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Effect of Acid Mine Water on *Escherichia coli*: Structural Damage

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Abstract. *Escherichia coli* B/5 12-h cultures were exposed to filter-sterilized acid mine water (AMW), fixed in situ, and examined for morphological changes by transmission electron microscopy, scanning electron microscopy, and x-ray spectrometry. Thin sections showed that layers of the Gram-negative envelope were altered and often lacking. Additionally, polar regions of the cell were frequently devoid of cytoplasm. AMW-exposed cells were distorted and had an amorphous substance associated with them. Spectra obtained by x-ray spectrometry suggested that this amorphous substance was cytoplasm rather than a mineral precipitate from AMW. Morphometric analyses of control and AMW-exposed populations showed significant differences in mean volume, length, and width of cells stressed in AMW; this indicates that smaller cells were selectively destroyed by the action of AMW. We concluded that loss of cytoplasm and cell lysis were the consequence of AMW damage to the bacterial envelope.

Significant amounts of reduced sulfur compounds, such as pyrite, are often associated with Appalachian coal deposits. In the presence of *Thiobacillus ferrooxidans*, pyrritic materials are oxidized to form sulfuric acid and soluble metal ions [18]. These waters, with low pH values and high metal ion concentrations, are referred to as acid mine water (AMW).

The production of AMW by abandoned coal mines and refuse piles is one of the most persistent industrial pollution problems in the USA [10]. Streams receiving AMW often serve as repositories for human wastes. Waters from such streams are frequently involved in other uses such as fishing, swimming, and consumption. Assessing the level of fecal contamination in streams receiving AMW is difficult because of the injury sustained by indicator bacteria [15]. Significant reductions in viable organisms [7] and increased resuscitation periods [6, 21] indicate that the injury sustained by *Escherichia coli* exposed to AMW is extensive. This injury, which has such a detrimental effect on viability, could be expected to produce changes in cell morphology. The present study was performed to investigate the morphological alterations of *E. coli* that result from exposure to AMW.

Materials and Methods

Test organisms. The effects of AMW on *E. coli* B/5 (from P. Snustad, University of Minnesota) and *E. coli* 12435 (isolated from an AMW stream) were examined. Results were identical for both isolates. Only micrographs from *E. coli* B/5 are presented here, as it was felt that use of a strain isolated from AMW might select for an organism resistant to its effects and bias any resulting injury.

Exposure and fixation. Four streams were monitored to locate a stable source of AMW representative of our geographical area. However, the physical–chemical characteristics varied so widely as a function of meteorological events that these waters were deemed unsuitable. AMW collected from the emergence of an underground stream that drained an area of active mining was used, because it demonstrated stable physical–chemical parameters. At the time of collection, the sample pH, temperature, specific conductance, and acidity were measured. Mean values (n = 66) for these parameters were: pH, 3.02, SD = 0.7; temperature, 16.1°C, SD = 0.5; specific conductance, 3079 μmhos/cm, SD = 167; and acidity, 1755 mg/liter as $CaCO_3$, SD = 262. Concentrations of metals commonly associated with AMW were determined by atomic absorption [2]. Mean values were (n = 11): Al, 133.2 mg/liter, SD = 7.1; Cd, not detectable; Cu, 0.11 mg/liter, SD = 0.02; Fe, 231.15 mg/liter, SD = 13.49; Mn, 5.60 mg/liter, SD = 1.09; Pb, 0.002 mg/liter, SD = 0.001; and Zn, 2.7 mg/liter, SD = 0.7. The osmolarity of AMW as determined by freezing-point depression (Osmette Precision Osmometer, model 2007) was 0.03 *M*.

Escherichia coli 12-h cultures grown in trypticase soy broth with 0.3% yeast extract were harvested by centrifugation, washed twice, and resuspended in 0.1% peptone buffer, pH 7.1 [2]; 5 ml of washed cells was injured by exposure to AMW by the method described by Wortman and Bissonnette [21]. Cells were exposed to AMW at 16.5, 12, 8, and 4°C; no differences were observed at these temperatures. Therefore, only micrographs from 16.5°C exposures are presented, as this value closely approximated the mean AMW temperature. Exposed bacteria were

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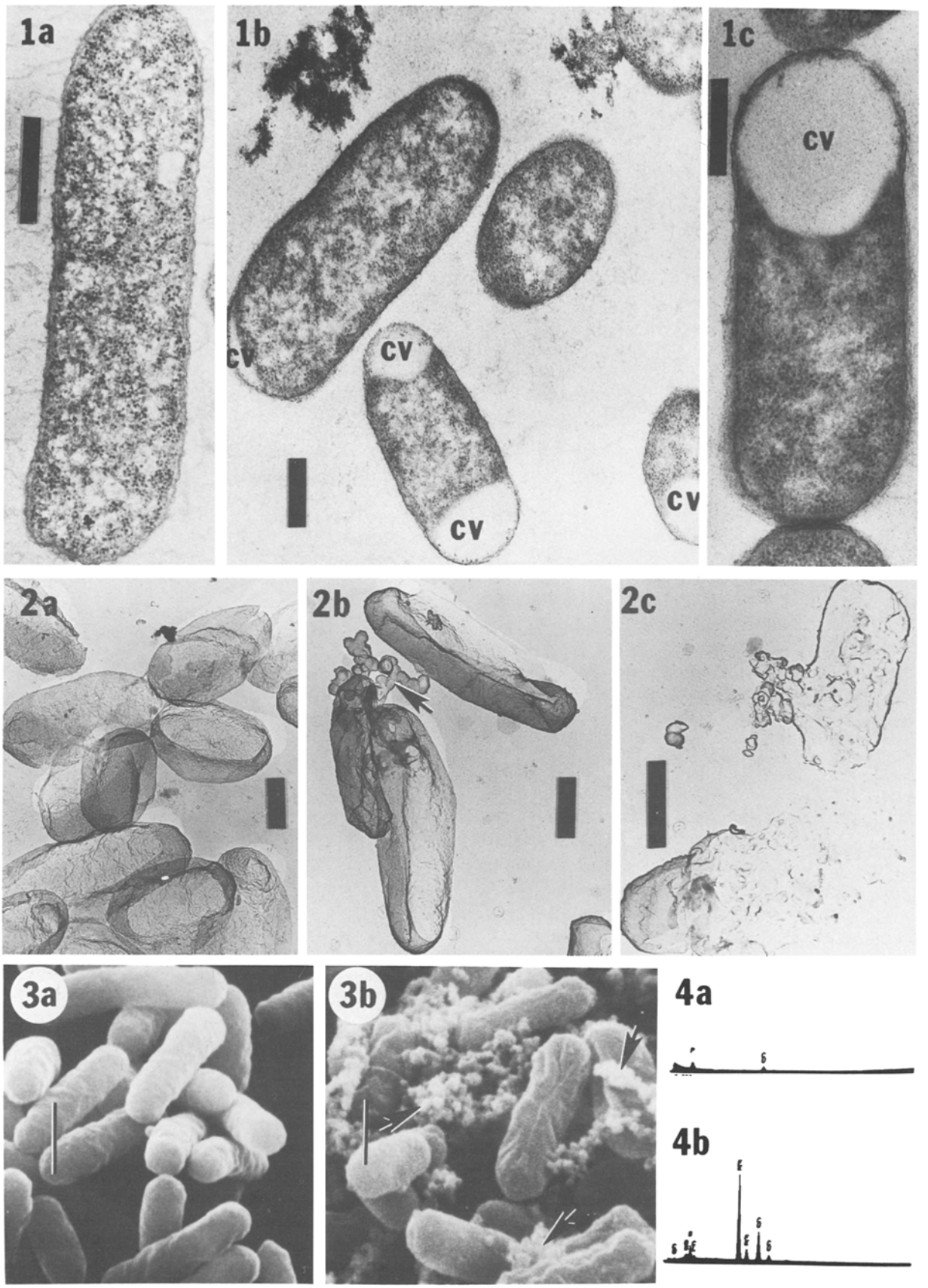
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fixed in the AMW by the method of Kellenberger et al. [9]. Controls were treated identically, except that the exposure and fixation were performed in 0.1% peptone buffer rather than AMW. Peptone buffer was chosen as a control because of its neutral pH and osmolarity, which closely approximated that of the AMW.

Transmission electron microscopy (TEM). Fixed and dehydrated preparations were embedded in Epon 812 and hardened at 60°C. Thin sections were cut with a Sorvall MT-2 ultramicrotome and collected on carbon-stabilized, nitrocellulose-coated copper grids. Sections were poststained for 5 min in a saturated solution of uranyl acetate, followed by 1 min in Reynolds' lead citrate [14]. Sections were examined at 100 kV with an RCA EMU-3G electron microscope.

Scanning electron microscopy (SEM). Fixed cells were dehydrated in an alcohol series, placed on a dished aluminum stub, and critical point dried with liquid CO_2. Dried cells were rotary coated with carbon, followed by a layer of gold–palladium (60 : 40). Specimens were viewed in a Cambridge Stereoscan S4-10 scanning electron microscope at 20 kV.

Cells used in the x-ray microanalyses were prepared by the procedure described for SEM except that dehydrated cells were placed on carbon stubs prior to critical-point drying. After drying, cells were coated with carbon.

The AMW precipitate used for microanalysis was prepared by adding 3 drops of 30% H_2O_2 to 25 ml of sterile AMW, then heating to 95°C to ensure complete precipitation of soluble metal ions. The precipitate was fixed and dehydrated by the procedure described for SEM, then placed on a nylon grid. Microanalysis was performed in a JEOL 100 CX electron microscope equipped with a Tracor Northern TN 2000 x-ray spectrometer and a Kevex 30-mm^2 detector; determinations were performed at 40 kV.

Ultraviolet absorption. A total of 10 ml of a washed 12-h culture of *E. coli* B/5 (1.2×10^9 CFU/ml) was placed in 90 ml of filter-sterilized AMW. After 1 min, bacteria were removed by filtration through a 0.22-μm filter. The filtrate was placed in a 1-cm quartz cuvette in a Perkin-Elmer Hitachi 124 spectrometer; filter-sterilized AMW was the blank. The absorbance of the sample was scanned from 350 to 180 nm.

Morphometric analyses. Replicas were prepared by the method of Ahmadian et al. [1] and viewed by TEM. Measurements for morphometric analyses were made from replica preparations with a digitizing pad (Micrographics). To calculate cell volume, assumptions were made as to cell shape. Two idealized shapes, an elongated ellipse and a cylinder with semicircles at the ends of the longitudinal axis, were tested to determine which best fit the actual form of the bacteria. For control and treated bacteria, calculated values for the ellipse were significantly different from actual values, whereas values for the cylinder with semicircular ends were not. Therefore, bacterial volume was determined using the equation: $(3L - W)(\pi/12)W^2$, where L = length and W = width.

Lengths and widths of the control populations were normally distributed; lengths and volumes for the AMW-exposed populations were not. The nonnormal distributions necessitated the use of the nonparametric rank sum method [5] to compare mean cell dimensions ($\alpha = 0.05$).

Results and Discussion

Transmission electron microscopy. Control cells exhibited normal morphology (Fig. 1a). Cytoplasm filled the entire cell, was homogeneous in appearance, and contained numerous ribosomes. The envelope of the Gram-negative bacterium appeared unaffected by the fixation procedure. Bacteria exposed to AMW had a markedly altered appearance (Fig. 1b and c). The most obvious feature was the presence of cytoplasmic voids at the cell poles. The outer and cytoplasmic membranes were altered or missing. Every AMW-exposed cell examined sustained visible alterations, ranging from slight changes in envelope morphology to complete loss of cytoplasm.

Two explanations could be advanced to explain the presence of the cytoplasmic voids. Plasmolysis could have occurred, with the voids then forming as the cytoplasm retracted, or cytoplasm could have leaked out of the cell through a damaged envelope. The molarity of AMW was 0.03 *M*, whereas the molarity of *E. coli* cytosol is 0.27 *M* [4, 12]. The solute concentration of the cytosol was ninefold greater than that of AMW; this finding effectively suggests that plasmolysis did not occur and indicates the leakage of cytoplasm.

Replicas. Control cells appeared robust and lacked distinctive surface features (Fig. 2a). AMW-ex-

Fig. 1. Electron micrographs of *Escherichia coli* B/5. (a) Control cell showing intact envelope and homogeneous cytoplasm. (b) Cells exposed to AMW, demonstrating cytoplasmic voids (CV) and membrane disruption. (c) AMW-exposed cell showing a large void (CV) and structural damage to the entire envelope. Bar = 0.5 μm.

Fig. 2. Electron micrographs of replica preparations of *Escherichia coli* B/5. (a) Control cells not exposed to AMW. (b) AMW-damaged bacteria showing surface distortions and amorphous material (arrows) which appears to have leaked out of the cells. (c) AMW-damaged cells demonstrating apparent lysis. Bar = 0.5 μm.

Fig. 3. Scanning electron micrographs of *Escherichia coli* B/5. (a) Control cells showing relatively smooth surface of unexposed bacteria. (b) Bacteria after exposure to AMW, demonstrating cell distortion and the amorphous material (arrows), which was observed only in preparations exposed to AMW. Bar = 0.5 μm.

Fig. 4. Spectra produced by energy-dispersive x-ray spectroscopy. (a) Spectrum of amorphous material associated with AMW-exposed *Escherichia coli*. (b) Spectrum of precipitate from AMW.

posed cells were distorted, often appearing partially or totally collapsed, with chains of globular material adhering to them (Fig. 2b). Cells that appeared to have undergone lysis were also observed (Fig. 2c). Tuttle et al. [17] observed a similar morphology in the acidophilic *T. ferrooxidans* after exposure to organic acids and concluded that this damage was caused by destruction of membrane integrity, which allowed the loss of cellular components.

The number of cells present in 25 random fields was determined for control and AMW-exposed preparations. Though equal numbers of bacteria were used in both preparations, a significant difference ($p < 0.005$) was found between the mean number of exposed cells per field (4.6, SD = 1.3) and control cells per field (17.3, SD = 4.1). On the assumption that all cells in the preparations sedimented during centrifugation, 76% of the AMW-treated cells underwent lysis, which could account for the large amount of amorphous materials in exposed preparations.

UV absorption. The filtrate from AMW-exposed cells absorbed significant amounts of UV radiation at 260 and 280 nm. Absorbance values were converted to concentration of protein (0.105 mg/liter) and nucleic acid (0.002 mg/liter) by calculations according to Warburg and Christian [19].

Scanning electron microscopy. Micrographs of control cells showed the typical rod-shaped morphology of *E. coli* (Fig. 3a). The cell surface had gentle ripples but did not appear significantly altered; bacteria exposed to AMW were folded and distorted (Fig. 3b). Large amounts of an amorphous substance, absent from control populations, were associated with AMW-exposed bacteria.

The elemental compositions of the amorphous material, unexposed cells, and AMW precipitate were analyzed by x-ray spectrometry. Spectra from the AMW precipitate contained peaks for Cu, Os, Cl, and Fe (Fig. 4b). The spectra of the control cells, amorphous substance, and exposed cells were identical (Fig. 4a). These spectra contained small peaks indicating the presence of Cu and P, which were generated by the specimen mount. A significant qualitative difference existed between the composition of the AMW precipitate and the amorphous material, indicating that these materials were different. Quantitation of these substances was not possible because of their globular nature and varied thicknesses. The lack of spectral peaks from the amorphous material suggested that elements making up this material were not present in sufficient quantities to be detected or that it was comprised of elements not detectable by this method of microanalysis (elements with atomic weights <23). If this material was cytoplasmic and composed primarily of C, H, O, and N, then the elements therein would not produce spectral peaks.

Morphometric analyses. AMW-exposed cells varied significantly ($p < 0.001$) from control cells in length, width, and volume. Treated organisms had a greater mean length (3.06 μm) than the control cells (2.48 μm); however, control cells had a greater mean width (1.24 μm) and volume (2.72 μm^3) than did exposed cells (width, 0.93 μm; volume, 1.98 μm^3). It seemed unlikely that the bacteria experienced a 23% length increase following AMW exposure. Rather, this shift represented a selective loss of part of the initial population; such a change would be observed if the shorter cells in the population were lysed. This hypothesis was supported by the observation that lysis and reduced cell numbers were observed in replica preparations.

The data indicated that smaller bacteria were preferentially lysed. Though length cannot be directly related to age, it has been shown that under slow growth conditions older cells are larger than younger ones [3, 11, 20]; this suggests that younger cells would be more sensitive to the effects of AMW. This is not surprising, since stresses such as freezing, starvation, and UV irradiation affect young cells more severely [13]. Physiologically young bacteria might be more prone to lysis because of their greater osmotic pressure. Mitchell and Moyle [12] reported that the osmotic pressure of young Gram-negative cells is 5–6 atm, as opposed to 2–3 atm in older bacteria. Osmotic pressure decreases during elongation but increases during constriction and septation, indicating that older, nondividing cells have less osmotic pressure [16]. If envelope damage were equal for all cells, then young bacteria should lyse more frequently because of their greater internal pressure.

Protons and heavy metals are components of AMW and could promote cellular damage. The proton is highly mobile and could have rapid effects on the cell because it is expected to pass freely through the outer membrane [4]. It was unlikely that the H^+ concentration was high enough to cause hydrolysis of membrane macromolecules; the normality of the acid in the AMW, as determined by NaOH titration, was 0.024, on the assumption that H_2SO_4 was the only acid species present. This suggests that the

initial damage could be due to conformational changes in membrane proteins brought about by the low pH. Lysis might be caused by activation of autolysins by increased H^+ concentrations [12] or from the AMW affecting the maintenance of the energized membrane and this reduced electrochemical potential stimulating autolytic activity [8].

Conclusions

This study showed that *E. coli* exposed to AMW experienced considerable changes in morphology. The bacterial envelope appeared to be affected, since leakage of cytoplasm and lysis often followed exposure to AMW. Morphometric determinations showed that older bacteria were able to withstand the effects of AMW better than younger, smaller cells. These results portray the severe nature of AMW injury and help to explain the increased number of sublethally injured cells and the reduced viability levels observed when *E. coli* are enumerated from streams containing AMW [6, 7].

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