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INJURY AND REPAIR OF *ESCHERICHIA COLI* DAMAGED BY ACID MINE WATER*

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Abstract—Pure culture suspensions of *Escherichia coli* B/5 were stressed by exposure to filter-sterilized acid mine water (AMW). Sublethally injured survivors were examined for their ability to repair in several resuscitation media under different conditions of pH, temperature and oxygen availability. The repair process was monitored as a function of time by periodically removing samples from the repair media and simultaneously plating on nonselective and selective media. *E. coli* was severely damaged by AMW; however, sublethally injured survivors repaired when placed under favorable conditions. Optimal repair occurred in trypticase soy broth supplemented with 0.3% yeast extract (TSYB) at pH 7.0 and 35°C. Resuscitation did not occur in TSYB at pH 9.0, at an incubation temperature of 20°C, or in the absence of oxygen. Lauryl tryptose broth (LTB), which is recommended for the presumptive isolation of fecal coliforms, was unable to facilitate repair of injury. The presence of the surfactant, sodium dodecyl sulfate, as well as the nutrient composition of LTB, appeared to be responsible for the inability of this medium to permit recovery of AMW-stressed *E. coli*.

Key words—*Escherichia coli*, acid mine water, sublethal injury, resuscitation

INTRODUCTION

Viability of allochtonous and authochthonous bacteria in aquatic environments is affected by a range of conditions which include nutrient availability (Ray and Speck, 1973), ultraviolet radiation (Kapuscinski and Mitchell, 1981; Ray and Speck, 1973), temperature (Beuchat, 1977; Jost and Johnson, 1978), pH extremes (Carlson-Gunnoe *et al.*, 1983; Double and Bissonnette, 1980; Hackney and Bissonnette, 1978; Kralovic and Wilson, 1969; Smith and Palumbo, 1978), heavy metals (Cook and Wilson, 1971), and chlorine (Camper and McFeters, 1979). Organisms exposed to such stresses can become sublethally injured. Sublethal injury is functionally defined as the temporary loss of an innate capability or structure which reduces the ability of damaged cells to form colonies in the presence of selective compounds incorporated into recommended media (McCoy and Ordal, 1978; Przybylski and Witter, 1979; Roth and Keenan, 1971). Such debilitated microorganisms are not recovered on selective media designed for their detection, resulting in an underestimation of the number of viable cells present (Bissonnette *et al.*, 1975; Braswell and Hoadley, 1974). Lack of detection of microorganisms of public health significance, such as *Escherichia coli*, could lead to erroneous conclusions concerning the level of fecal contamination present and the suitability of waters for human contact or consumption. The problem of reliably assessing the sanitary quality of freshwaters polluted with acidic discharges generated during the mining of coal has been documented (Carlson-Gunnoe *et al.*, 1983; Double and Bissonnette, 1980; Hackney and Bissonnette, 1978).

Appalachian coal deposits often contain a significant amount of reduced sulfur compounds such as pyrite. Bacteria of the genus *Thiobacillus* oxidize the reduced sulfur compounds and thereby contribute to the formation of a solution containing elevated concentrations of H^+, Fe^{2+}, Fe^{3+} and SO_4^{2-} (Kleinmann and Crerar, 1979). This solution, which has a low pH and high concentrations of metal ions, is termed acid mine water (AMW). AMW from underground coal mines and coal refuse piles is one of the most persistent industrial pollution problems in the United States (Kim *et al.*, 1982). Although the Federal Water Pollution Control Act was passed more than a decade ago, recent reports indicate that there has been little or no reduction in the number of streams adversely affected by AMW (Kim *et al.*, 1982).

Streams affected by AMW are often impacted by municipal and industrial waste discharges. When AMW enters a stream, it can cause a drastic reduction in the overall population of heterotrophic bacteria (Kralovic and Wilson, 1969) as well as specific groups of organisms including sanitary indicator bacteria (Double and Bissonnette, 1980; Hackney and Bissonnette, 1978). Joseph and Shay (1952) found that every stream surveyed which received fecal contamination and AMW contained detectable, al-

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beit reduced, numbers of indicator bacteria. Sublethal injury is reversible since damaged cells may repair their injury on a nonselective, nutritionally complete medium and regain the ability to grow on appropriate selective media. Cells injured by various stresses can be resuscitated by brief preincubation in a nonselective medium prior to exposure to selective media (Bissonnette *et al.*, 1975; McFeters *et al.*, 1982; Ray, 1979; Warseck and Speck, 1973).

At present, little is known about the extent of damage or the effects of various cultural conditions used for the detection of *E. coli* from AMW-containing streams. The purpose of the current research was to examine the extent of injury and the ability of *E. coli* to resuscitate following contact with AMW, as well as to determine those conditions which encourage repair. Data indicated that the incidence of death and debilitation was high following AMW exposure. Resuscitation required 3–4 h and was significantly affected by the pH, temperature and nutrient composition of the repair medium.

MATERIALS AND METHODS

Acid mine water

Initially, four streams were monitored to locate a single, stable source of AMW. The physical-chemical characteristics varied so extensively as a function of meteorological events that these waters were deemed unsuitable for *in vitro* exposure studies. AMW collected from the emergence of an underground stream was used because it demonstrated a high degree of physical-chemical stability and caused a consistent level of injury. At the time of collection, sample pH, temperature, specific conductance and acidity were measured by standard methods (APHA, 1980). Mean values and standard deviations were ($n = 66$): pH, 3.02, SD = 0.07; temperature, 16.1°C, SD = 0.5; specific conductance, 3079 μmhos cm^{-2}, SD = 167; acidity, 1755 mg l^{-1} as $CaCO_3$, SD = 262. The concentrations of metals commonly associated with AMW were determined by atomic adsorption (APHA, 1980). Mean values and standard deviations were ($n = 11$): Al, 133.20 mg l^{-1}, SD = 7.10; Cd, not detectable; Cu, 0.11 mg l^{-1}, SD = 0.03; Fe, 231.15 mg l^{-1}, SD = 13.49; Mn, 5.60 mg l^{-1}, SD = 1.09; Pb, 0.002 mg l^{-1}, SD = 0.001; Zn, 2.71 mg l^{-1}, SD = 0.67. AMW was sterilized by passage through a membrane filter with a pore size of 0.45 μm, diluted with sterile, distilled deionized water (3:1, AMW:dH_2O), and held at 16.5°C. AMW was used within 2 h of collection.

Test organisms

E. coli 12435 (isolated from an AMW-polluted stream) and *E. coli* B/5 (generous gift of P. Snustad, University of Minnesota) were used. Results obtained with these organisms were almost identical. Only data acquired with *E. coli* B/5 are reported since use of a bacterium isolated from AMW might select for an organism which was resistant to its effects and, thereby, inaccurately represent any resulting injury.

Cultures were maintained at 4°C on slants of trypticase soy agar supplemented with 0.3% yeast extract (TSYA). One hundred ml of trypticase soy broth supplemented with 0.3% yeast extract (TSYB) was inoculated with a test culture and incubated at 35°C. Six h later, two loopfuls of the culture were transferred into 100 ml of TSYB and incubated for 12 h at 35°C on a rotary shaker operating at 125 cycles min^{-1}. Cells were harvested by centrifugation at 3020 ***g*** for 10 min and washed twice with an amount of 0.1% peptone buffer (APHA, 1980) equal to the volume of culture harvested. The final suspension contained 1.2×10^9 CFU ml^{-1}. Two ml of this suspension was then exposed to AMW.

Exposure and repair of cells

Initially, bacteria were exposed to AMW from several area streams and their levels of viability and injury were followed. Maximum survival under *in vitro* conditions was 6 h. The amount of injury sustained by a culture on a given day varied significantly as a function of the meteorological events preceding the collection of the water sample. A stable source of AMW was obtained by collecting samples from the emergence of an underground stream which drained an area of active strip-mining. This water was more heavily contaminated than any other waters tested, having ion concentrations and acidity values which were four to seven times greater than other streams found in the area. However, the physical-chemical parameters of the water from this underground source demonstrated a degree of stability not found in any other source, since its flow was not influenced by meterological events, thereby greatly increasing the reproducibility of the exposure procedure. Because this water was more concentrated than other sources, various dilutions and exposure times were examined to determine the conditions of exposure which produced levels of death and debilitation approximating that incurred in a more typical water during a 3–6 h exposure (i.e. 98% death and 98% injury). A 1-min exposure to a 3:1 dilution of AMW to distilled water produced the requisite amount of death and injury.

Two milliliters of the washed cell suspension was placed in 98 ml of the AMW preparation and agitated vigorously. After 1 min, 2 ml of the inoculated AMW was withdrawn and placed in 98 ml of various repair broths. This exposure procedure resulted in a mean injury rate of 98.3% (SD = 3.1, $n = 81$) and a mean death rate of 98.3% (SD = 2.6, $n = 81$) which is the level of injury and death observed during a 3–6 h exposure in typical regional streams (Hackney and Bissonnette, 1978). TSYB, lauryl tryptose broth (LTB), tryptose broth (LTB without sodium dodecyl sulfate), TSYB + 0.25% glucose, TSYB + 0.25% lactose and glucose salts medium were used as repair broths.

Broths inoculated with AMW-exposed cells were incubated at various temperatures (20, 35 and 44.5°C) and pH values (6.0, 7.0, 8.0 and 9.0 $\pm$ 0.1) to determine the effect of these conditions upon repair. The pH of the medium was adjusted with 1 N NaOH or 1 N HCl. Broths were constantly agitated during the repair period with a magnetic stirrer. At regular time intervals, samples were removed, diluted in 0.1% peptone buffer, and surface-plated in triplicate onto 10.0 ml of nonselective and selective media. The nonselective medium was TSYA, whereas the selective medium was TSYA supplemented with 0.05% sodium deoxycholate (TSYDA). Plates were incubated for 24 $\pm$ 2 h at 35°C; colonies were then counted on a Quebec dark-field colony counter.

Repair in the presence of 2,4 dinitrophenol (DNP)

AMW-exposed cells were added to flasks of TSYB containing a series of 2-fold dilutions of DNP. Cell viability was monitored on the nonselective medium. The highest concentration of DNP which did not cause a decrease in cell viability during the first 3 h of incubation was considered inhibitory but not lethal; this level was used to examine the role of proton motive force on repair.

Repair in an N_2 atmosphere

Bottled, 100% N_2 gas was passed through a silica gel desiccant and sterile glass wool to remove moisture and particulate contaminants. The gas was then bubbled through the TSYB at an empirically determined rate which maintained the E_h below the methylene blue end point (*Manual of Methods for General Bacteriology*, 1981). Flasks of TSYB purged with N_2 were inoculated with AMW-

exposed cells and repair was followed as previously described. The broths were continuously purged with N_2 throughout the entire repair period.

Assumptions and calculations

All viable cells (injured and noninjured) are capable of producing colonies on the nonselective TSYA, whereas only repaired or uninjured cells can grow on the selective medium containing sodium deoxycholate (TSYDA). Increased counts observed on the selective medium without a concomitant increase on the nonselective medium are due to repair of damaged bacteria. Simultaneous increases in TSYDA and TSYA counts are due to cell division (Ray and Speck, 1973).

Calculations were conducted as follows:

% Dead = [(nonselective count before AMW-exposure − nonselective count after AMW-exposure)/(nonselective count before AMW-exposure)] × 100.

% Injured = [(nonselective count after AMW-exposure − selective count after AMW-exposure)/(nonselective count after AMW-exposure)] × 100.

Statistical analysis

Data were analyzed to determine the amount of time needed by *E. coli* to repair injury sustained during AMW-exposure. The General Linear Models Procedure of the Statistical Analysis System was used to fit quadratic equations to the data points. Two equations were produced for each data set, one from counts made on the nonselective medium and a second from counts made on the selective medium. A 95% confidence interval was generated for each equation. The time needed for repair was determined to the nearest 0.1 h as that point where the counts were not significantly different (overlapping of 95% confidence intervals).

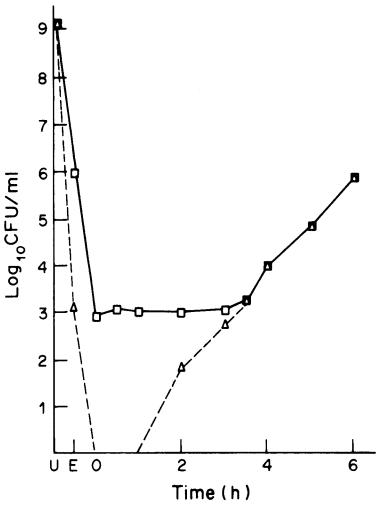

Fig. 1. Repair of *E. coli* B/5 following exposure to AMW. A washed 12-h culture was exposed for 1 min to a 3:1 (AMW:dH_2O) dilution of sterile AMW, placed in TSYB at pH 7.0, and incubated at 35°C. Solid line represents counts made on TSYA (nonselective medium); broken line represents counts made on TSYDA (selective medium). U—cell counts prior to AMW exposure. E—cell counts after 1 min exposure to AMW but prior to addition to repair broth. O—cell counts from TSYB immediately after inoculation with AMW-exposed bacteria.

RESULTS

Repair in TSYB

The ability of *E. coli* to repair following exposure to AMW was initially examined (Fig. 1). Contact with AMW severely stressed test bacteria. Greater than 98% of exposed cells did not form colonies on TSYA and were assumed to be nonviable. Of the viable cells present, 98% were sublethally injured as reflected by the inability to produce colonies on selective TSYDA. Although the death rate was high, significant numbers of cells were viable after a 1 min exposure (10^5–10^6 CFU ml^{-1} on TSYA). Counts made on TSYA remained constant between 1 and 4 h while TSYDA counts increased rapidly after 1 h, indicating that repair was occurring. Cell multiplication began within 4 h, demonstrating that cells damaged by the action of AMW had undergone resuscitation.

Control cells which were exposed to 0.1% peptone buffer rather than AMW were not sensitive to the selective medium. This lack of injury was evidenced by identical counts on selective and nonselective plating media and by the resumption of cell division within 15 min of being placed in the repair broth.

Effect of temperature and pH on repair

The resuscitation temperature significantly affected the ability of stressed organisms to repair. Flasks of media containing the AMW-stressed bacteria were incubated at 20, 35 and 44.5°C (Fig. 2). Resuscitation occurred within 3.1 h when damaged cells were incubated at 35°C and within 4.3 h when incubated at 44.5°C. Repair was not observed at 20°C.

The effect of repair broth pH on resuscitation was examined by adjusting the pH to 6.0, 7.0, 8.0 and 9.0 ± 0.1 unit, respectively (Fig. 3). All flasks were incubated at 35°C. Repair was not observed in TSYB adjusted to pH 9.0 as evidenced by the lack of viable cells in the repair broth. Resuscitation occurred when damaged bacteria were placed in pH 8.0 TSYB, but only after 99% of the cells initially present became nonviable during the first 2 h of the incubation period. Repair occurred within 4.1 h at pH 7.0, with cell numbers remaining relatively constant during resuscitation.

Counts made on the nonselective medium increased throughout resuscitation at pH 6.0. The increase of the nonselective counts prior to completion of repair could have been the result of an increased proton motive force supplied by the higher H^+ concentration of the medium, or due to a reduced amount of heat generated during neutralization of AMW by TSYB. To further investigate this observation, the uncoupling agent 2,4-denitrophenol (DNP) was added to a flask of TSYB at pH 6.0 to abolish any proton gradient imposed on the bacteria by the repair broth. AMW-exposed cells were then added to DNP-supplemented TSYB as well as to a control flask lacking DNP. The presence of DNP did not affect the counts obtained on the nonselective

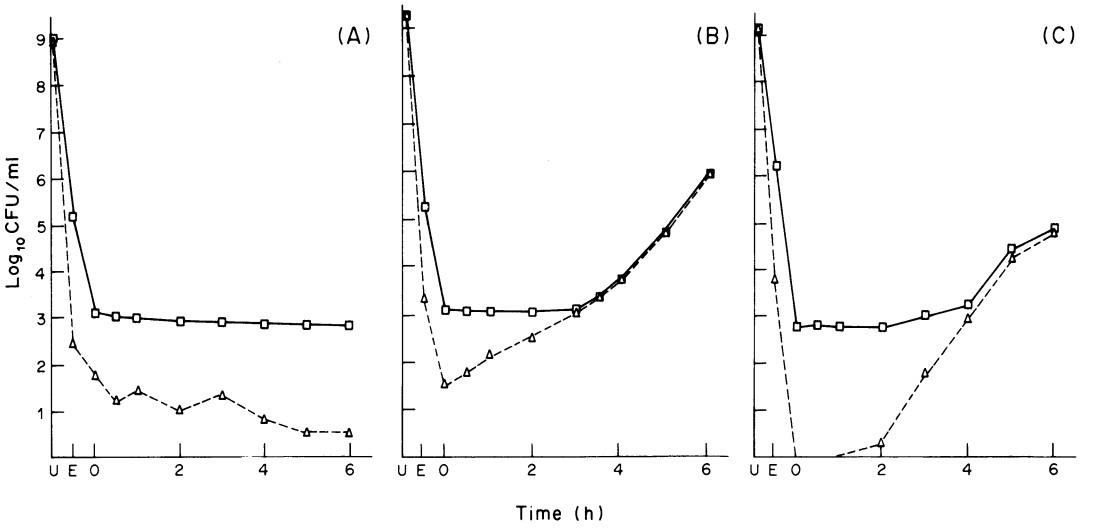

Fig. 2. Effect of incubation temperature on the ability of *E. coli* B/5 to repair damage sustained during AMW-exposure. A washed 12-h culture was exposed for 1 min to a 3:1 (AMW:dH_2O) dilution of sterile AMW, placed in TSYB at pH 7.0 and incubated at the indicated temperature. (A) 20°C; (B) 35°C; (C) 44.5°C. See Fig. 1 for explanation of symbols.

medium during the intial hour of repair; both broths showed substantial increases in cell numbers. In fact, counts increased faster during the first hour when injured cells were placed in the presence of the uncoupler than did the control. Hence, increased cell numbers in the pH 6.0 repair broth were not due to an artificially applied proton motive force. The enhanced repair might have been related to smaller pH changes during AMW neutralization at pH 6.0 than in higher pH broths.

Effect of nutrient composition of repair broth on resuscitation

Repair failed to occur in the glucose salts minimal medium. Addition of 0.25% (w/v) glucose or lactose to TSYB increased the amount of time required for the AMW-exposed cells to repair. The control culture (TSYB only) completed resuscitation in 2.8 h, whereas the culture supplemented with 0.25% lactose repaired in 3.0 h and the culture containing 0.25% glucose resuscitated in 3.4 h.

LTB is recommended by *Standard Methods* (APHA, 1980) for the presumptive isolation of coliforms from water. This broth, which contains the surfactant sodium dodecyl sulfate (SDS), was evaluated for its ability to support repair of AMW-injured *E. coli*. Repair was followed in LTB, LTB without SDS and TSYB at 35°C (Fig. 4). Repair was not observed in LTB at 20, 35 or 44.5°C as evidenced by a rapid decline in the number of viable cells soon after inoculation. When damaged bacteria were placed in LTB lacking SDS, repair took 4.1 h as compared to 3.2 h in TSYB.

Effect of atmospheric composition on repair

Repair did not occur when AMW-exposed cells of *E. coli* B/5 were placed in TSYB and incubated in an atmosphere of 100% N_2. Counts made on the nonselective medium showed a slight increase during the 6 h incubation period, but the absence of growth on the selective medium indicated a lack of repair. Similarly, uninjured cell populations (buffer-exposed) did not increase rapidly during the N_2 incubation. Since growth and repair in TSYB under anoxic conditions could have been inhibited by lack of a fermentable carbohydrate, these experiments were also performed in TSYB supplemented with 0.25% glucose or lactose. Repair was not observed in the presence of the added carbohydrates. However, rapid cell division occurred when unexposed control cells (exposed to buffer only) were placed in media containing glucose or lactose.

DISCUSSION

E. coli was able to repair under appropriate cultural conditions even after extensive damage caused by *in vitro* exposure to AMW. Damage incurred during AMW exposure was greater than that produced by acetic acid (Przybylski and Witter, 1979) or HCl (Roth and Keenan, 1971), as indicated by the greater death rate and longer time required to achieve repair. The additional damage could be due to the presence of toxic ions in addition to the effect of protons.

The shortest repair period was observed at 35°C, whereas resuscitation at 44.5°C required an additional 1.2–1.8 h. When injured cells were incubated at 20°C, nonselective and selective media plate counts fluctuated slightly, but repair did not occur. Therefore, synthetic activity might be required for resuscitation, since recovery occurred more rapidly at temperatures where biosyntheses proceed optimally. This is in contrast to damage caused by exposure of *E. coli*

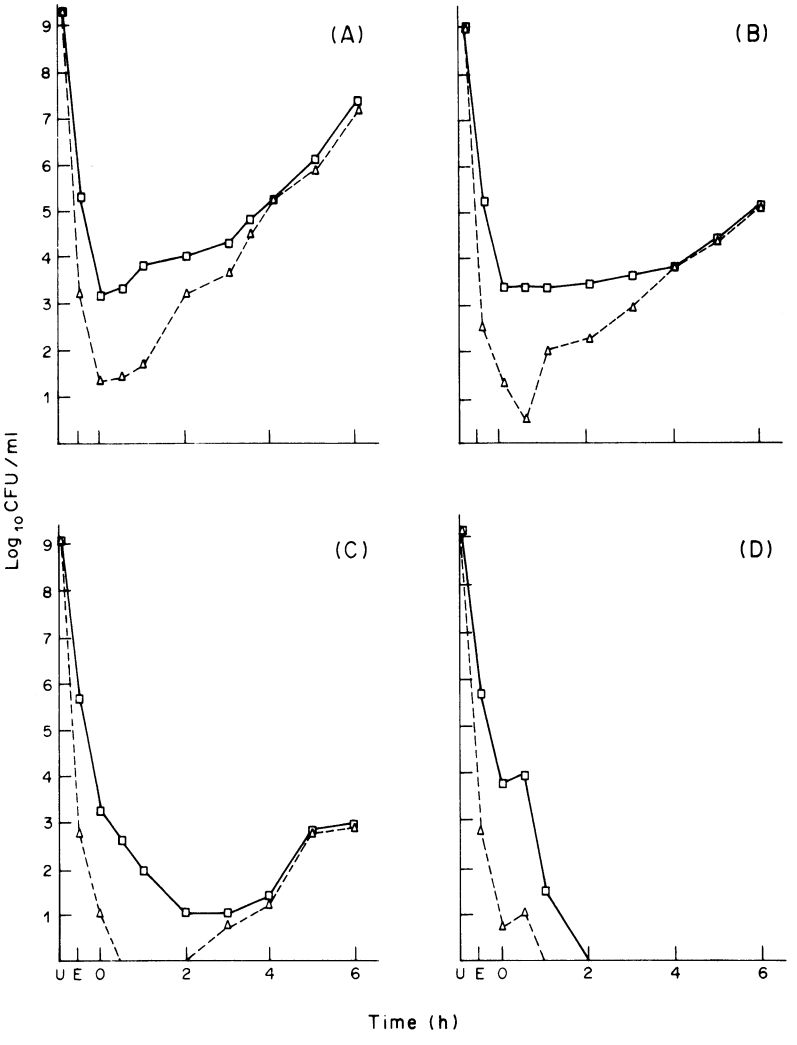

Fig. 3. Effect of repair broth pH on the ability of *E. coli* B/5 to repair damage sustained during exposure to AMW. A washed 12-h culture was exposed for 1 min to a 3:1 (AMW:dH_2O) dilution of sterile AMW, placed in TSYB with its pH previously adjusted to 6.0, 7.0, 8.0 or 9.0 and incubated at 35°C. (A) pH 6.0; (B) pH 7.0; (C) pH 8.0; (D) pH 9.0. For explanation of symbols see Fig. 1.

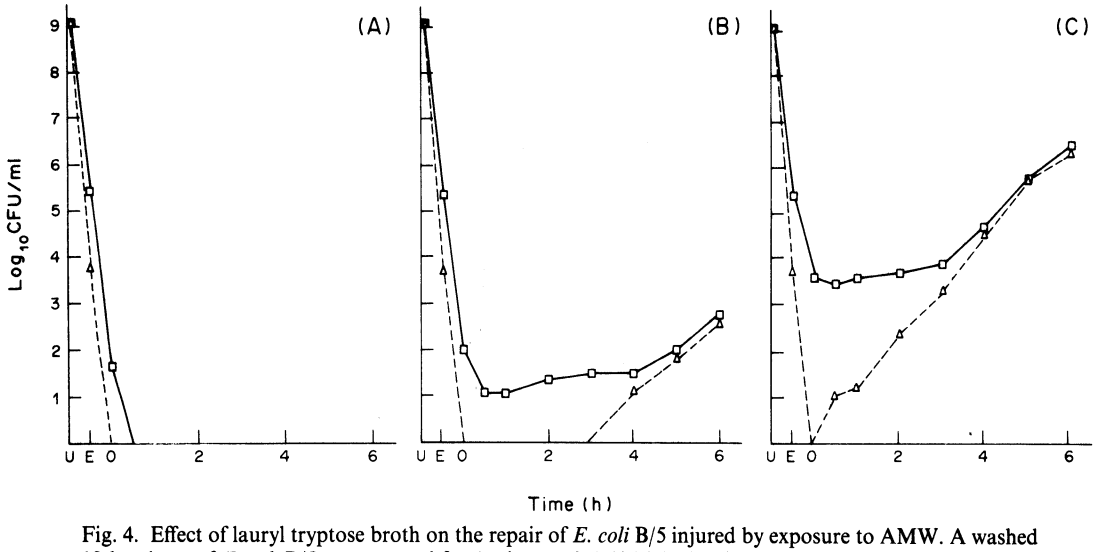

Fig. 4. Effect of lauryl tryptose broth on the repair of *E. coli* B/5 injured by exposure to AMW. A washed 12-h culture of *E. coli* B/5 was exposed for 1 min to a 3:1 (AMW:dH_2O) dilution of sterile AMW, placed in repair broth, and incubated at 35°C. Repair broths were: (A) lauryl tryptose broth; (B) tryptose broth (lauryl tryptose without sodium dodecyl sulfate); and (C) TSYB. For explanation of symbols see Fig. 1.

to solutions of acetic acid (Przybylski and Witter, 1979) for which no synthetic activity was required and repair occurred within 40 min.

E. coli is capable of growth over a pH range of 4.5–9.0 (Mitchell, 1951); however, the pH of the repair broth had a pronounced effect upon repair. Of the pH values examined, pH 6.0 and 7.0 facilitated resuscitation of injured cells wereas repair was adversely affected at pH 8.0 and inhibited at pH 9.0. When bacteria were removed from the AMW stress and placed in repair broth, there was a 50-fold dilution in cell population. However, the decrease in cell counts following this transfer was often greater than 50-fold. These results suggested that bacterial viability continued to decline after the cells were placed in the repair broth. Furthermore, this decrease was greatest when injured bacteria were placed in broths with a pH of 9.0 and least in broths at pH 6.0, suggesting that repair broth pH influenced the continued reduction of cell numbers. Broths with lower pH values might have bolstered the cell's proton motive force, increasing ATP production and transport of substrates (Sjogren and Gibson, 1981). However, the role of proton motive force in repair did not appear to be significant. The presence of DNP, which eliminates the bacterium's proton motive force (Harold, 1970), did not affect recovery in a pH 6.0 repair broth.

Heat generated when AMW was neutralized by the broth could have caused additional damage to the injured bacteria. Our results are in agreement with this hypothesis since the change in pH, and therefore the heat generated, would be greatest when the cells were placed in a pH 9.0 broth and least when added to a pH 6.0 medium. Other researchers suggest similar hypotheses (Kralovic and Wilson, 1969; Przybylski and Witter, 1979).

In addition to appropriate pH and incubation temperature, the resuscitation process required the presence of oxygen. AMW-injured cells failed to repair in TSYB under a 100% N_2 atmosphere, regardless of whether additional fermentable carbohydrates were present. Since unstressed control cells did initiate cell division under the anoxic conditions, it appears that repair of AMW-injury cannot proceed satisfactorily without oxygen.

The ability of *E. coli* to repair AMW-induced damage was influenced by the nutrient composition of the repair broth. Damaged cells were unable to resuscitate in glucose salts medium indicating that AMW-stressed bacteria experienced metabolic as well as structural injury (Ray and Speck, 1973). Addition of lactose or glucose to TSYB increased the time needed for repair even though unexposed control cultures produced greater numbers of cells when lactose was added. The reason for repair inhibition by fermentable carbohydrates was not apparent; however, the presence of fermentable carbohydrates encourages acid production which could be detrimental to injured cells.

The inability of AMW-damaged cells to repair in LTB was due, in part, to the presence of the surfactant SDS. Injured cells resuscitated in LTB when the surfactant was omitted, but repair was still not as rapid as in TSYB. This suggested that LTB had a less than ideal nutrient composition for the repair of AMW-stressed coliforms. TSYB contains vitamins, cofactors and peptides in the form of yeast extract and papaic digest of soybean meal which are not components of LTB; this might be responsible for the greater repair in TSYB. Reduced repair in LTB lacking SDS could also have resulted from the greater concentration of glucose and ionic salts which have been reported to inhibit resuscitation of acetic acid-injured *Salmonella bareilly* (Blankenship, 1981). These results raise question as to the recommended standard method (APHA, 1980) of using LTB either as a presumptive medium in the multiple-tube fermentation technique or as an enrichment broth in the two-step membrane filtration procedure for detection of total coliforms from streams polluted with AMW.

Blankenship (1981) reported that the best medium for resuscitation of acetic acid damage was a 0.5% broth of casamino acids. McFeters *et al.* (1982) reported that protein based buffers were more protective of stressed cells than were mineral based buffers. Results from these workers and the present study suggest that optimal recoveries of AMW-damaged bacteria could be achieved with a peptone or phytone broth which lacks fermentable sugars and mineral buffering.

Pollution of freshwater streams in coal mining regions by AMW and organic wastes is, unfortunately, common occurrence. The results presented in this study indicate that *E. coli* was severely stressed by exposure to AMW. Such exposure resulted in a reduced ability of recommended selective media to detect sanitary indicator organisms, thus complicating the assessment of the public health safety of these waters. Though damage was extensive, resuscitation proceeded when cells were placed under favorable conditions of pH, temperature, oxygen and nutrient availability. LTB, as recommended by *Standard Methods* (APHA, 1980), was unsatisfactory for the recovery of AMW-damaged *E. coli*. The presence of SDS, as well as the nutrient composition of LTB, appeared to be responsible for the inability of this medium to permit recovery of AMW-stressed *E. coli*. The use of LTB to evaluate the public health safety of AMW-contaminated streams could result in erroneous assessments of bacterial water quality due to poor indicator recovery. Valid sanitary evaluations of waters receiving AMW and organic wastes are strongly dependent upon optimizing the sensitivity of recovery procedures for detection of bacterial indicator organisms.

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