STARVATION EFFECTS ON BACTERIAL RESPONSES TO STRESS

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Abstract: Microorganisms in aquatic environments are subject to nutrient starvation, which may influence sensitivity to a wide range of secondary stresses. A Gram-negative polar flagellated rod, isolated from a Colorado mountain stream, and a culture of *Escherichia coli* were starved in phosphate buffer to determine their responses to transient heating stress (52 C for 5 minutes) and the short-term presence of nutrients (glucose, peptone, acetate) under starvation conditions. These microorganisms had markedly different responses to starvation, secondary stress and nutrient presence. At the beginning of the starvation time, the water isolate was more sensitive to glucose presence, and was extremely sensitive to the secondary warming stress. These responses were markely diminished after a three-week starvation period. *E. coli*, under similar conditions, responded differently, in that with increasing starvation time it exhibited progressively greater sensitivity to the secondary stress. The differences in sensitivity to secondary stress and short-term nutrient availability showed by *E. coli* and this indigenous aquatic microorganism may reflect major physiological differences which lead to exclusion of *E. coli* from aquatic euvironments.

INTRODUCTION

The survival of vegetative bacteria during starvation has been studied for many years and by many investigators, including Clifton (1966), Cohen (1922), Ensign (1970), Garvie (1955), Harrison (1960), Jacobson and Gillespie (1968), Mackelvie, Campbell and Gronlund (1968), Morrison, El Bagoury and Fletcher (1956), Postgate, Crumpton and Hunter (1961), Postgate and Hunter (1962, 1963), Shearer (1917), Strange (1961), Strange, Dark and Mess (1961), Sykes (1963), Cook and Wills (1958), Winslow and Falk (1923a, 1923b), Winslow and Haywood (1931), Winslow and Dolloff (1928).

When a physical or chemical agent (or factor) is applied to an individual microbial cell, it can fail to make a satisfactory adaptation, causing physiological stress which may contribute to death or nonviability. In microbiology, survival is related to starvation resistance, which can influence the fate of organisms which are placed under conditions unfavorable to their growth. Sykes (1963) described resistance as implying an active state of opposition to an attack on the cell by an outside agent, whereas survival connotes a more passive state of endurance induced by a physical or chemical agent.

In natural environments, such as in soil and water, microorganisms encounter varied stresses, including the deficiency of essential element or nutrients, exposure

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to adverse temperature, pH, moisture, lethal radiation, salinity, etc. For example, bacteria are subject to nutrient starvation which may influence sensitivity to a wide range of secondary stresses such as chemical and physical agents (Postgate 1967, Russell and Morris 1973).

Postgate (1967) pointed out that "bacteria subject to stress become hypersensitive to secondary stress," however, hypersensitivity to secondary stresses may not occur in all kinds of bacteria. This phenomenon may be limited to specific groups of organisms, such as the enteric microorganisms.

The effects of starvation stress on two different types of microorganisms, *Escherichia coli*, which is not considered as indigenous to waters, and an indigenous water bacterium, have been studied to determine if there are differences in starvation resistance and susceptibility to secondary stress between representatives of these two groups of microorganisms.

Information on microbial starvation and secondary stress sensitivity relationships may provide us with a better understanding of the nature of the aquatic microbial environment.

LITERATURE REVIEW

Starvation and Release of Materials

As early as 1917 Shearer studied the survival of starved bacteria in an aqueous suspension. He reported that physiological saline was more toxic to meningococci than distilled water or a 1.5% solution of sodium chloride, and calcium or potassium antagonized this toxicity. In an experiment carried out by Cohen (1922), *Bact. coli* (*E. coli*) was suspended in a phosphate buffer at pH 7.1, and the death rate increased as temperature increased from 0, 10, 20, to 30 C. At lower pH values, the cells were more sensitive to the increased temperature. Winslow and Falk (1923a) noted that *Bact. coli* viability in 0.0145, 0.0725, 0.145 molar (0.1, 0.5 and 1.0 isotonic respectively) sodium chloride solutions was greater than in distilled water. These workers (Winslow and Falk, 1923) also cited the effect of calcium and sodium ions on the viability of *Bact. coli* populations. Calcium chloride is more toxic than sodium chloride. If NaCl is mixed with $CaCl_2$ mutual antagonism resulted, which permitted bacteria to survive in undiminished numbers for nine to twenty-four hours. Winslow and Dolloff (1928) and Winslow and Haywood (1931) studied the viability effects of certain cations which, when present in very low concentrations, allowed viability retention (associated with increased permeability) but inhibited the bacterial viability (associated with decreased permeability) when the cation was presental in nutrient media at higher concentration.

Morrison, El Bagoury and Fletcher (1956) observed that 10 or 100 μg per ml of chloramphenicol maintained the viability of *E. coli*. Cook and Wills (1958) observed that suspensions of *E. coli*, consisting of washed cells suspended in a phosphate buffer solution, maintained a higher viability and higher resistance to a chemical stress (phenol) than suspensions either of unwashed cells or washed cells suspended in water.

Holden (1958) showed that ribonucleic acid fragments were excreted by starved *Lactobacillus arabinosus*. Higuchi and Uemura (1959) also observed that the degradation of ribonucleic acid and release of ribonucleate from yeast cells did not result in the death of the cells or even in degradation of the cell protein.

Strange, Dark and Ness (1961) showed that the survival characteristics of *Aerobacter aerogenes* populations were influenced by the constituents of the bacterial growth medium, the growth phase and the period during which the organisms had been in the stationary phase. The degradation of polymeric cell constituents such as protein, polysaccharide, ribonucleic acid and the excretion or release of fragments of these polymers was suggested as causing the death of the populations.

Strange (1961) reported that utilization of cell polymers such as protein, ribonucleic acid and glycogen by starving stationary phase *A. aerogenes* held at 37 C as a buffered suspension with aeration, depleted these polymers and markedly reduced the subsequent ability of the bacteria to form induced enzymes. The reduction of enzyme synthesis in these starved but still viable bacteria was apparently not due to a deficiency of adenosine triphosphate. Brdar, Kos and Drakulic (1965) observed that bacteria incubated under starvation conditions or in a deficient medium preferentially utilized nucleic acid precursor pool materials for DNA synthesis.

Dawes and Ribbons (1965) showed that RNA is degraded by the *E. coli* cells immediately upon starvation, that ribose is oxidized, and that ultraviolet-absorbing materials are released to the suspending medium. Clifton (1966) also observed significant ribonucleic acid fraction decreases with aging of *E. coli*. The rates of endogenous and exogenous (glucose) respiration decreased much more rapidly than did the viable count during the first 24 hours of incubation.

Mackelvie, Campbell and Gronlund (1968) also reported that ribonucleic acid and protein decreased by similar amounts, and deoxyribonucleic acid incrased in endogenously respiring cell suspensions of *Pseudomonas aeruginosa*. Postgate (1967) found that the general starvation result was the breaking down all of the major polymeric constituents in starved microorganisms except lipid and deoxyribonucleic acid, including such materials as poly-β-hydroxybutyric acid or sulphur. The rates and orders of degradation of these components depend on the type of organism and its nutritional status. The extent of individual substrate degradation appears to depend on their initial concentrations in the cells, which are determined by the nutritional status of the organism (Dawes and Ribbons, 1964). Boyd, Nixon, Gillespie and Gillespie (1968) observed that the DNA and protein of *E. coli* remain unchanged during starvation, but that two-thirds of the RNA is lost. At least 95% of the detectable ribosomes disappear, and they are degraded to functional ribosomal protein and RNA fragments and nucleotides. During starvation studies carried out by Jacobson and Gillespie (1968), the DNA content per viable *E. coli* cell remained constant, but the RNA content was drastically reduced, tending to confirm the prior observations of Holden (1958).

Starvation of *Streptococcus lactis* in phosphate buffer can cause RNA and protein degradation. Thomas and Batt (1969) emphasized that RNA breakdown rates and subsequent cell death were reduced by the addition of Mg^{++}. These workers concluded that although considerable RNA may be degraded without affecting viability, the ribosome stability is important for survival.

Ensign (1970) reported that endogenous respiration decreased 80-fold during the first 2 days of starvation of *Arthrobacter crystallopoietes*, which may be an important factor in the unique starvation resistance of this organism. Survival for a period of time greater than 20 times that of other bacteria has been noted, implying that the lower the rate of endogenous respiration, the longer the starved bacteria can survive. Boylen and Ensign (1970) observed that a starving bacterium can utilize

RNA as a substrate for endogenous metabolism and a cell can loss about 85% of its RNA content and still remain viable. The results of Chapman, Fall and Atkinson (1971) showed that starved *E. coli* cells excrete a considerable amount of AMP into the medium, affecting the energy charge value of the organism.

Weinberg (1974) reported the formation of secondary metabolites which serve as disposal packages of primary metabolites that have accumulated in microbial cells. If the packaging process is completed successfully, the cells can possess long-term viability. On the other hand, interruption of distortion of the packaging process may result in early death of the microorganisms. Thus, primary metabolites may have some toxic or detrimental effects on the non-dividing microbial cells, and the removal of these substances thus can directly affect the cell viability.

Heat Stress Effects on Microorganisms

Heat stress, including pasteurization, is one of the most widely used mothods for destruction of spoilage and pathogenic bacteria. However, mild heat treatment does not instantaneously kill bacterial cells, but instead may cause varying degrees of injury. After heating treatment the cell will die or recover, depending on the degree of injury and on the conditions under which they are maintained. Measurement of the degree of injury usually is shown by a loss of ability to reproduce or divide on a given cultural medium (Moats, 1971). Sherman and Albus (1923) reported that heating cells to relatively low temperatures more rapidly destroyed young bacterial cells than older ones. Rahn (1945) and Wood (1956) summarized the possible mechanisms proposed for heat destruction of bacteria: (1) coagulation of enzymes, (2) inactivation of enzymes, (3) disruption of cellular lipids and (4) damage to the microbial genetic apparatus. In 1964 Strange and Shon suggested that RNA depletion is probably not the primary cause of death of *Aerobacter aerogenes* at 47 C, b ut that effect of a rapid increase in endogenous pool constituent resulting from RNA degradation may contribute to the lethal effects.

Bacteria which have encountered sublethal heat injury may have modified cell membranes, allowing soluble cellular components to leak into the heating menstruum. Iandolo and Ordal (1966) presented evidence that RNA which was degraded by heat treatment can also leak through the injured membrane. Allwood and Russell (1969) showed that heated bacteria exhibit a prolonged lag phase of growth in recovery media. With higher heating temperatures longer lag phases were noted.

Heating vegetative bacteria above 45 C may render cells sensitive to post-treatment recovery conditions. Klein and Wu (1974) observed that heterotrophic microorganisms in water samples are susceptible to the short term heating stress of warmed agar used in the standard methods pour plate procedure. Microbial recoveries with a spread plate technique were almost three-fold higher than those from pour plates (using 42 C agar). Stapert, Sokolski and Northam (1962) and Zobell and Conn (1940) also mentioned that temperature of warmed agar used in pour plates can cause lower microbial recovery. According to Postgate and Hunter (1962) the death rate of a starving population will increasing incubation temperature.

Substrate-accelerated Death

Substrate-accelerated death is defined as the effect of the substrate that limited growth of a microbial population accelerating its death when present in non-growth starvation conditions. Ryan (1959) showed that if glucose is available to starved *E. coli* (histidineless) cells, it accelerated bacterial death at a rate approximately 1% per hour. Harrison (1960) reported that glucose addition at a concentration of

less than 1 part per million has a beneficial effect and allowed prolonged survival. He concluded that glucose is used as a source of energy by the cells. Ryan (1959) observed the release of small amounts of histidine by lysis of bacteria which caused an increased death rate.

Strange, Dark and Ness (1961) observed that glucose addition to an aerated microorganism suspension (*A. aerogenes*) in buffered saline at 37 C caused 50% viability loss after 4 hours of incubation. They explained that the glucose-accelerated death of the organisms was due to the degradation or denaturation of internal substances such as enzymes.

Postgate and Hunter (1962, 1963a) noted that glycerol, glucose (the energy source for growth), and intermediates in glycerol metabolism such as pyruvate, succinate, and malate caused accelerated death of bacteria starved in a non-nutrient buffer. Postgate and Hunter (1963b) reported that glucose or ribose did not accelerate the death of glycerol-limited *A. aerogenes* cells. Although both compounds were utilized by the organism, they accelerated the death of glucose and ribose-limited population respectively. These workers also observed glucose- or pyruvate-accelerated death of starved populations obtained from a complex medium (Postgate and Hunter, 1964). The survivors of substrate-accelerated death have a long lag period. This suggests that reclamation of the lost materials during starvation after transfer to the recovery medium was a slow process (Postgate and Hunter, 1964).

Several suggestions have been advanced to explain the basis of stubstrate-accelerated death. Calcott and Postgate (1971), Calcott, Montague and Postgate (1972) suggested that the phenomenon of substrate-accelerated death may be related to intracellular catabolite repression-derepression processes since they found that cyclic AMP or its butyryl derivative prevented substrate-acclerated death but did not abolish the prolonged lags characteristics of survivors of this stress.

Population Effects on Survival

Harrison (1960) observed that various cells densities of exponentially-grown *Aerobacter aerogenes* kept at growth temperature in the absence of added nutrients showed rates which were related to the cell densities. In this work, he noted that best survival was shown by populations of 10^8 cells/ml. At populations above or below this level lesser viability retention was observed. The surviving micro-organisms maintain viability at the expense of material derived from dead or dying cells, commonly termed cryptic growth.

Postgate (1967) established that the sparse populations die more rapidly than denser ones by measuring direct cell viabilities, where the higher the original cell population the greater percentage of viable cells which can be recovered at any assay time.

Postgate and Hunter (1962) noted that the dying bacterial populations showed rapid initial breakdown of intracellular ribonucleic acid, with release of phosphate and base fragments into the medium. Intracellular protein also was degraded. Their experimental results of population effects confirmed the observation of Harrison (1960).

Stress Sensitivity

Postgate (1967) stated that bacteria subjected to a primary stress can become hypersensitive to secondary stresses. Jacobs and Harris (1960) reported that *E. coli* and *Staphylococcus aureus* damaged by phenol were very sensitive to their environ-

ments. These workers (1961) also observed that bacteriological agar can be toxic towards these organisms. Arber (1963) found that *E. coli* starved in 0.01 M $MgSO_4$, was infected by bacteriophage λ with almost 100% probability at 37 C. Thus, starvation can also influence microbial susceptibility to bacteriophage infection.

In summary, the available literature indicates that microorganisms can respond to starvation by utilization of various intracellular and extracellular materials. Based on the available literature, there is little information on starvation effects on microbial responses to secondary stress. Regarding mild heating as a particular stress, this is described in the literature (Postgate, 1967), but only in general terms and not in relation to specific prior starvation of microorganisms.

MATERIALS AND METHODS

Cultures

Water samples were collected from Arthur's ditch on Colorado State University campus with a sampling bottle. Immediately after being brought back to the laboratory, four 400 ml aliquots of the water samples were measured into 750 ml volume sterilized Erlenmeyer flasks. One flask was used for the zero time assay, and the remaining flasks were placed on a shaker (150 RPM, NBS Gyrotory shaker, Model G-25) at 25 C for assay after 1, 2, and 3 weeks of incubation, respectively.

The flasks were weighed weekly so that the amount of water lost through evaporation during the course of incubation could be determined, and this was replenished with sterilized distilled water.

A water microorganism was isolated from a water sample by spreading an appropriately diluted sample on nutrient agar plates. *E. coli*, which is not considered as indigenous to water, was obtained from our laboratory stock culture. These microorganisms were grown on nutrient agar slants for later use.

Preparation of Cells

The Gram-negative, polar flagellated rod isolated from Arthur's ditch on Colorado State University campus, and a culture of *E. coli* were grown in peptone broth (0.5% w/v) on a shaker at 25 C for 24 hours, washed with phosphate buffer twice and then starved in phosphate buffer (pH 7.2) using Erlenmeyer flasks. These flasks were kept on the shaker at 25 C for three to four weeks. Water loss through evaporation was replenished by addition of sterilized distilled water.

Viability Determination

Plating The enumeration of viable microorganisms was carried out using nutrient agar plates, completed by adding 0.1 ml portions of the appropriately diluted bacterial suspension in a four-fold replication, followed by spreading with a precooled alcohol-flamed glass rod. The agar plates were incubated in an inverted position at 25 C for 5-7 days.

Direct Viability Assay The percent viability of starved *E. coli* were determined by a slide culture technique (Postgate, Crumpton and Hunter, 1961; Casida, 1969).

Stress Sensitivity Evaluation

The determination of bacterial responses to a transient heating stress (52 C for 5 min) with or without one hour prior presence of glucose or peptone was carried out as described below:

Five ml of a cell suspension was pipetted into test tubes and heated in a water

bath at 52 C for five minutes with shaking, cooled immediately in tap water and diluted appropriately using phosphate buffer blanks and nutrient agar spread plates.

The normal procedure for evaluation of nutrient addition effects on microbial sensitivity to stress involved addition of the substrates (glucose, peptone, acetate or yeast extract) at 15 mg/l for one hour. This substrate level was chosen to represent a possible maximum which might be found in polluted aquatic environments, and the contact time was chosen to provide a maximum practical period for the organism to respond to nutrient presence.

In later experiments, varied contact times at a constant substrate level were tested, together with varied substrate levels at a constant contact time to determine possible minimum substrate level-contact times needed to sensitize these organisms to the uniform secondary stress.

Monitoring of Culture Starvation

Turbidity The turbidity of the cell suspensions at the beginning of starvation time were measured by using Klett-Summerson photoelectric colorimeter with filter 66 (Model 800-3, Klett MFG. Co., INC., N. Y.).

Dry Weight To determine the cell dry weight, 10 ml of washed cell suspensions were measured into preweighed aluminum planchets (blank buffer solutions also were measured), followed by drying in a hot air oven at 110 C for 60 minutes to constant weight, and weighing again after cooling.

Preparation of Cells and Supernatants for Analyses Cell suspension supernatants were collected in 270 ml volume plastic centrifuge bottles by centrifugation (17300× g, Sorvall Superspeed centrifuge, using a SS-34 centrifuge head). These supernatants were saved by freezing for later analysis. After the supernatants were collected, the remaining cell pellets were resuspended and washed in sterile distilled water. After re-centrifugation, the cells were transferred to serum bottles using Pasteur pipettes, frozen immediately, and lyophilized.

pH Determination The pH of phosphate buffer and the media used were measured with Corning pH meter, Model 7 (Scientific Instruments, Corning Glass Works, Corning, New York).

Microscopic Observations Cells suspensions were observed using a wet mount method under phase contrast conditions (American Optical Co., Scientific Instrument Division, Buffalo, N. Y.). For routine studies, *E. coli* and the water microorganism were grown on nutrient agar slants and observed by means of Gram staining under phase contrast or bright field conditions.

Characterization of the Water Microorganism Motility: Motility was determined by hanging drop and semisolid agar deep stab methods (Am. Soc. Bacteriologists, 1957) and by a flagella stain method using the following staining solutions:

A. 5% phenol solution 10 ml
   Tannic acid 2 gm
   $AlK(SO_4)_2 \cdot 12H_2O$ Solution 13.5% w/v 10 ml
B. Crystal violet in ethanol 10% (w/v)
C. Working solution: mix 10 parts of solution A with 1 part of solution B.

The staining method used involved the following steps:

(1) Cells from a young slant culture (24 hours) were suspended in sterilized distilled water.
(2) The bacterial suspension was placed carefully on a clean slide, and the cells were spread by tilting before being air dried.

(3) The cells were stained with the prepared working solution for 2–3 minutes, washed with tap water, dried in air and examined under oil immersion.

Physiological Characteristics of Organisms

Glucose Incorporation Glucose incorporation by *E. coli* and the water microorganism was measured by using C–14 labeled glucose at an activity of 0.1 μCi/ml. The cell suspension concentrations were adjusted to a turbidity of 40 Klett units, and 50 micrograms of chloramphenicol per ml was added to inhibit protein synthesis. The mixtures were prepared by pipetting 12.0 ml of cell suspension into 25 ml Erlenmeyer flasks, and a mixture of 1.0 ml of 1 mM C–12 glucose and 0.13 ml of C–14 glucose (activity at 10 μCi/ml) were added. After the glucose addition, 1 ml aliquots of cell suspensions were filtered using a millipore filter (pore size 0.45 μm) at 1, 3, 6, 10, 15, and 20 minutes during shaken 25 C incubation and washed with 10 ml of chloramphenicol-containing phosphate buffer. The filters were then placed in liquid scintillation counting vials. After being dried in a hot air oven (110 C for 40 min) the filters were counted in a liquid scintillation counter (Model LS–113), Beckman Instruments, Fullerton, Calif.). The scintillation cocktail contained 4 grams of omnifluor (New England Nuclear, Pilot Chemicals Division, 575 Albany St., Boston, Mass.) in 1 liter of toluene. The cell dry weight per ml also was measured to allow calculation of glucose uptake per mg cells dry weight per minute.

Oxygen Uptake Measurement Starvation effects on oxygen usage by *E. coli* and the water microorganism were measured with a biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Cell suspensions (3 ml at 40 Klett units) were saturated with oxygen, and endogenous oxygen usage was measured. After 15 minutes, 50 lambda of 1 mM glucose was added, and the oxygen depletion rates were recorded.

Chemical Composition of Cells

The cell chemical compositions including carbohydrate, protein and ribonucleic acid were determined using procedures summarized by Keleti and Lederer (1974) and Herbert, Phipps and Strange (1971).

Statistical Analysis

Statistical analyses were carried out using procedures described in Snedecor and Cochran (1971). For all experiments, the results of at least three separate runs were averaged.

RESULTS

Physiological Characteristics of the Water Microorganism

The morphological and physiological characteristics of the aquatic microorganism are shown in Tables 1 and 2.

From the results shown in Table 1 and Table 2, and according to Bergey's Manual of Determinative Bacteriology (Breed, Murray and Smith, 1957) and Eddy (1960), the monotrichous, Gram-negative water microorganism (Figure 1) can be considered as an *Aeromonas* species.

Population Effects on Starvation Susceptibility

Population Effects on Stress Sensitivity As noted in Table 3, populations of *E. coli* at 1×10^8 cells/ml are not particularly sensitive to glucose addition and heating stress. If the *E. coli* cell population is in the range of 10^7 cells/ml, it appears to be signi-

Table 1. Morphology and biochemical reactions of the aquatic microorganism

Character	Reaction	Character	Reaction
Form of cell	Short rod	Indole	Positive
Size (μM)	0.7-0.8×1.5-1.7	MR	Negative
Motility	Positive	VP	Negative
Flagellation	Monotrichous	Citrate	Growth
Spore	None	Glucose	Acid, gas
Growth in broth	Turbid	Lactose	Slightly acid
Gelatin stab	Growth, no liq.	Sucrose	Acid, gas
Gram stain	Negative	Mannitol	Acid, gas
Hemolysis	Non-hemolysis	Litmus milk	Reduction
Amylase	Negative	Catalase	Positive

Table 2. Fermentative-oxidative metabolism of carbohydrates by the aquatic microorganism (Hugh and Leifson, 1953)

Sugar	Open		Covered	
	Autoclaved	Filtration	Autoclaved	Filtration
Glucose	A	A	A	A
Lactose	—	—	—	A (slightly)
Maltose	AG		AG	
Sucrose	—	—	A	—
Fructose		A		AG
Control	—	—	—	—

Legend: A means acid reaction;—means neutral or alkaline reaction; AG means acid and gas reaction.

Table 3. Population effect on stress sensitivity of *E. coli*

Treatment	Colonies Counted on Nutrient Agar Plates		
	$\times 10^6$/ml	$\times 10^5$/ml	$\times 10^4$/ml
Control	98±5[a]	213±38	40±5
Heat[b]	86±5	137±6	9±0.6
Glucose[c]	77±3	123±5	18±0.7
Heat and glucose	69±3	2±0.4	$<10^2$ cells/ml

[a] Standard deviation.
[b] Heated in water bath at 52 C for 5 minutes.
[c] Glucose level at 15 mg/l.

ficantly more sensitive to glucose presence followed by a mild heating treatment. If the cell population is as low as 4×10^5 cells/ml, the microorganism appears to be extremely sensitive to the heat stress. Based on this observation, cell stress sensitivity studies were carried out using cell populations of approximately 10^7 cells/ml.

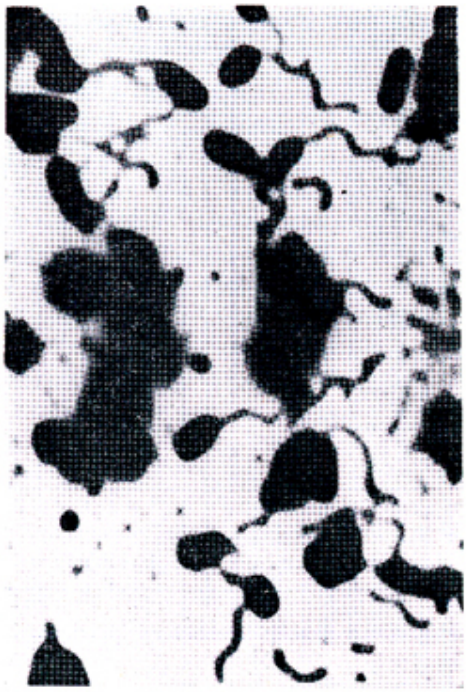

Figure 1. Flagella stain of *Aeromonas* species.

Table 4. Population effect on viability of starved *E. coli*

Population	Viabilities %		
	Trial	Mean	S. D.
0.98×10^8	39.18 38.89 38.04 36.23	38.08	1.33
2×10^7	38.04 26.08 30.10 35.48 27.27	31.39	5.19
4×10^5	18.18 25.00 20.83	21.33	3.44

Population Effect on Viability In Table 4, the viability assays for three different cell population levels are shown. With higher cell populations of three-week starved *E. coli*, higher viable counts were obtained, confirming the population effect which has been observed by other workers.

Stress Sensitivity Evaluation

Mixed Aquatic Microbial Population The unstressed mixed aquatic microorganism population maintained fairly constant cell numbers with increased starvation time, whereas the stressed samples tended to show increased resistance to mild heating treatment (Figure 2). The microbial population appeared to shift to those organisms which were resistant to heating stress during the course of starvation. The addition of exogenous substances, including glucose, peptone, acetate and yeast extract, gave higher viable counts on both stressed and unstressed water samples. Yeast extract showed especially strong increases when added to the stressed water samples.

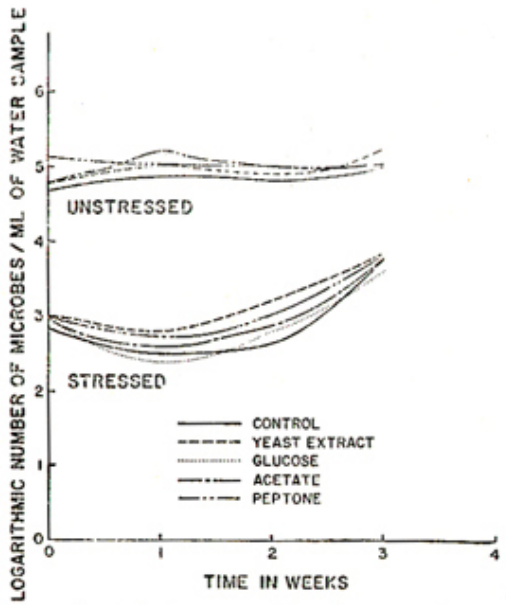

Figure 2. Starvation effects on aquatic microorganism sensitivity to mild heating (52 C for 5 min) with and without presence of glucose, peptone, acetate or yeast extract (15 mg/l).

It is important to note that glucose has negligible effects when added to a mixed aquatic population, indicating that these organisms adapted to this environment are generally not specifically sensitized to the heating stress by this substrate.

Aeromonas species The starvation effects on susceptibility of the water derived bacterium to mild heating stress with and without prior presence of glucose or peptone is shown in Figure 3. At the beginning of the starvation time the organism was very sensitive to the heating stress and prior glucose presence. However, peptone gave some degree of protection against the heating stress. These responses were markedly decreased after a three-week starvation period. The starved unstressed cells were slightly less sensitive to glucose presence than the unstarved cells.

Escherichia coli Under similar starvation and heating conditions, *E. coli* responded differently from the *Aeromonas* species with increasing starvation time as shown in Figure 4. In relation to starvation time, it gradually appeared to have a greater sensitivity to heat and glucose presence especially when glucose was present in the stressed culture suspensions.

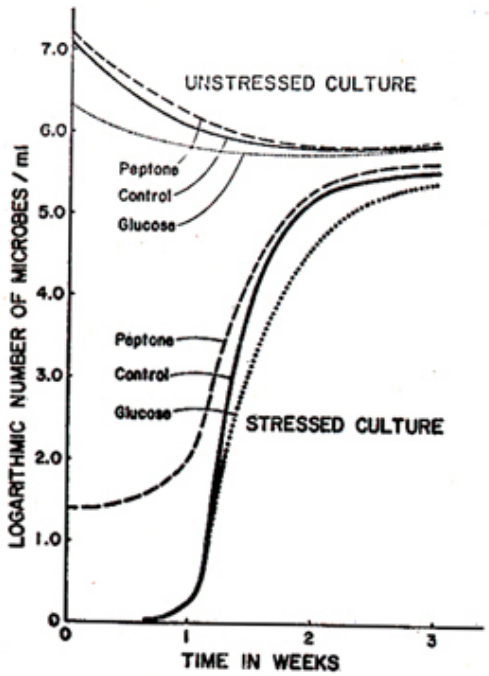

Figure 3. Starvation effects on susceptibility of a water-derived bacterium to mild heating (52 C for 5 min) with and without one hour prior presence of glucose or peptone (15 mg/l).

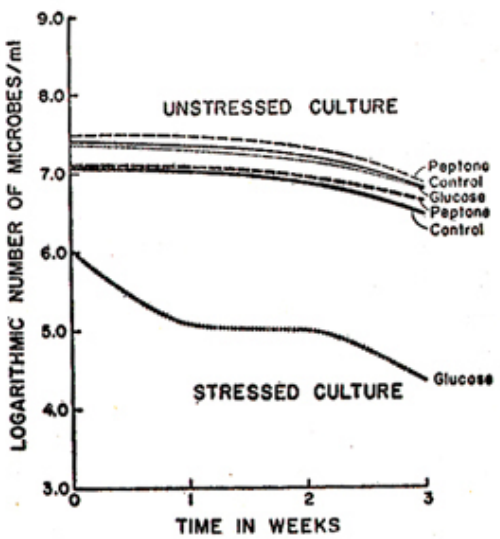

Figure 4. Starvation effects on *E. coli* susceptibility to mild heating (52 C for 5 min) with and without one hour prior presence of glucose or peptone.*

* Glucose or Peptone added at 15 mg/l.

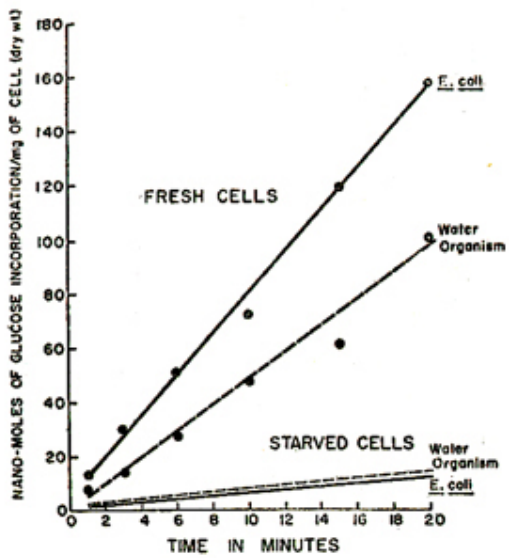

Figure 5. Starvation effects on glucose incorporation by *E. coli* and a water-derived bacterium.

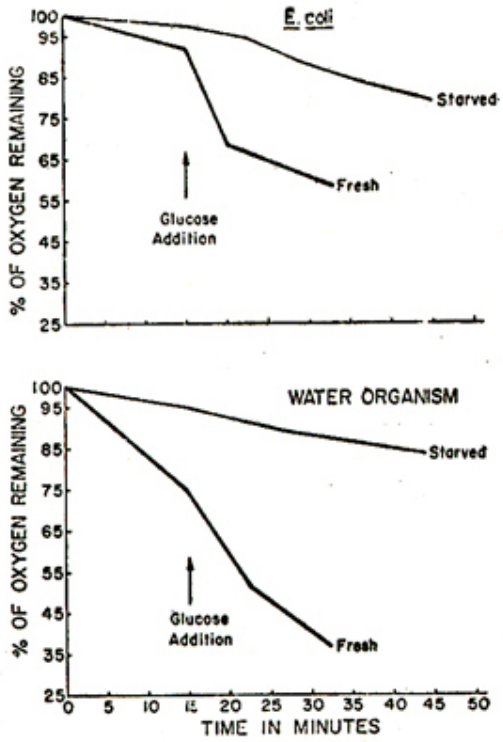

Figure 6. Starvation effects on oxygen usage by *E. coli* and a water-derived bacterium.

Physiological Characteristics of Fresh and Starved Cells

Glucose Incorporation The level of glucose incorporated by fresh cells of *Aeromonas*, as shown in Figure 5, was lower than that of *E. coli* cells. Glucose incorporated by starved cells of both organisms was essentially similar.

Oxygen Usage The starvation effects on oxygen usage by *E. coli* and *Aeromonas* as shown in Figure 6. The endogenous oxygen usage of the fresh *Aeromonas* culture was faster than that of the *E. coli* cells. After 15 minutes, 50 lambda of 1 mM glucose was added to 3 ml of cell suspensions, under these conditions, the oxygen consumption rate of the fresh *E. coli* cells was greater than that of the *Aeromonas* culture. In the starved cultures, after glucose addition the *E. coli* culture utilized oxygen at a rate greater than endogenous over the 50 minute experiment, while the *Aeromonas* culture showed only a slight response to the glucose addition before returning to the original endogenous rate.

Starvation Effects on Chemical Composition

Cell Analyses The differences in cell composition between fresh and starved cells of *E. coli* and *Aeromonas* are shown in Figure 7. The starved cells of both microorganisms contained lower carbohydrate, protein, and ribonucleic acid than those of the fresh cells. The starved cells of *E. coli* had carbohydrate and RNA losses of about 50%, whereas the *Aeromonas* culture lost around 30% and 80% respectively of those constituents. The major difference between these organisms is the increased RNA loss by the water organism (80%) in comparison with the lesser loss (50%) shown by the *E. coli* culture.

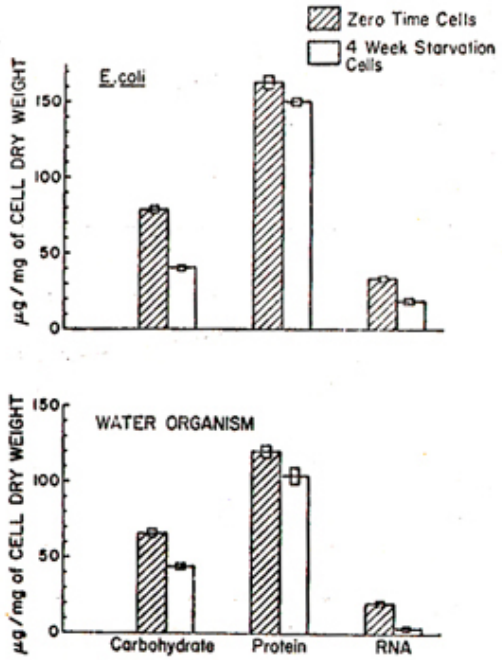

Figure 7. Starvation effects on cell composition of *E. coli* and a water-derived bacterium.

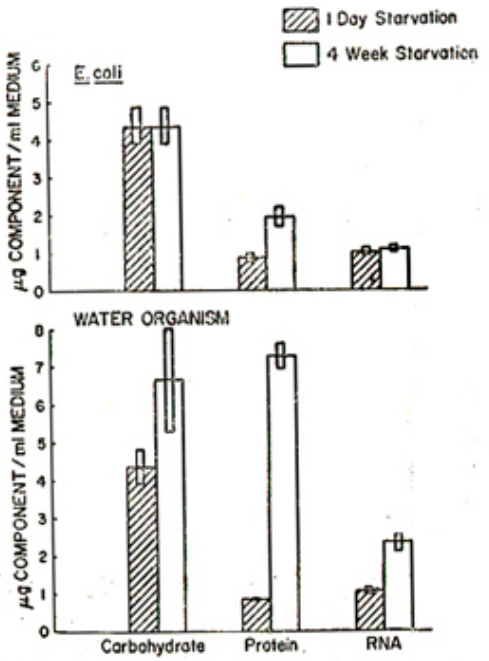

Figure 8. Starvation effects on cell component excrction by *E. coli* and a water-derived bacterium.

Supernatant Analyses The starvation effects on cell components excretion by *E. coli* and *Aeromonas* are shown in Figure 8. The starved *Aeromonas* cells generally tended to release more materials into the starvation medium than did the *E. coli* culture.

Effects of Varied Glucose Levels and Contact Times on Escherichia coli Sensitivity to Secondary Stress

A major question in analysis of these results centers upon the role of specific substrate level and contact time effects on sensitivity of the organisms to the uniform secondary heating stress. The choice of one hour contact at 15 mg/l of substrate was made, as discussed previously, to be equivalent to a possible maximum contact time before stress, and a maximal level of substrate which these organisms might encounter in aquatic environments. These experiments were carried out to determine the minimum time and substrate level which might be needed to sensitize an organism to a secondary stress.

Contact Time Effects In Figure 9, the effects of glucose contact (at 15 mg/l) over varied times with mild heating (52 C for 5 min) on starved *E. coli* are shown. Cell recovery was decreased even with glucose contact times as short as 1 minute, however after this contact time the cell numbers remained fairly constant up to 5 minutes. After 5 minutes, the number decreased again markedly until 20 minutes, and then remained at approximately 10^6 cells/ml.

Glucose Level Effects Figure 10 shows the effects of varied glucose levels on starved *E. coli* recovery at constant contact time (60 min) after mild heating. Glucose effects were observed at a 1 mg/l addition level, and the cell viability was

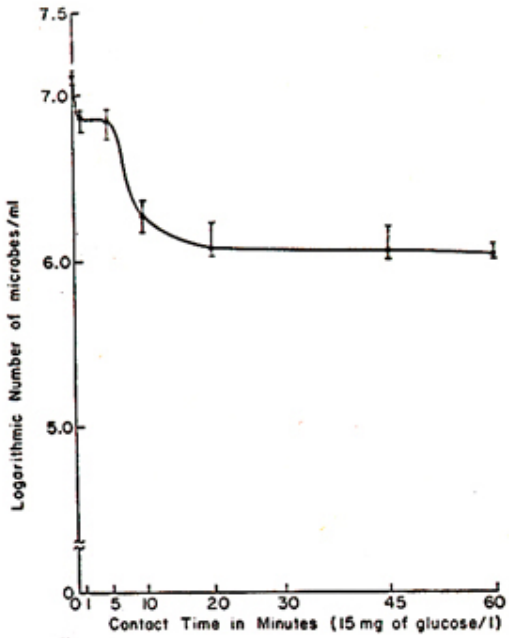

Figure 9. The effects of varied glucose contact time at 15 mg/l on starved *E. coli* sensitivity to a mild heating (52 C for 5 min) stress.

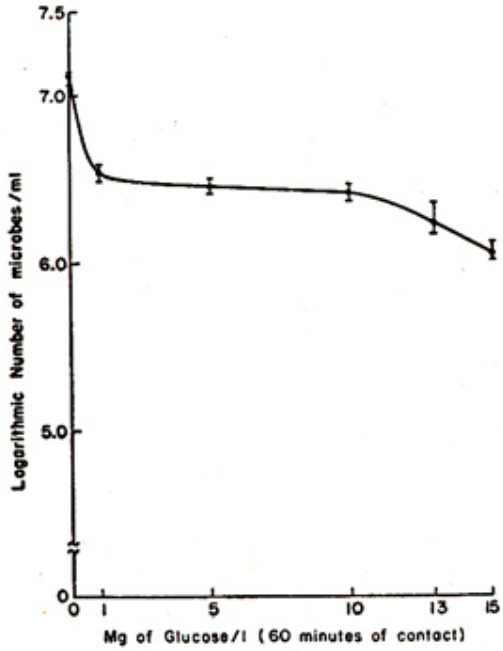

Figure 10. The effects of varied glucose levels on starved *E. coli* at contact time (60 min) with mild heating (52 C for 5 min).

significantly decreased in comparison with controls. As the glucose level increased by tenfold no major increase in *E. coli* sensitization was seen. Increasing the glucose level up to 15 mg/l only caused slightly increased susceptibility to the heating stress.

DISCUSSION AND CONCLUSIONS

The death rate of a starved microbial population is dependent on the density of the population. As shown by Harrison (1960) the maximum survival of starved *Aerobacter aerogenes* is at a particular cell density, and populations above or below this will have decreased relative viabilities. Postgate (1967) also emphasized that sparse bacterial populations can die off more rapidly than denser ones. As seen in the present study, starved *E. coli* suspensions exhibited a greater resistance to glucose and heating treatment at a concentration of 10^8 cells/ml than at 2×10^7 cells/ml. At cell populations of 4×10^5 cells/ml, starved *E. coli* cells are very sensitive to glucose and a mild heating treatment. This phenomenon of decreased sensitivity with higher populations may be due to some cells excreting cellular materials, and lysis cell providing substrates for survivors from which new cells can be formed. Thus the bacterial viabilities tend to remain fairly constant. Ryan (1955) termed this as "cryptic growth," a concept which has many applications in starvation and stress studies. A distinct population effect on starved *E. coli* viability is seen, as more viable cells are observed with higher initial populations.

In relating this work to more natural ecological conditions, *E. coli* cell populations in water can be expected to be lower than 10^7 cells/ml, the level used in these stress sensitivity studies. The reasons for not choosing lower cell populations for stress sensitivity studies are mainly procedural, as at densities which might be expected in natural water, microbes become much more rapidly sensitized to secondary stress, and one might have some difficulties in viability assays and in harvesting cells for using in assays. The *Aeromonas* showed especial sensitivity, rendering initial stress assays extremely difficult to carry out.

The starvation effect on the sensitivity of mixed aquatic microorganisms to mild heating treatment with and without the presence of exogenous substrates shows that these cells tend to become more resistant to secondary stress with increasing starvation time. The *Aeromonas* species isolated from the water sample also exhibited decreased sensitivity to glucose presence and mild heating stress with increasing starvation time. Thus, in the stress sensitivity experiments, *Aeromonas* might be considered as being representative of the predominant microorganisms in the waters which were tested.

The starved *E. coli*, in contrast, shows increased sensitivity to the secondary stress with increasing starvation time, becoming especially sensitive to glucose and heating stress at the end of the three-week starvation period.

The physiological investigations carried out in this study supported the differences seen between these two organisms in initial starvation stress sensitivity experiments.

In comparing the incorporation of glucose by *E. coli* and *Aeromonas*, the glucose level incorporated by fresh *E. coli* cells was significantly higher than that of the fresh *Aeromonas* cells, but after starvation both cultures showed decreased but similar glucose incorporation rates.

In addition, the endogenous respiration rate of the *E. coli* cells is relatively less than that of the *Aeromonas*, and *E. coli* starved in phosphate buffer solutions survive

longer. This corresponds to Ensign's (1970) observation of a relationship between slower microbial endogenous respiration rates and longer organism survival in starvation conditions.

With addition of glucose, *E. coli*, on the other hand, shows a faster rate of respiration, becoming less resistant to secondary stress than the *Aeromonas*.

The major difference in degradation of cellular constituents between these two different kinds of microorganisms during starvation is in the extent of ribonucleic acid degradation. The *Aeromonas* lost 80% of its RNA, whereas the starved *E. coli* still maintained approximately 50% of its original RNA. RNA can be utilized as a substrate for endogenous metabolism by a starving bacterium. Boylen and Ensign (1970) observed that a cell can less about 85% of its RNA and still remain viable. Thus, the decrease in RNA levels in the *Aeromonas* might not influence the viability or ability to respond to change environmental conditions which might allow growth.

The glucose sensitivity of the early and late cultures of *Aeromonas* and *E. coli*, even at levels of 1.0 mg/l, must be considered in light of glucose levels which have been found in natural aquatic environments. Vaccaro, Hicks, Jannasch and Carey (1968) found that the glucose concentration in seawater varied from 1.8 μg/l to 180 μg/l. Thus, the highest value they observed for a natural water was one-fifth the concentration of glucose used in the special glucose contact experiment. Wright and Hobbie (1966) reported that glucose and acetate concentrations in natural waters were found to be 1 to 10 μg/l. If the glucose concentration increased up to levels approaching 1 mg/l with contact for 60 minutes, or at 15 mg/l for 1 minute, this could significantly sensitize *E. coli* cells. Additional studies to establish specify minimal substrate levels and contact times are needed to confirm the broad ecological concepts which appear to be evident from this study.

The *Aeromonas* isolated from an aquatic environment appeared to release more materials into the medium, and tended to become less sensitive or susceptible to heating treatment and added glucose. *E. coli*, on the other hand, become more susceptible. Thus Postgate's statement of stress increasing sensitivity to secondary stresses may need to be qualified.

This research also revealed that this water microorganism may have the ability to decrease its pool of internal components to minimize maintenance requirements. *E. coli* may not have this ability, thus tending to hold more materials and causing greater maintenance energy requirements.

There are many aspects of the stress problem which will require additional investigations, such as the application of different kinds of stress (acidity or alkalinity, salinity, radiation) to microorganisms. In addition, different combinations of sensitizing substrates could be used to better duplicate stress conditions in natural environments, and to determine if the glucose sensitizing effect could be overcome by the presence of other compounds. The addition of *E. coli* to natural water samples to determine the responses of this organism to secondary stress with and without glucose could provide additional informations of direct ecological interest. It could also be important to understand the transport, pathways and physological effects of sensitizing agents by means of radiotracer techniques.

Concepts of energy charge and energy charge effects on starvation resistance should also be considered, especially in regard to glucose additions as they may be related to substrate-accelerated death. Chapman, Fall and Atkinson (1971) reported that the adenylate energy charge of *E. coli* during normal growth is about 0.8;

however, it dropped to 0.5 when the organism was in a starved condition. The viability of *E. coli* cells is maintained at adenylate energy charge values between 0.8 and 0.5, and microorganisms may die at values below 0.5. It could also be important to determine is there any relationship between these values and the susceptibility to secondary stress, especially in regard to the marked effect which glucose has on secondary stress sensitivity.

SUMMARY

Microorganisms in aquatic environments are subject to nutrient starvation, which may influence sensitivity to a wide range of secondary stresses. An *Aeromonas* species isolated from a Colorado mountain stream (Arthur's ditch), and a culture of *Eschorichia coli* were grown in peptone broth (0.5% w/v) on a shaker at 25 C for 24 hours, washed with phosphate buffer, and starved in phosphate buffer to determine their responses to transient heating stress (52 C for 5 min) and the short-term presence of nutrients under starvation conditions.

These two microorganisms had markedly different responses to starvation, secondary stress and nutrient presence. At the beginning of the starvation time *Aeromonas* cells were very sensitive to the mild heating stress and one hour prior glucose presence. However, peptone gave some degree of protection against the heating stress. These responses were markedly decreased after a three-week starvation poriod. The starved unstressed cells were slightly less sensitive to glucose presence than the unstarved cells. In contrast, *E. coli*, under similar conditions, responded differently from the *Aeromonas* species with increasing starvation time, as it gradually appeared to have a greater sensitivity to heat and glucose presence, especially when glucose was present in the stressed culture suspensions.

The level of glucose incorporated by fresh cells of *Aeromonas* was lower than that of *E. coli* cells, however the endogenous oxygen usage of the fresh *Aeromonas* culture was faster than that of *E. coli* cells. In the starved cultures, after glucose addition the *E. coli* culture utilized oxygen at a rate greater than endogenous over the 50 minute experiment, while the *Aeromonas* culture showed only a slight response to the glucose addition before returning to the original endogenous rate.

The starved cells of both microorganisms contained lower carbohydrate, protein and ribonucleic acid levels than fresh cells. The starved *E. coli* cells had carbohydrate and RNA losses of about 50%, whereas the *Aeromonas* culture lost around 30% and 80% respectively of these constituents. The major difference between these organisms is the increased RNA loss by *Aeromonas* (80%) in comparison with the lesser loss (50%) shown by the *E. coli* culture. The starved *Aeromonas* cells generally tended to release more materials into the starvation medium than the *E. coli* culture. Thus, *Aeromonas* may have the ability to decrease its pool of internal components to minimize maintenance requirements. *E. coli* may not have this ability and may hold more materials and have greater maintenance energy requirements.

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