" cell membranes. However, it should be emphasized that vesicles are produced under reducing conditions (dithiotreitol) in order to maintain the reduced state of electron-transport components (9). Indeed, a reducing environment is important to obtain vesicles with active dehydrogenases (3, 11, 29). In the environment (under non-reducing conditions) ETS components would be expected to oxidize, degrade, and become nonfunctional. However, reduction of CTC or INT by membrane-bound ETS components (in the absence of viable cells) in reducing environments such as sediments cannot be ruled out.

CTC and INT Reduction Under Anaerobic Conditions

Tetrazolium reduction in whole cells was assayed under anaerobic conditions which induce 4 different electron transport pathways: Glucose fermentation, formate dehydrogenase-fumarate reductase (> +30 mV), glycerol dehydrogenase-fumarate reductase, and nitrate reductase (+20 to +220 mV) under endogenous respiration (Figs. 5A and B) (20). R2A-P was added as a general source of substrates. Glucose fermentation and R2A-P medium (which contains glucose and pyruvate) stimulated reduction of both tetrazolium salts over 100%, indicating that the various dehydrogenases postulated to be active during glucose fermentation reduce CTC, and particularly INT. Induction of the formate dehydrogenase-fumarate reductase pathway increased CTC

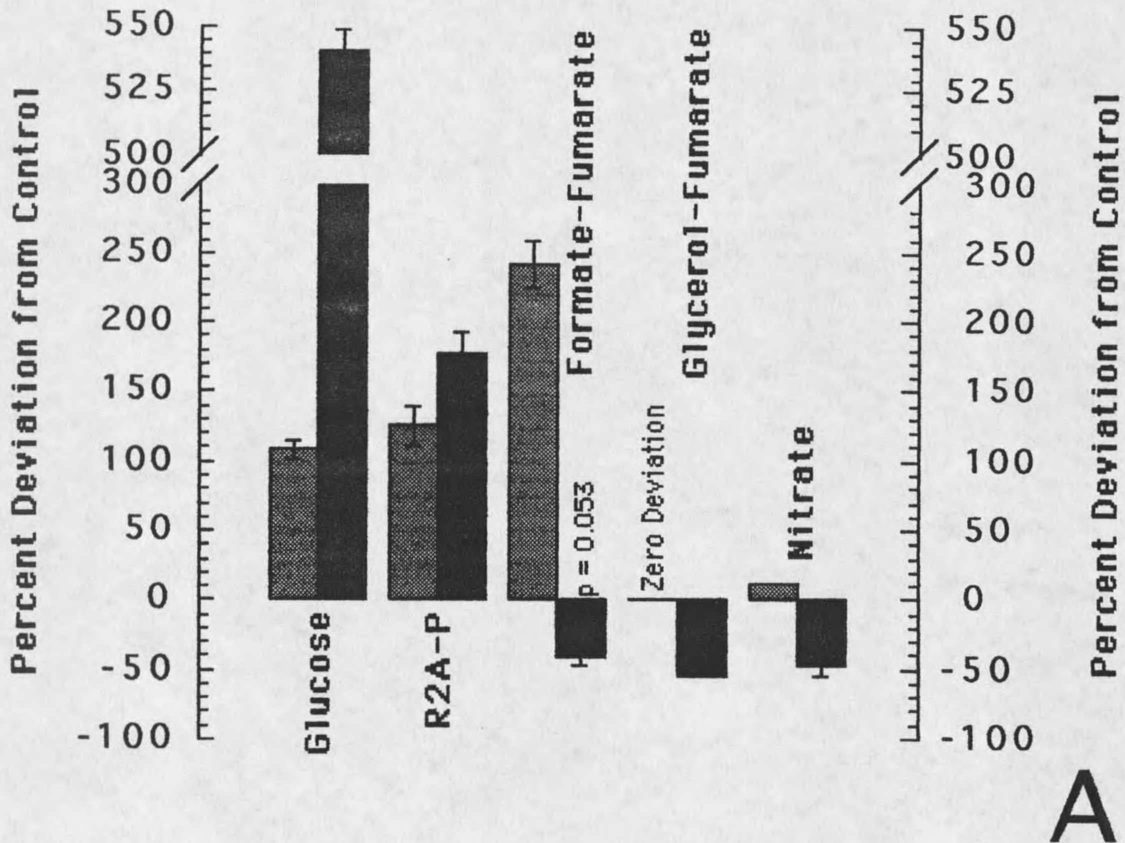

Figure 5(A). Effects of various substrates and electron acceptors on CTC (shaded bars) and INT (solid bars) reduction by whole *E. coli* cells under anaerobic conditions. Formazan production values are expressed as a percentage deviations from control without substrate(s) (A). P-values are ≤0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (B). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent standard deviations (n = 2). See materials and methods for calculations and abbreviations.

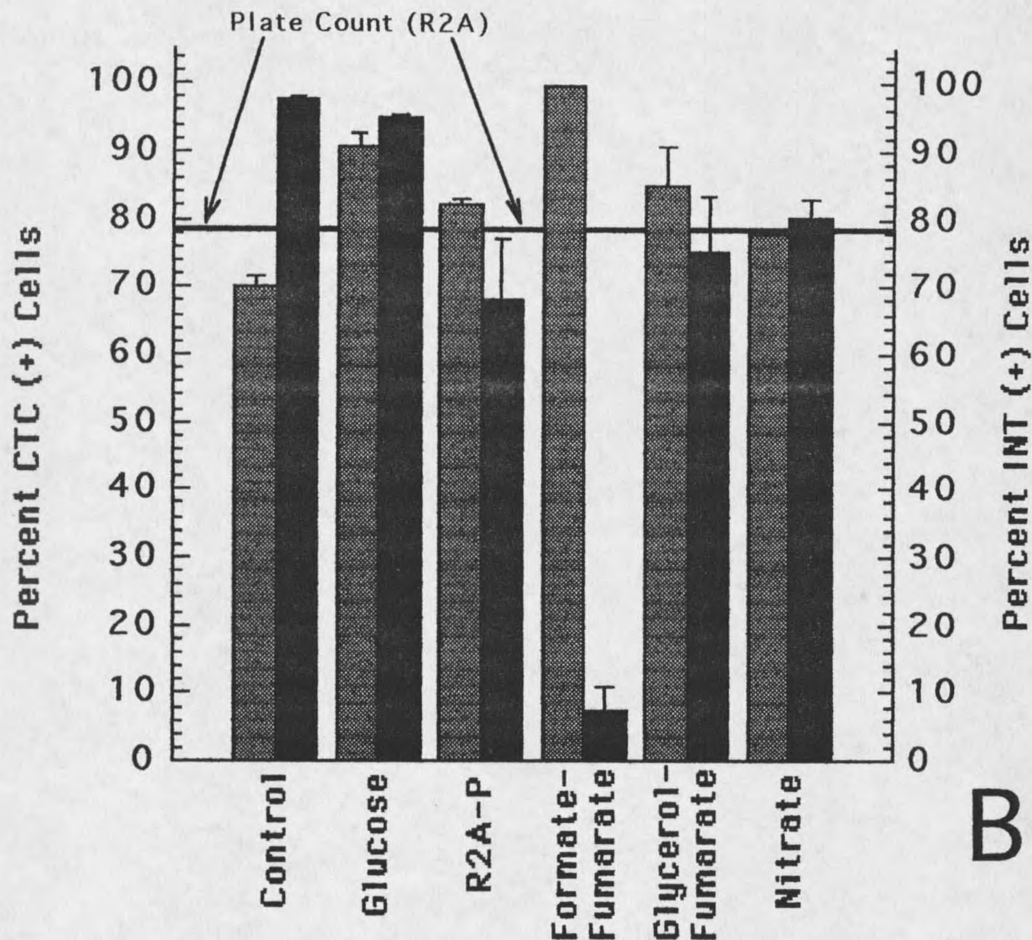

Figure 5(B). Effects of various substrates and electron acceptors on CTC (shaded bars) and INT (solid bars) reduction by whole *E. coli* cells under anaerobic conditions. Formazan production values are expressed as a percentage deviations from control without substrate(s) (A). P-values are ≤0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (B). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent standard deviations (n = 2). See materials and methods for calculations and abbreviations.

reduction to the greatest degree (Fig. 5A and B). CTC is most likely reduced by formate dehydrogenase (-145 to -360 mV) in this system (20). Stimulation of CTC reduction by formate is similar to results found with aerobic *Campylobacter jejuni* cells (7). Glycerol-fumarate and nitrate had little, or no effect on CTF formation, but CTC (+) cell numbers with glycerol-fumarate increased 5-10% over control values (Figs. 4A and B). In contrast, INF formation, and INT (+) cell numbers decreased with fumarate or nitrate as the terminal electron acceptor (Fig. 5A). Numbers of CTC (+) and INT (+) cells were above ca. 70% under all anaerobic incubation conditions tested (except INT in the presence of formate-fumarate) (Fig. 5B). Glucose and formate-fumarate produced the largest numbers of CTC (+) cells, while INT (+) numbers were greatest in the control and glucose samples (Fig. 5B). With the exception of the control, CTC (+) numbers were equal to, or greater than plate counts on R2A in all cases. Thus, substrate addition appeared necessary for CTC (+) counts to exceed plate counts when using 1 mM CTC. INT (+) cells numbers were 5-20% greater than plate counts in the control, glucose, and nitrate samples (Fig. 5B). INT and CTC were reduced to significant degrees under all anaerobic conditions employed in this study, with glucose particularly stimulating formazan production.

In summary, studies using both inverted membrane vesicles and whole cells indicated CTC and INT were reduced by both SDH and NADH-D (Fig. 2). However, the specific sites involved in the reduction of CTC and INT were somewhat different since CTC appeared to be reduced by dehydrogenases other than SDH in whole cells. In addition, INT could be reduced by ubiquinone, possibly cytochrome $b_{555,556}$, superoxide anion, and perhaps other primary dehydrogenases which donate electrons to ubiquinone (Fig. 2). These results

are consistent with reduction sites inferred from INT and CTC mid-point redox potentials, as well as being similar to sites determined for eukaryotic mitochondrial electron transport chains, and *Micrococcus luteus* (Fig. 2, Table 2) (14, 26, 27, 30, 37). Both CTC and INT were reduced under most anaerobic conditions tested, particularly glucose fermentation (Fig. 5A). Coupling of tetrazolium reduction to respiration (as measured by oxygen consumption) differed between whole cells (CTC > INT) and inverted membrane vesicles (INT > CTC), but in general ranged between 10 and 40 pairs of respiratory electrons for every tetrazolium molecule reduced. CTC reduction appeared more sensitive to effectors of intracellular redox potential than INT in whole cells, and appeared to produce a weakly-, or non-fluorescent formazan at redox potentials higher than ca. -200 mV. Also, use of inhibitors of terminal oxidases (i.e. cyanide, azide), and uncouplers may not be useful to maximize whole cell prokaryotic CTC reduction assays.

Information obtained using CTC and/or INT to assess respiratory activity in prokaryotic cells will vary depending on the organism and assay conditions. For instance, it appears some active bacteria do not reduce INT, or CTC under certain conditions (40, 47). This could be related to low tetrazolium salt substantivity (association with cell components, permeability), unique respiratory pathways, or low cellular $\Delta\mu_{H^+}$ (particularly using CTC) in some bacteria (37). To date CTC reduction has been reported in *E. coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Enterococcus faecalis* (40), *Klebsiella pneumoniae* (52), *Paracoccus denitrificans* (41), *Micrococcus luteus* (14), *Pseudomonas fluorescens* (13), *Pseudomonas putida* (33), *Campylobacter jejuni* (7), *Listeria monocytogenes* (8), *Listeria innocua* (41), *Shigella flexneri*, and *Vibrio cholerae* (non-01) (49). However, considering the relatively variable

nature of prokaryotic respiratory chains compared to eukaryotes, differences in tetrazolium reduction between organisms are to be expected. Therefore, selection of an appropriate tetrazolium salt for an application, and interpretation of data should take into account the histochemical properties of these indicators of respiration in prokaryotic systems.

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CHAPTER 3

EFFECTS OF SUBSTRATES AND PHOSPHATE ON INT (2-(4-IODOPHENYL)-3-(4-NITROPHENYL)-5-PHENYL TETRAZOLIUM CHLORIDE), AND CTC (5-CYANO-2,3-DITOLYL TETRAZOLIUM CHLORIDE) REDUCTION IN *ESCHERICHIA COLI*

Introduction

The tetrazolium salts INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) and CTC (5-cyano-2,3,ditolyl tetrazolium chloride) have often been used as indicators of bacterial respiratory activity and viability (3, 4, 5, 7, 10, 11, 13, 20, 21, 23, 26, 29, 30, 35, 36). Both salts are reduced to their insoluble red (INF), or fluorescent-orange (CTF) formazans by components of the prokaryotic respiratory chain (16, see chapter 2). Formazan crystals can subsequently be viewed under epifluorescence microscopy, or extracted in an organic solvent (commonly ethanol) and quantified spectrophotometrically. CTC appears to be reduced by the primary dehydrogenases (succinate, NAD(P)H, and possibly others) in *Escherichia coli*, while INT may also be reduced by ubiquinone, and possibly cytochromes *b*555,556 (see chapter 2). These sites of reduction are somewhat similar to those suggested for eukaryotic cells (15, 16, 17, 24, 27, 28). The redox state of cell membranes also appears to play a role in CTC reduction to fluorescent formazan crystals (see chapter 2). The amount of electron flow through the respiratory chain is dictated by the availability and quality of oxidizable

substrates, whether exogenous or endogenous. Indeed, it appears that several organisms increase tetrazolium reduction in response to increased nutrient concentrations (3, 5, 20, 21,23, 29). Considering the increased use of INT and CTC as indicators in studies of prokaryotic cell physiology and viability, an understanding of which substrates promote optimal tetrazolium reduction is needed.

Studies were undertaken to examine the effects of substrate addition on CTC and INT reduction in aerobic *Escherichia coli*, a prokaryote with relatively well described dehydrogenase and respiratory chain components (2, 6, 18, 34, Fig. 2). Substrates for the primary aerobic *E. coli* dehydrogenases (*sn*-glycerol-3-phosphate, succinate, formate, lactate, formate, and D-amino acid), as well as glucose were added at equimolar concentrations to cultures in saline, and gross formazan production, as well as INT and CTC (+) cell numbers compared. This laboratory has also noted a significant inhibitory effect of inorganic phosphate on CTC reduction to visible, fluorescent CTF deposits in some bacterial strains (Pyle *et al.* submitted). This inhibition results in significant underestimations of actively respiring cell numbers when certain buffers and/or media are used for CTC reduction assays. In order to investigate this phenomenon we correlated formazan production, oxygen consumption, and CTC/INT (+) cell numbers with phosphate concentrations commonly used in biological buffers and growth media.

Materials and Methods

Chemicals

5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was purchased from Polysciences, Inc., Warrington, Pa., U.S.A. 4',6-diamidino-2-phenylindole (DAPI), acridine orange (AO), disodium succinate, glucose, lactic acid, glycerol, and potassium phosphate were all purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A. R2A medium and casamino acids were obtained from Difco Laboratories, Detroit, MI, U.S.A.

Growth Conditions

Escherichia coli K-12 was grown aerobically with vigorous aeration at 25°C to early-stationary phase in glycerol (8 g/l)-mineral medium supplemented with 1 μmol l^{-1} selenic acid and sodium molybdate (6). Growth on glycerol will support production of all the primary *E. coli* dehydrogenases (18). Cells were washed 3 times and resuspended at 10^{10} cells/ml in 0.85% NaCl (pH 6.5). Plate counts were performed by spot-plating subsamples (exposed for the same period, in the same buffer as respective assays) onto R2A medium and incubation for 48 h at 25°C.

Incubation of Cells with CTC and INT

One-tenth ml of resuspended cells from above were added to 0.8 ml of saline containing 10 mM of either glucose, glycerol, succinate, formate, lactate,

or 0.2% casamino acids (final concentrations). Cells were also resuspended in saline containing 10, 25, 50, 75 and 100 mM potassium phosphate buffer, pH 6.5. Suspensions were shaken vigorously at 250 rev/min for 30 min, then freshly prepared CTC or INT was added to a final concentration of 1 mM. This concentration was chosen to maximize differences in tetrazolium reduction between substrates, rather than to optimize CTC (+), or INT (+) cell numbers (5, 21). Incubations were performed in duplicate at 25°C with vigorous aeration for 2 h in the dark. This time of incubation was found to give optimal CTC and INT (+) cell numbers. Reactions were stopped by addition of 3.7% formaldehyde (final concentration) and immersion in an ice bath. Formaldehyde killed (15 min exposure) controls were performed for all assays.

Examination of Cells for CTC and INT Reduction

Subsamples for CTC-reduction assays were diluted in filter-sterilized saline, filtered through 0.22 μm pre-blackened Nuclepore polycarbonate membranes (no. 110656), and counterstained with 10 μg/ml DAPI for 10 min. Subsamples for INT assays were diluted as above in distilled water and air-dried, heat-fixed smears prepared using 20 μl aliquots. These were counterstained with 0.01% acridine orange (AO) in 2 mM Tris buffer (pH 7.0) for 3 min and viewed under both epifluorescence and bright field microscopy. A similar bright field technique was used to visualize total (i.e. fluorescent and non-fluorescent) CTF deposits. However, DAPI was used as the fluorochrome. Samples for epifluorescence direct counts were viewed using a Leitz Ortholux II microscope equipped with Leitz filter blocks B2, N2.1, and H3 to visualize DAPI, CTC, and INT/AO, respectively. INT (+) cells contained red INT-formazan deposits when viewed under bright-field microscopy, while CTC (+) cells

contained fluorescent-orange CTC-formazan deposits. At least 600 cells in ≥10 fields were counted at 1,250 X for each filter. Error was calculated as described by Jones (9).

Measurement of Formazan Production

Samples for spectrophotometric determination of formazan (0.9 ml) were centrifuged in a high-speed microcentrifuge (5,000 X g, 10 min). Supernatants were then discarded, and pellets resuspended in 95% EtOH and disrupted by sonication at high power for 15 s using a sonicator microtip (Fisher Scientific Sonic dismembrator 50) at 30% power and stored at 4°C overnight. Suspensions were then centrifuged (9,000 X g, 10 min) and absorbance of supernatants determined at 450 nm for CTC, and 485 nm for INT using an HP 8452A spectrophotometer. Cell pellets retained no visible formazan after the above extraction procedure. Molar extinction coefficients were determined in 95% ethanol by dissolving INF and CTF at known concentrations. These values were subsequently used to convert optical density measurements to moles of formazan.

Measurement of Oxygen Consumption

Oxygen consumption was measured using a Gilson 5\6 oxygraph with a Clark-type electrode. All experiments were performed in a 2 ml- water-jacketed cell at 25°C. Oxygen saturation was measured using double-distilled water and corrected for altitude and pressure (1). All assays were carried out in saline (pH 6.5) alone, or with added phosphate as described above. Replication was achieved by reaerating the sample 3 times with a 5-ml syringe

and 22 gauge needle. The resuspended cells from Formaldehyde (3.7%) treated controls showed no detectable oxygen consumption.

Results

Effect of Substrates on Formazan Production

In all cases CTF production was considerably greater than INF by a factor of 30-150, depending on the substrate (Fig. 6A). In order of effectiveness in promoting CTF formation substrates were: lactate > formate > glucose > succinate ≈ glycerol > casamino acids > control. In order of production of INF substrates were: formate >> casamino acids > lactate > succinate ≈ control > glycerol ≈ glucose.

Effect of Substrates on Direct Counts

All substrates tested resulted in at least 85% CTC (+) cells, with formate, lactic acid, and casamino acids yielding 100% of cells showing fluorescent CTF deposits (Fig. 6B). All CTC (+) counts were 30-50% greater than plate counts with the most effective substrates in the order lactate ≈ formate ≈ casamino acids > succinate ≈ glycerol > glucose > control. CTF crystal size and intensity of fluorescence varied considerably between substrates. In general, succinate, lactate, and casamino acids produced the largest, most brightly fluorescent crystals, with most cells containing one deposit. Glucose, formate, glycerol, and endogenous respiration resulted in somewhat smaller CTF crystals. These cells often contained 1-2 deposits per cell, some of which were poorly

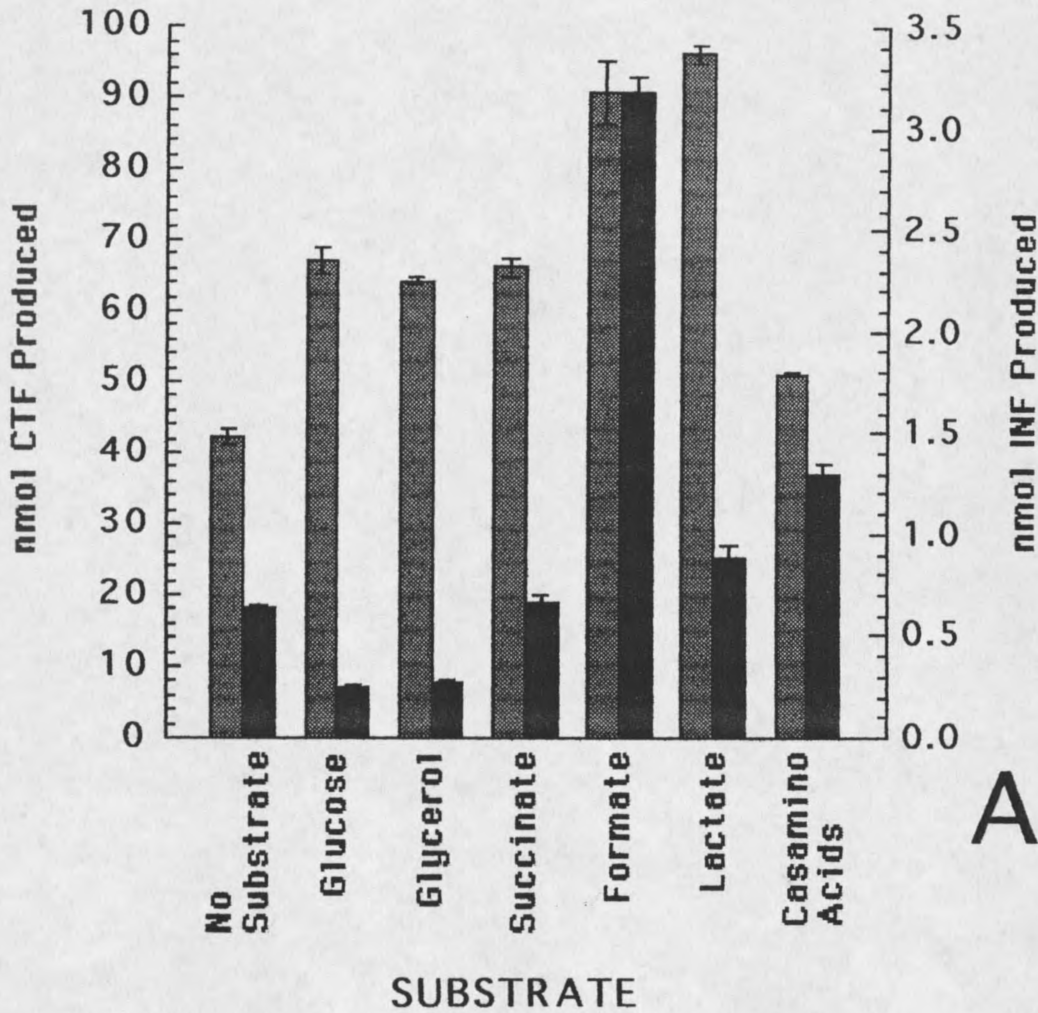

Figure 6(A). Effects of different substrates on CTC (shaded bars), and INT (solid bars) reduction to ethanol extractable formazan (A), and percent CTC/INT (+) cells by epifluorescence microscopy (B). Plate counts were on R2A agar. Error bars represent standard deviations (n = 2).

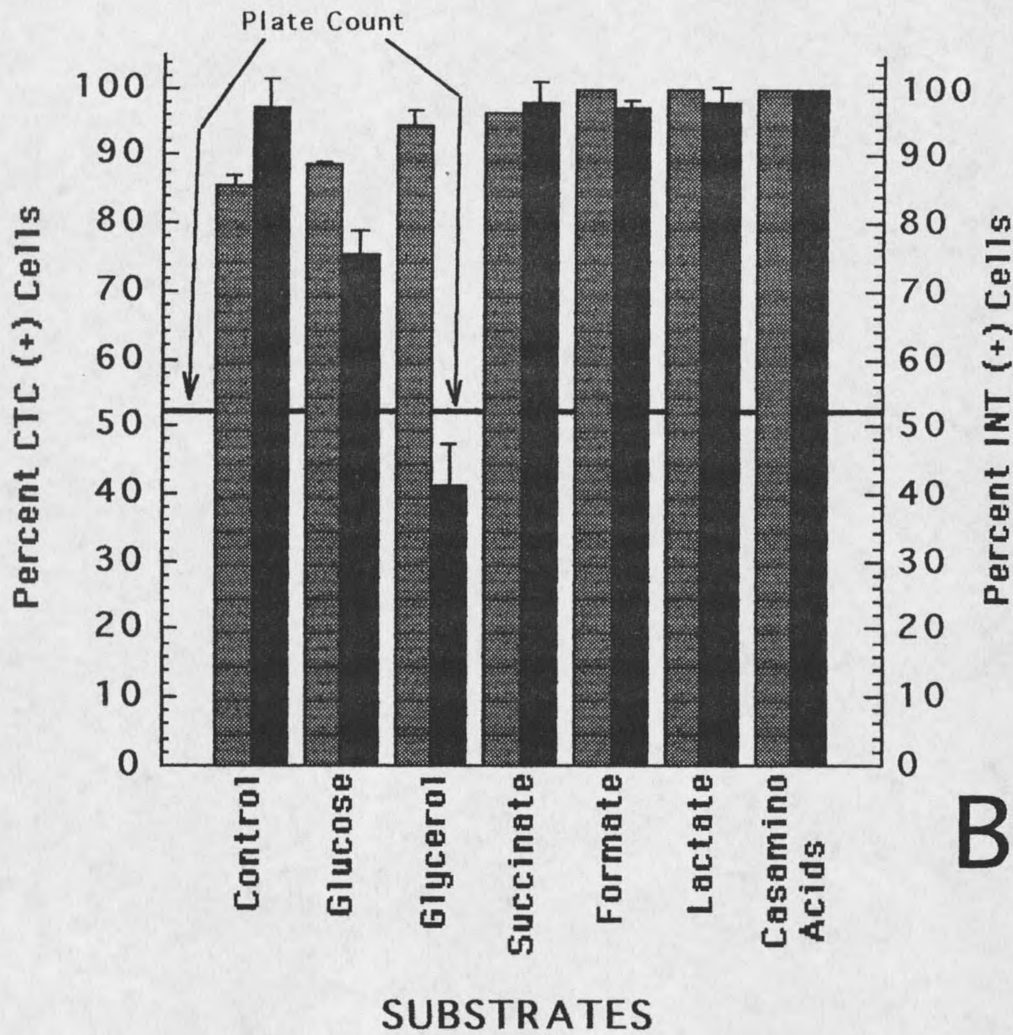

Figure 6(B). Effects of different substrates on CTC (shaded bars), and INT (solid bars) reduction to ethanol extractable formazan (A), and percent CTC/INT (+) cells by epifluorescence microscopy (B). Plate counts were on R2A agar. Error bars represent standard deviations (n = 2).

fluorescent (Fig. 6B). This effect was not as noticeable for INF deposits, except that crystal size was noticeably larger in samples with formate and casamino acids. Glycerol resulted in the least INT (+) cells, 10% below plate counts (Fig. 6B). INT (+) counts with other substrates were all 25-50% greater than plate counts with the most effective substrates in the order casamino acids > succinate ≈ lactate ≈ formate ≈ control > glucose > glycerol. Glucose resulted in INT (+) counts 15% below that of the control, which were approximately 95%.

Effects of Phosphate on CTC and INT Reduction

Phosphate increased CTC and INT reduction at 10 mM compared to controls containing no phosphate (Figs. 7A and B). However, it decreased both INT and CTC reduction above 10 mM. INT and CTC (+) cell counts also increased 5 and 10% with 10 mM phosphate compared to saline alone (Fig. 7A and B). CTC (+) cell numbers decreased with increasing phosphate concentration, declining sharply above 50 mM to less than 5% at 100 mM (Fig. 7B). In contrast, INT (+) cells remained between 90 and 95% up to 75 mM phosphate, with counts dropping to 85% at 100 mM (Fig. 7A). Bright field microscopic examination of CTF deposits showed that, while fluorescent plus nonfluorescent CTF (+) cell numbers decreased 20% with increasing phosphate, this effect was much less pronounced than the reduction in CTC (+) (i.e. fluorescent CTF crystals) cell numbers (Fig 7B).

Effect of Phosphate on Oxygen Consumption

Increasing phosphate concentration decreased rates of respiration as measured by oxygen consumption, although this was not seen with mid-log

phase cells (Table 4). This effect was most pronounced above 50 mM phosphate with O_2 consumption decreasing approximately 40%.

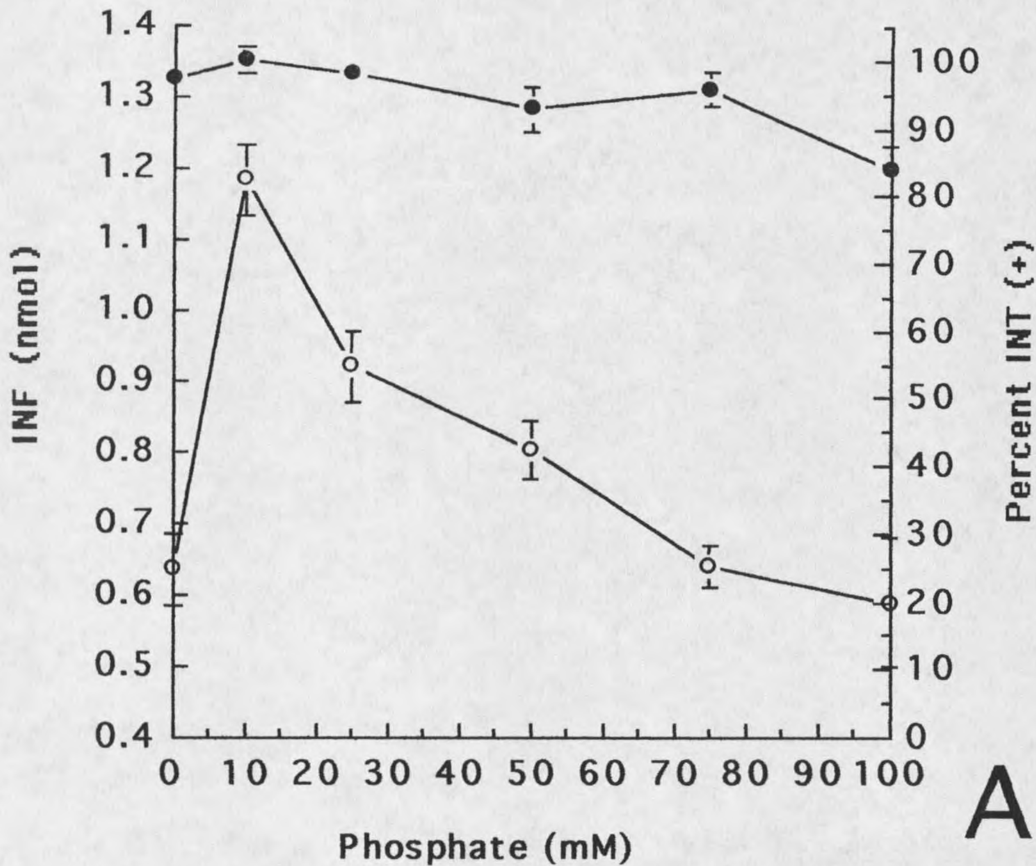

Figure 7(A). Effects of phosphate on CTC (+) (A), and INT (+) (B) cell numbers, and total formazan production.. Symbols: Percent CTC (+) or INT (+) cells (solid circles), total formazan produced (open circles), total CTF deposit containing cells (fluorescent + nonfluorescent) (solid triangles). Error bars represent standard deviations (n = 2).

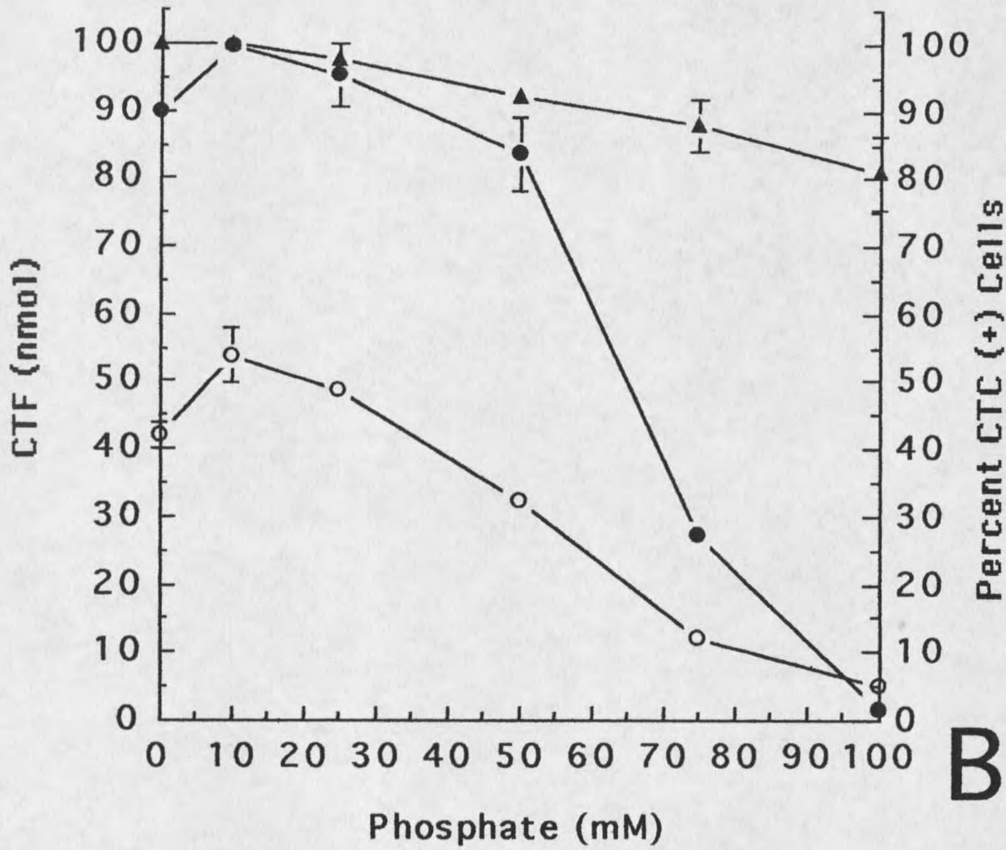

Figure 7(B). Effects of phosphate on CTC (+) (A), and INT (+) (B) cell numbers, and total formazan production. Symbols: Percent CTC (+) or INT (+) cells (solid circles), total formazan produced (open circles), total CTF deposit containing cells (fluorescent + nonfluorescent) (solid triangles). Error bars represent standard deviations (n = 2).

Table 4. Effect of phosphate concentration on respiration rates in *Escherichia coli.*

Phosphate (mM)	[a] Respiration rate (nmol O/ml min^{-2})	
	[b] Stationary phase	Log phase
0	11.23 ± 0.92	10.38 ± 1.71
10	8.47 ± 0.84	8.79 ± 0.11
25	9.87 ± 0.83	11.41 ± 1.35
50	9.02 ± 0.59	13.11 ± 0.13
75	5.63 ± 0.83	12.93 ± 0.60
100	4.99 ± 0.23	4.99 ± 0.23

[a] Errors represent standard deviations ($n \geq 3$).
[b] Cells used in tetrazolium reduction assays.

Discussion

Effect of Substrates on CTC and INT Reduction

The production of 30-150 times less INF compared to CTF on a molar basis indicated reduction of INT to formazan was significantly less tightly coupled to respiratory activity than CTC in whole *E. coli* cells. This was true regardless of substrate addition or type (Fig. 6A). However, roughly equivalent INT and CTC (+) cell numbers were noted, even though much less INF than CTF was produced (Fig. 6B).

Addition of nutrients to endogenously respiring *E. coli* cells increased reduction of CTC to CTF, and CTC (+) cell numbers in all cases (Fig. 6B). Thus it

appears that provision of exogenous substrates enhanced CTC reduction relative to endogenous respiration. While addition of nutrients also generally increased INT reduction to INF, and INT (+) cell numbers, glucose and glycerol both decreased these values (Fig. 6B)). These substrates are both glycolytic pathway intermediates, and glucose is known to repress the activity of the tricarboxylic acid (TCA) cycle (18). One of the suggested sites of reduction of INT is succinate dehydrogenase (SDH) in the TCA cycle (13, 15, 17, 29). Thus, inhibition of TCA activity by glucose may lead to decreased INT reduction. Glycerol is acted upon by *sn*-glycerol-3-phosphate dehydrogenase, and this substrate appears to repress reduction of INT compared to endogenous respiration (18). Succinate, the substrate of the enzyme suggested to mediate both INT and CTC reduction, had no effect on INT reduction, while increasing CTC reduction near values for glucose and glycerol (15, 21). Formate produced the greatest general stimulation of formazan production by INT and CTC, and is acted upon by formate dehydrogenase in *E. coli* (Fig. 6A) (18). Increased tetrazolium reduction could be mediated through the activity of this enzyme directly, or by its ability to translocate protons effectively (4 H+/formate oxidized), generating a lower intracellular redox potential (18). Lactate increased reduction of both CTC and INT, the former to the highest value of all substrates tested (Fig. 6A). Lactate is oxidized by lactate dehydrogenase (LDH) in *E. coli*, producing both NADH and pyruvate. CTC may be reduced directly by LDH activity, or through increased NADH dehydrogenase (NADH-D), or TCA cycle activities (18). Casamino acids stimulated both CTC and INT reduction and increased CTC and INT (+) cells numbers to 100% (Fig. 6A). Amino acids are acted upon by D-amino acid dehydrogenase in aerobic *E. coli* cells (18). In general, it appeared the most effective substrates for increasing CTC and INT

reduction and CTC/INT (+) cell numbers were formate, casamino acids, and lactate. Formate has been previously found to promote tetrazolium reduction in *Campylobacter jejuni* (4).

Effect of Phosphate on CTC and INT Reduction

Inorganic phosphate had a repressive effect on CTC and INT reduction above 10 mM. This effect has also been noted in *Klebsiella pneumoniae*, and *Salmonella typhimurium* (19). These results were consistent with decreases in respiration rates as measured by oxygen consumption, particularly above 50 mM phosphate (Table 4). However, addition of 10 mM phosphate increased INT and CTC (+) cell numbers as well as CTF and INF formation in *E. coli*, while respiration rates decreased slightly (Fig. 7, Table 4). Little effect was noted on INT (+) cell numbers up to 75 mM phosphate. Levels of fluorescent CTC (+) cells paralleled gross CTF production trends over 0-100 mM phosphate. However, levels of nonfluorescent plus fluorescent [CTF (+)] cells only decreased from 100 to 80%, while fluorescent CTC (+) cells numbers decreased to < 5% with increasing phosphate concentrations (Fig. 7B). This suggests that, while CTF formation and rates of respiration were decreased by increasing phosphate, formation of *fluorescent* CTF crystals was inhibited to a greater degree. Phosphate therefore appears to inhibit CTC reduction to fluorescent formazan either directly, or indirectly by lowering rates of respiratory activity. Production of a weakly fluorescent, or more diffuse CTF has been observed using meldola blue as an artificial electron donor in both prokaryotic and eukaryotic cells (5, 28). This response was also seen in previous work (see previous chapter) using several inhibitors of electron transport and oxidative phosphorylation. Pyle *et al.* (19) have also shown that

CTC reduction to fluorescent formazan is inhibited to some degree above pH 6.5 in *E. coli, K. pneumoniae,* and *S. typhimurium,* and that the effect is both phosphate and pH dependent. Since the CTC reduction technique is dependent upon the production of visible, intracellular fluorescent-formazan crystals, it is suggested that phosphate concentration be kept at or below 10 mM during CTC assays with some bacteria. Buffers and growth media for bacteriological use commonly contain phosphate at approximately 1-150 mM (Table 5). Phosphate concentrations in unpolluted fresh waters generally range from approximately 10 nM to 2 μM, while those in seawater range between 1-5 nM (8, 33). However, it should be noted that seawater pH is commonly 8.2-8.4, and freshwater pH can be significantly alkaline, which may also have an effect on CTC and INT reduction.

Table 5. Concentrations of inorganic phosphate in natural waters as well as commonly used buffers and growth media.

Medium	Phosphate (mmol l^{-1})
M9	64
M63	100
MOPS	1.32
R2A	1.7
TSB	14.4
Phosphate Buffered Water	0.62
Phosphate Buffered Saline	10 - 150 mM
Seawater	10 nM - 2 μM
Freshwater (unpolluted)	1-5 nM

Although INT (+) cell numbers paralleled the concentrations of INF produced, increasing phosphate did not reduce INT (+) numbers to the same degree as with CTC (Figs. 7A and B). At 100 mM phosphate INT (+) cell numbers were only decreased 10% from maximum values. This indicated that formation of visible INF crystals and formazan reduction were less sensitive to phosphate concentration, decreased respiration rate, and reduced formazan production than CTC. In addition, it appeared that INT formed visible intracellular crystals at lower formazan concentrations than CTC. As noted above, this may be due to the need for production of *fluorescent* CTF crystals, which apparently was inhibited by phosphate and several other agents known to affect intracellular redox processes (see previous chapter).

Due to the variable nature of prokaryotic respiratory dehydrogenase activities, and electron transport chains in various bacteria, differences in CTC and INT reduction are to be expected. This is particularly true with different organisms, and physiological conditions (31). It should be emphasized that tetrazolium-formazan systems are, in the most basic sense, redox indicators with insoluble reduction products. While it appears that reduction of individual tetrazolium salts is mediated primarily by specific reductants (i.e. dehydrogenases and ETS components), they can also be reduced by electron donors such as ascorbate, dithionite, thiosulfate, and some undefined medium components (4, 5, see previous chapter). Conditions which strongly affect intracellular and transmembrane redox potential would also be expected to effect tetrazolium reduction. In addition, for maximal CTC (+) cell numbers, substrate addition appeared to promote CTC reduction to fluorescent CTF compared to endogenous respiration. Use of selected substrates, and avoidance of high inorganic phosphate concentrations (> 50 mM) may thus help to

maximize reduction of INT and particularly CTC to visible, intracellular formazan deposits.

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CHAPTER 4

SURVIVAL, PHYSIOLOGICAL RESPONSE, AND RECOVERY OF ENTERIC BACTERIA EXPOSED TO A POLAR MARINE ENVIRONMENT

Introduction

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 highly attenuated and seasonal (38). In addition, community primary and secondary production are 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 culturing using selective media, the accurate 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.

Considering that the minimum temperature at which growth of *Escherichia coli* has been reported is ca. 7.5°C, the question with very cold marine environments is not 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 injured or VBNC 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 VBNC state of enteric bacteria under polar marine conditions. Experiments were performed by using diffusion chamber exposure of two indicator (*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* TX-432 (078:H12), *S. typhimurium* SL320, *Y. enterocolitica* 0:8, 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_{640}. Cells were pelleted at 3,000 X g for 10 min and washed twice at ca. 20°C in filter-sterilized (1- and 0.22-μm-pore-size filters), aged sea water (FSASW) collected weekly from Winter Quarters Bay, Antarctica. Cells were then resuspended at a concentration of ca. 10^9 cells/ml (10^{10} 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, 1240-liter circulating aquarium (D [dilution rate] = 3.92 h^{-1}) at McMurdo Station, Antarctica (166° 40' E, 77° 51' S) during the austral summer 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 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 using 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 used 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, 72, and 144 h, or until CFU's no longer increased. Atypical or slowly growing colonies which formed were periodically picked from plates after various periods of exposure and streaked onto either Tergitol-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 ($CFU_{nonselective}$- $CFU_{selective}$/$CFU_{nonselective}$) X 100 as described previously (45). T_{90} and T_{99} decay rates (time 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 FSASW at -1.8°C and assayed for substrate responsiveness 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% naladixic 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 (AODC) were enumerated by epifluorescence microscopy.

CTC Reduction

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 as above at -1.8, 8, 20, and 37°C with and without initial addition of 0.1 X TLY (final concentration, ca. 3.3 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 using 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 (Optiquip 1500). Zeiss filter set no. 48-77-09 (KP 490, FT 510, and LP 520) for AO was used for DVC counts, while 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 8 fields were counted for each sample, and cell size was noted by using a calibrated ocular grid. Error was calculated as described previously (22).

Diffusion Rates

In order to determine diffusion rates of metabolites across chamber membranes in situ, rhodamine diffusion across the chamber membranes was assayed 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 A_{546} of subsamples. Readings were corrected for dilution due to sampling and plotted against time in situ. A Beckman DU 7400 spectrophotometer was used for all absorbance measurements.

Chemicals, Media, and Equipment

Rhodamine 6G, α-lactose, Tris-base, Tris-HCL, DAPI, AO, 37% formaldehyde, naladixic 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 purchased 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 (Figs. 8-12). 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).

E. coli recoverability on TLYD decreased rapidly throughout the 54-day exposure, with a T_{99} of approximately 336 h (Fig. 8). Recoverability on TLY

indicated a T_{99} 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. 12).

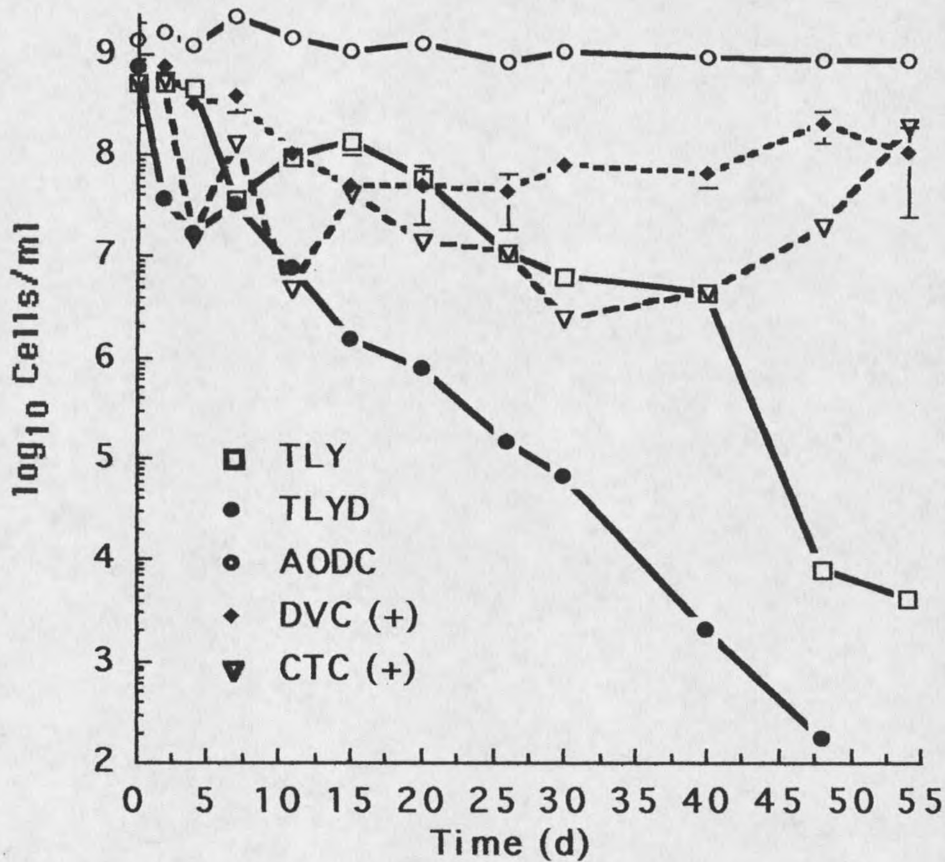

Figure 8. Plate (n = 5), CTC (+), DVC (+), and total direct counts for *E. coli* exposed to a polar marine environment for 54 days. Error bars represent standard deviations.

S. typhimurium recoverability on TLYD and TLY decreased by 1 log unit after 4 and 11 days exposure, respectively. This was followed by continuing decreases in recoverability on both media (Fig. 9). T_{99} 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. 12). After 54 days of exposure, plate counts on both TLY and TLYD were approximately 5 X 10^3 CFU/ml.

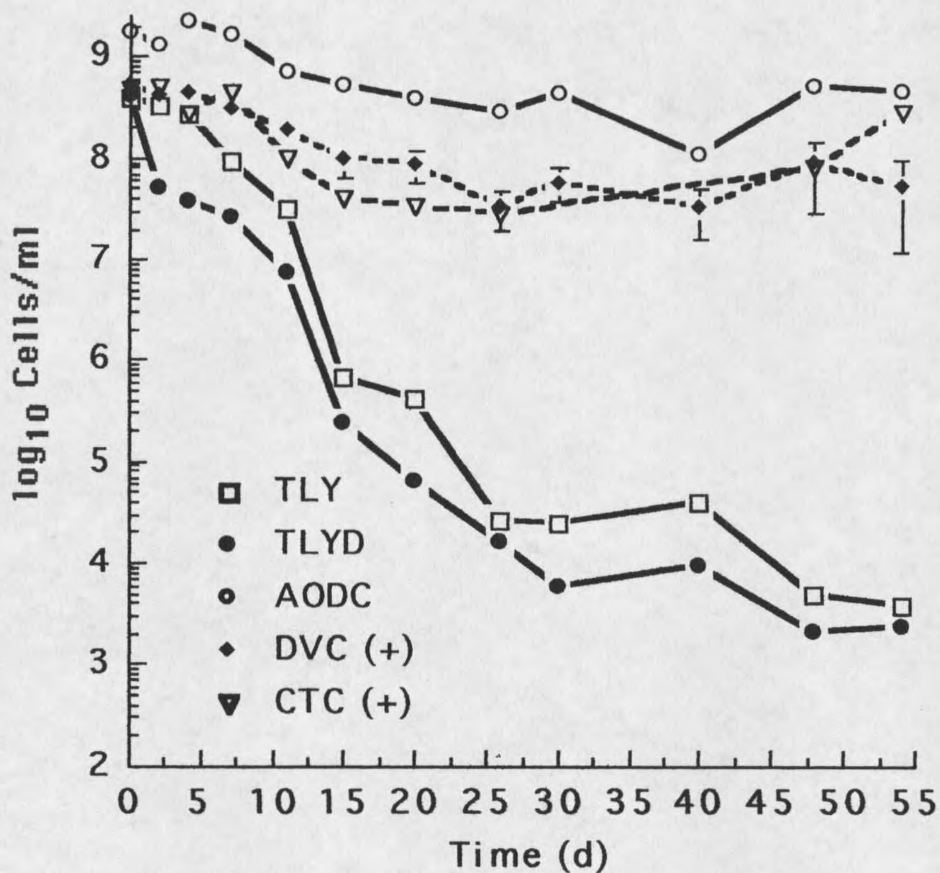

Figure 9. Plate (n = 5), CTC (+), DVC (+), and total direct counts for *S. typhimurium* exposed to a polar marine environment for 54 days. Error bars represent standard deviations.

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. 10). 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 T_{99} values of 360 and 504 h, respectively.

As with *E. coli* and *S. typhimurium, Y enterocolitica* 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. 12). After 54 days of exposure, plate counts on TLYD and TLY were 10^3 and 10^4 CFU/ml, respectively.

E. faecalis recoverability on BHI agar decreased 1 log 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. 11). In contrast, mE agar plate counts decreased 0.4 order of magnitude until 4 days, after which CFU's on this medium rapidly decreased to <0.2 CFU/ml by 27 days. Injury increased gradually to 99% after 1 week of exposure and remained effectively 100% after approximately 27 days (Fig. 12). T_{99} 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. 13). 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. 13B), while *Y. enterocolitica* showed greater colony-forming ability on this medium at these temperatures (Fig. 13C)

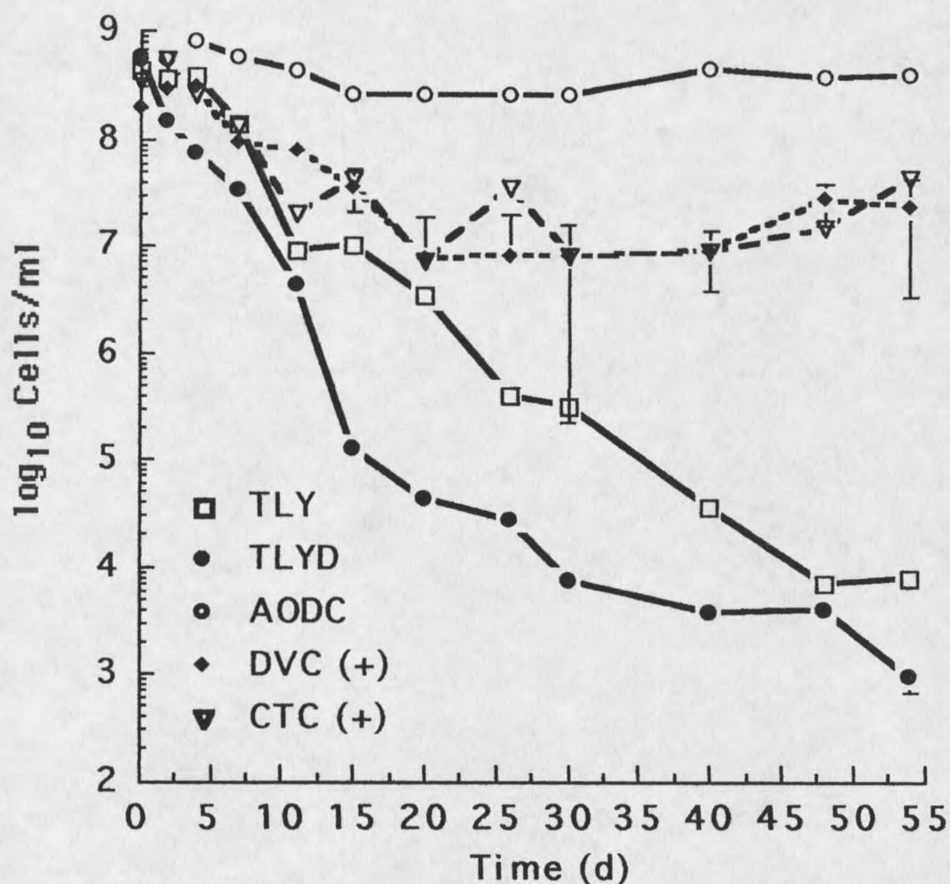

Figure 10. Plate (n = 5), CTC (+), DVC (+), and total direct counts for *Y. enterocolitica* exposed to a polar marine environment for 54 days. Error bars represent standard deviations.