Energy Dispersion X-Ray Analysis by Scanning Electron Microscopy for Measuring Celluar Elemental Composition in Bacterial Cells

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The use of scanning electron microscopy in conjunction with energy dispersion analysis of X rays is presented as a technique in a novel application to in situ studies of cellular elemental composition. The method has been applied, in the example of its use reported here, to examining the effects of copper-chrome-arsenic wood preservative on growth and element uptake of selected bacteria in agar cultures.

Unlike the well-known electron probe microanalyser (see review by A. Läuchli, 1972) energy dispersion analysis in scanning electron microscopy has been used seldom in physiological research. The technique provides a nondestructive means of in situ qualitative and semiquantitative analyses; accurate quantitation of results from biological materials is both difficult and unreliable (J. C. Russ, Proc. 5th Annu. SEM Symp., p. 73–80, 1972; J. C. Russ and M. W. Barnhart, Proc. 6th Int. Congr. X-ray Optics and Microanal., 1971). However, a great advantage of the technique, especially when compared with the electron probe microanalyser, is the precise correlation of element distribution with biological fine structure (2). Furthermore, the low electron beam intensities of the scanning electron microscope avoid over-heating and subsequent migration of elements and destruction of the specimen. In this investigation a Cambridge Instrument Co. Ltd. Stereoscan Mk 2a scanning electron microscope was employed in conjunction with an attachment from Edax International for energy dispersion analysis of X rays, in studying the response of wood-attacking bacteria when growing on a medium containing copper-chrome-arsenic (CCA) preservative. It was hoped to determine whether the preservative components are incorporated by the bacterial cells, and to ascertain the influence of CCA on the movement of other essential elements from the medium to the cell cytoplasm.

Pseudomonas aeruginosa Migula, two strains of Bacillus subtilis Cohn, and Streptococcus salivarius Andrews and Horder were grown in Merck Standard nutrient agar no. 1, containing 0, 0.080, 0.225, and 0.675% CCA. S. salivarius was included in the study since polysaccharides, present in the large mucoid colonies of this bacterium, complex readily with elements such as copper. After 14 days of growth bacterial cells were harvested, washed in deionized water, and dried on aluminium specimen holders. Sterile agar was dried directly onto separate holders. The preparations were coated with carbon by vacuum evaporation, prior to examination and analysis in the scanning electron microscope.

CCA inhibited bacterial growth at the two highest concentrations, although viability tests showed that the preservative was bacteriostatic rather than bactericidal. Growth rates of the spore-forming B. subtilis were much reduced in the presence of 0.225 and 0.675% CCA, whereas characteristic pigment formation by P. aeruginosa was inhibited.

Inhibition of growth may be a result of direct metabolic interference by components of the wood preservative, although the analyses showed a significant difference in elemental composition between bacterial cells growing on nutrient agar alone and the same microorganisms growing on agar containing CCA (Table 1). This suggests that CCA may affect the permeability of cell membranes, or cell transport systems, or both, producing abnormal levels of certain elements within the cell. It is also possible that the presence of CCA would affect the type of ionic compound in the medium, producing more or less motile ionic
Table 1. Comparison of elemental composition of growth media and bacterial cells

| Culture          | CCA concn (%) | Element |
|------------------|---------------|---------|
|                  |               | Na  | P  | S  | Cl | K  | Ca | Cu | Cr | As |
| **B. subtilis no. 1** |               | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0                | 794           | 1,239 | 1,232 | 7,344 | 842 | 4,394 | 0   | 0   | 0   | 0   |
| 0.080            | 700           | 1,317 | 1,122 | 6,407 | 777 | 5,149 | 0   | 277 | 0   | 0   |
| 0.225            | NT*           | NT  | NT  | NT  | NT  | NT  | NT  | NT  | NT  | NT  |
| 0.675            | 541           | 5,154 | 1,712 | 4,438 | 1,980 | 601 | 526 | 2,883 | 1,078 |
| 0.850            | 600           | 6,061 | 610 | 7,295 | 724 | 3,005 | 0   | 0   | 0   | 0   |
| **B. subtilis no. 2** |               | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0                | 702           | 944  | 1,425 | 10,099 | 711 | 3,065 | 0   | 296 | 0   | 0   |
| 0.225            | 693           | 3,044 | 1,210 | 6,061 | 881 | 799 | 118 | 3,618 | 30   |
| 0.675            | 563           | 3,650 | 1,311 | 4,671 | 1,596 | 475 | 415 | 2,783 | 840  |
| **P. aeruginosa** |               | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0                | 1,582         | 6,188 | 1,018 | 8,798 | 351 | 4,589 | 0   | 0   | 0   | 0   |
| 0.080            | 1,139         | 5,486 | 1,286 | 2,314 | 272 | 2,483 | 0   | 650 | 0   | 0   |
| 0.225            | 966           | 3,836 | 1,172 | 9,069 | 1,079 | 1,523 | 130 | 1,577 | 60   |
| 0.675            | 1,785         | 2,381 | 1,228 | 9,753 | 2,978 | 336 | 604 | 2,605 | 227  |
| **S. salivarius** |               | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0                | 1,231         | 4,438 | 929  | 8,385 | 600 | 4,370 | 0   | 0   | 0   | 0   |
| 0.080            | 1,084         | 5,163 | 1,164 | 5,544 | 535 | 5,015 | 0   | 1,029 | 0   | 0   |
| 0.225            | 1,203         | 1,977 | 846  | 10,627 | 1,248 | 468 | 0   | 3,700 | 0   |
| 0.675            | 1,050         | 2,828 | 840  | 4,894 | 1,950 | 392 | 633 | 3,628 | 1,098 |
| **Uninoculated medium** |           | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0                | 1,808         | 832  | 2,097 | 31,110 | 2,813 | 739 | 0   | 0   | 0   |
| 0.080            | 992           | 819  | 2,450 | 24,049 | 3,203 | 745 | 124 | 387 | 12   |
| 0.225            | 1,152         | 771  | 3,127 | 29,797 | 5,938 | 928 | 258 | 1,798 | 71   |
| 0.675            | 1,679         | 792  | 6,283 | 29,025 | 10,648 | 1,369 | 907 | 7,255 | 1,758 |

*a* Counts per 100 s, background corrected. Averages of three measurements.

As the preservative concentrations increased in the medium, Na⁺, Ca²⁺, and Cl⁻ uptake decreased in the two cultures of *B. subtilis*. Phosphorus, however, was accumulated by the cells, and K⁺ levels doubled from growth at 0% CCA to growth at 0.675% CCA. *P. aeruginosa* and *S. salivarius* responded like the bacillus with respect to Ca²⁺ and K⁺, but intracellular Na⁺ and sulphur in these species remained relatively steady on all media, whereas phosphorus decreased with increasing CCA concentrations, and Cl⁻ showed no apparent relationship to preservative presence. Differences in metabolic pathways between *Bacillus* cells and *Pseudomonas* or *Streptococcus* cells probably contribute toward these differences in elemental uptake, especially PO₄³⁻. In the case of the two strains of *B. subtilis*, the levels of phosphorus increased with increasing concentration of CCA (Fig. 1). Since arsenates are known to interfere with oxidative phosphorylation in the living cell (4, 7), it is probable that the bacterial cells when growing in the presence of arsenic accumulate phosphorus as a means of competitive inhibition of the interference mechanism. Da Costa (1) showed that phosphorus acts as an antidote to the effect of arsenic in growth experiments with *B. subtilis*, *P. aeruginosa*, and a number of fungi. The increased K⁺ uptake detected for all cultures when growing on increasing concentrations of CCA may be similarly accounted for; the role of K⁺ in phosphate transfer and ATP hydrolysis has long been known (6). Thus, the effect of arsenic on this vital cycle may not only be countered by increasing PO₄³⁻ levels in the cell, but also by K⁺ ions.

All three bacterial species incorporated chromium from the test media. Intracellular concentrations, at the highest levels, were equivalent to about 2% (0.500 µg) of the medium content. Copper and arsenic were absorbed by the pseudomonad and both strains of *B. subtilis* from 0.225 and 0.675% CCA agar, whereas uptake of these metals by *S. salivarius* was limited to agar containing the highest concentration of CCA. No evidence was found to confirm the affinity for metals of polysaccharides in the slime produced by this bacterium, although levels of copper, chromium, and arsenic were all higher in *S. salivarius* than in cultures of the other bacteria.

A detailed presentation and discussion of this work has been made elsewhere (3), but the summary of results shown here are presented to
Fig. 1. Elemental analysis of B. subtilis cells grown on, (a) 0.080% CCA-containing agar, (b) 0.675% CCA-containing agar. The displays clearly show the difference in cellular elemental composition, especially phosphorus. The unlabeled peak between As and P is A2 from the specimen holder.

illustrate that physiological activities of living cells may be closely followed by in situ examinations by using the scanning electron microscope together with an energy dispersion X-ray analyzer.

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