Copper Toxicity in Erwinia amylovora: An X-Ray Microanalytical Study

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COPPER TOXICITY IN ERWINIA AMYLOVORA: AN X-RAY MICROANALYTICAL STUDY

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Abstract

The effect of Cu\(^{2+}\) ions on the growth and elemental composition of the phytopathogenic bacterium Erwinia amylovora was investigated by in vitro culture in nutrient broth supplemented with CuSO\(_4\). No detectable inhibition in bacterial growth occurred with medium supplemented at 10\(^{-4}\) M Cu (compared to control medium with no added Cu), but partial inhibition occurred at 10\(^{-3}\) M Cu (limiting toxicity) and complete inhibition at 10\(^{-2}\) M Cu. Under conditions of limiting toxicity, incubation in Cu\(^{2+}\) ions leads to a reduction in the general synthesis of cell materials and the formation of abnormally large cells with a small dry mass. X-ray analysis revealed the uptake of small levels of Cu\(^{2+}\) under these conditions, plus wide-ranging changes in other major elements present in the bacterial cells. These changes included a reduction in the level of K and increased levels of the divalent cations Ca and Fe. The results obtained are consistent with the toxic effect of Cu being mediated via its effect on the cell membrane, with internal elemental changes resulting from a perturbation of membrane permeability.

Introduction

Erwinia amylovora is a phytopathogenic bacterium that causes fire blight disease of Rosaceous plants (van der Zwet & Keil, 1979). Effective chemical control of fire blight and certain other bacterial plant diseases is limited at present to the use of antibiotics (particularly streptomycin) and copper-containing compounds (Thind et al., 1984; Wilson et al., 1990). Little is known about the disease-control effect of Cu\(^{2+}\) ions, which may act by inhibiting bacterial infection at a number of points - including bacterial multiplication on the plant surface, entry of bacteria into the tissue or early stages of multiplication and spread within the host.

The effect of toxic concentrations of Cu\(^{2+}\) at the cellular level has received little attention in the case of plant pathogenic bacteria, though studies by Domek et al. (1987) have demonstrated a range of metabolic effects in Escherichia coli. In some situations, the cytoplasmic surface membrane appears to be the target organelle for Cu-mediated toxicity (Kohen & Chevion, 1988).

The major objective of this study was to investigate the effect of toxic levels of copper on the multiplication and elemental composition of Erwinia amylovora in vitro, and to provide information on the mode of action of this cation on cultured cells. The use of X-ray microanalysis in this context follows previous applications of the technique to plant pathogenic bacteria to determine optimal preparation conditions for cultured cells (Sigee et al., 1985), elemental changes during infection and disease development (El-Masry & Sigee, 1989), elemental composition of bacterial DNA (Sigee & El-Masry, 1986) and nickel toxicity in Pseudomonas syringae pv tabaci (Sigee & Al-Rabaei, 1986). A preliminary report on the effect of Cu\(^{2+}\) on elemental composition has been given previously (Sigee et al., 1990).

Key Words: Copper toxicity, X-ray microanalysis, Erwinia amylovora, Plant pathology.

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Materials and Methods

Culture of bacteria.

Erwinia amylovora strain Ea519, originally isolated from hawthorn (Wilson, 1989), was cultured in liquid nutrient broth (Oxoid Ind.) at 23 °C, with intermittent shaking. Cultures were grown for successive cycles of 24h and 16h (late log phase), and the final suspension used to inoculate the test media (with different levels of added Cu²⁺ ions).

Inoculation and sampling of Cu²⁺ media.

2ml aliquots of bacterial suspension were added to 98 ml of control medium (with no added Cu), plus nutrient broth supplemented separately with AnalaR CuCl₂ and CuSO₄ to give final levels of 10⁻⁴, 10⁻³, 10⁻² and 10⁻¹M Cu²⁺. Culture samples were taken at intervals of 0h, 3h, 6h, 9h, 12h and 24h post-inoculation to determine population counts, levels of Cu in the medium and for transmission electron microscopy.

Bacterial population. Counts of bacterial population (total cells/ml) were determined turbidimetrically using an SPB-100 series spectrophotometer, calibrated by reference to counts made with a Helber slide.

Cu levels in medium. Total levels of Cu in the culture medium were monitored throughout the experiment by atomic absorption spectrophotometry of filtered samples.

X-ray microanalysis of cryosections.

Bacterial cells were collected by centrifugation at 3000 XG for 15min, resuspended in distilled water and centrifuged again to form a dense pellet. This was then mounted on a metal stub and plunge-frozen in liquid propane. 80-100nm thick sections were cut at -70°C, mounted on carbon formvar-coated nickel grids, and allowed to freeze-dry overnight in a dry nitrogen atmosphere.

X-ray microanalysis of whole cell preparations.

Preparation of material. Bacterial cells were collected as previously, briefly washed in distilled water, then allowed to sediment on carbon formvar-coated nylon grids for five minutes. Grids were then drained of excess liquid and rapidly air-dried prior to electron microscopy.

Elemental mass fractions. X-ray microanalysis was carried out in a Corinth Analytical microscope (CORA) fitted with a Kevex detector and Link 860 (series 1) analyser. Whole cells were normally examined at a magnification of 20K, accelerating voltage of 60 kV, using an electron probe of similar size and shape to individual bacterial cells, with a beam current of 60nA. X-ray counts were normally obtained over a livetime of 200s, with a count rate from bacterial cells of about 150 cps. Mass fractions were determined using a computer programme based on Hall (1971) thin film quantitation, incorporating spectral information derived from AnalaR microcrystal standards. The accuracy of quantification was verified using metalloprotein thin film standards (El-Masry & Sigee, 1986). For each sample, mean mass fractions were calculated only for those elements that were routinely present (ie. in at least 15 out of 20 spectra). Significant differences between mean levels of elements were statistically assessed using Duncan's multirange test (95% significance level). Correlations between elements within single analyses were determined over 15 spectra by calculation of Pearson correlation coefficients.

Bacterial dry mass. The relative dry mass of bacterial cells was determined as the continuum X-ray count obtained (under standard conditions) during the course of elemental analysis. The continuum level for individual spectra was recorded over the energy range 15-20 keV, with background (adjacent formvar film) subtracted, and a mean corrected continuum obtained for each sample.

Results

Bacterial growth and Cu²⁺ level.

Changes in the population of Erwinia amylovora at different levels of CuSO₄ are determined using a computer programme based on Hall (1971) thin film quantitation, incorporating spectral information derived from AnalaR microcrystal standards. The accuracy of quantification was verified using metalloprotein thin film standards (El-Masry & Sigee, 1986). For each sample, mean mass fractions were calculated only for those elements that were routinely present (ie. in at least 15 out of 20 spectra). Significant differences between mean levels of elements were statistically assessed using Duncan's multirange test (95% significance level). Correlations between elements within single analyses were determined over 15 spectra by calculation of Pearson correlation coefficients.

Fig.1 Growth of bacteria in copper-supplemented nutrient broth.
Copper toxicity in \textit{Erwinia amylovora}

Table 1. Cu levels* in external media

<table>
<thead>
<tr>
<th></th>
<th>0h</th>
<th>3h</th>
<th>6h</th>
<th>9h</th>
<th>12h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (X 10^{-7})</td>
<td>7</td>
<td>16</td>
<td>13</td>
<td>7</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>10^{-4}M (X 10^{-4})</td>
<td>1.7</td>
<td>2.1</td>
<td>1.8</td>
<td>1.5</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>10^{-3}M (X 10^{-3})</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* expressed as molarity (M)

Fig.2 Transmission electron microscopy of cryosections of control bacteria (no added Cu).

Fig.2a. Freeze-dried cryosection of group of bacterial cells, showing external polysaccharide (P), cell wall complex (W) and internal protoplasm (R). Scale bar 1µm.

Fig.2b. X-ray emission spectra from cryosection, using a 0.1um-diameter probe. The Ni peak is derived from the grid bars.

shown in Fig.1. Closely similar changes occurred in nutrient broth cultures supplemented with CuCl$_2$, consistent with Cu$^{2+}$ being the toxic moiety.

Bacteria grown in nutrient broth control medium (without added Cu$^{2+}$) showed the typical sigmoid curve of batch-cultured organisms, with a log phase from approximately 3-12h after inoculation of medium. Cells grown in 10$^{-4}$M Cu showed no significant difference from the control. Major inhibition of growth occurred at 10$^{-3}$M Cu, with only a limited population increase up to 9h, followed by a marked decline. At 10$^{-2}$ and 10$^{-1}$M Cu, no significant population increase could be detected.

Atomic absorption determination of Cu levels in each medium throughout the course of the experiment (Table 1) showed no significant change in the level of cation, indicating that uptake by bacterial cells had no substantial effect on the external level of copper at either high or low external concentration.

Electron microscopy of cryosections is shown in Fig.2a and reveals three main specimen areas - extracellular polysaccharide, electron-dense cell wall complex (including the capsule) and internal protoplasm.

Characteristic X-ray emission spectra from cryosections of control cells (no added Cu) are shown in Fig.2b. In each
Typical Transmission electron microscope appearance of whole cells of *Erwinia amylovora*, 3h after inoculation in $10^{-3}$M Cu medium (Fig.3a), Control medium (Fig.3b) and $10^{-4}$M Cu medium (Fig.3c). Cells in Fig.3a and 3b are in late and mid-division respectively. The characteristically larger size of cells grown in $10^{-3}$M Cu is apparent. C - capsule. Scale bar - 2µm.

In most cases, the amount of bacterial matter within the probe area was small, and both continuum and specimen peaks were correspondingly small. The emission spectra reveal the presence of Na, P, Cl plus Ca within the wall complex and Na, P, Cl plus K in the internal protoplasm. No clear elemental peaks were observed in the extracellular polysaccharide. Preliminary investigation of Cu-treated cells revealed the presence of similar elements to those of control samples, with no clear presence of Cu yet detected. Quantitative assessment of elemental levels was not carried out with either control or copper-treated cells due to the small size and variability of the characteristic peaks.

Electron microscopy of whole bacterial cells.

The characteristic appearance of whole cell preparations viewed by transmission electron microscopy is seen in Fig.3. Bacterial cells typically appeared as electron-dense rod-shaped structures, surrounded by a thin peripheral zone of capsular material. Variation between cells was noted in terms of state of division, presence/absence of flagella and cell size.

The volume ($V$) of individual cells was estimated on the basis that they approximated to cylinders with hemispherical ends.

$$V = \pi (d/2)^2 \times (l-d) + 4/3 \pi (d/2)^3 ... (1)$$

where: $l$ = length of bacterium
$d$ = diameter.

Changes in mean cell volumes presented in Fig.4, and reveal major differences for bacteria cultured under...
Copper toxicity in *Erwinia amylovora*

toxic (growth-limiting) conditions.

Cells grown in nutrient broth control medium varied in mean size from approximately 0.4-0.8 µm³, with no significant changes during the course of the experiment. Mean volumes of cells grown at 10⁻⁴M Cu were approximately in the same range as control cells, and showed a significant decrease during log phase. At 10⁻³M Cu, bacteria were significantly larger than control and 10⁻⁴M Cu cells over most of the time course, increasing markedly in size up to 9h after inoculation, followed by a sharp decline to a constant level.

**X-ray emission spectra.**

Representative emission spectra, taken from whole bacterial cells, are shown in Figs. 5 & 6. Throughout the course of the experiment, cells grown in unsupplemented nutrient broth had qualitatively similar spectra to cells grown in 10⁻⁴M Cu, with the routine presence of major peaks of Na, P, Cl and K. The presence of small levels of Mg, S, Ca, Fe and Cu was also occasionally detected. Background spectra from formvar film adjacent to bacterial cells had occasional small extraneous peaks of Si, Al and Fe.

Cells grown in 10⁻³M Cu showed a clear spectral difference from control samples at 9h (Fig.5), with a much reduced continuum level, barely detectable peaks of Na, Cl and K, and the clear presence of Ca, Fe and Cu. Similar spectra were observed at 12h and 24h (Fig.6) after inoculation.

**Elemental mass fractions**

Changes in the total cell level (mean mass fraction) of routinely-detected elements in control, 10⁻⁴M and 10⁻³M cultures are shown in figures 7-11.

**Sodium.** The level of sodium varied considerably in relation to the growth phase of the bacterium (Fig.7). In control samples, there was a sharp initial rise, reaching a mass fraction of about 400 µmol/g during early log phase, with a rapid decline at about 9h after inoculation. Similar trends occurred at 10⁻⁴M and 10⁻³M Cu.

**Phosphorus.** In control samples, the phosphorus mass fraction varied from about 100-450 µmol/g with a significant rise during the log phase and a significant fall during the stationary phase (Fig.8). Cells cultured in 10⁻⁴M Cu showed closely similar changes, while growth in 10⁻³M Cu led to oscillations in the level of P, reaching a mass fraction of 450 µmol/g at 9h after inoculation.

**Sulphur.** Although small peaks of sulphur were frequently observed, this element was close to the limits of detectability of the technique. Highest levels were recorded at 24h in the control (mean mass fraction 23 µmol/g) and 10⁻³M Cu (27 µmol/g) samples.

**Chlorine.** This major anion varied in level from about 200-1200 µmol/g (Fig.9) and showed a close correspondence with the level of Na in all three treatments. In each case, the mean mass fraction of chlorine was at an optimum level during the log phase of growth.

**Potassium.** The occurrence of potassium in control and 10⁻⁴M Cu cultures showed a close correspondence, with a major peak between 6-12h (reaching 200µmol/g) and a second increase to 24h (Fig.10) in 10⁻³M Cu cultures, the mass fraction of potassium showed a sharp fall during the first few hours of culture, remaining at about 20 µmol/g for most of the experiment.

**Calcium.** In control cells, this element was routinely detected only in 12h and 24h cultures, where it was highly
Fig. 6 X-ray emission spectra from 24h samples. Spectra are from air-dried, whole-cell preparations.

Fig. 7 Changes in the mass fraction of sodium. Mean mass fractions (derived from at least 15 separate cells) are shown, with 95% confidence limits. Pairs of samples significantly different using the Duncan multiple range test (see Fig. 4) were:
- Control - 2(1,5,6); 3(1,5,6); 4(5,6)
- 10^-4M Cu - 4(1,2,5,6)
- 10^-3M Cu - 3(1,5,6); 6(2,3)

Fig. 8 Changes in the mass fraction of phosphorus. Legend as Fig. 7. Duncan multiple range test:
- Control - 2(1); 3(1,6); 4(1,6); 5(1,6)
- 10^-4M Cu - 3(1,2); 4(1,2); 5(1); 6(1)
- 10^-3M Cu - 2(1,3,5); 4(1,3,5); 6(1,3,5)

variable, and present at mean levels of about 25µmol/g. A similar pattern occurred in cells grown at 10^-4M Cu, with no routinely detectable calcium until the late log (12h) and stationary phase (24h) cultures. Cells grown at 10^-3M Cu showed no clear peaks of calcium until the 9h sample, when it had a mean mass fraction of about 100 µmol/g with a significant rise after 12h during stationary phase (Fig. 11).

Iron. In control preparations, iron was occasionally detected as a small peak in bacterial spectra but was not routinely present. Its occasional presence in background (formvar spectra) indicated a small extraneous contribution. Routine peaks were also absent from most of the samples grown in Cu-supplemented media, but significant levels of Fe were detected at 24h in the 10^-4M Cu sample (40µmol/g) and at 9h to 24h in the 10^-3M Cu sample (34-50µmol/g).

Copper. Although small copper peaks were occasionally observed, this element was not routinely detected in any of the control samples or in any samples taken from the 10^-4M Cu medium. Cells cultured in 10^-3M Cu did not show clear presence of
Copper toxicity in Erwinia amylovora

Changes in the mass fraction of chlorine. Legend as Fig. 7. Duncan multiple range test:
Control - 2(1,5,6); 3(1,5,6); 4(1,5,6)
$10^{-4}$M Cu - 3(1,2,5,6); 4(1,2,4,5,6)
$10^{-3}$M Cu - 3(1,4,5,6); 2(1,4,5,6)

This element until 9h after inoculation, when a Cu peak was present in all spectra, with a mean mass fraction of approximately 40 µmol/g - rising to 90 µmol/g at 24h (Fig. 11).

Bacterial dry mass.

Changes in the mean background corrected continuum of bacterial cells cultured in different media are shown in Fig. 12. Since the continuum level in an emission spectrum relates, under constant conditions of beam current and livetime, to the total mass of material within the probe area, these changes reflect changes in bacterial dry mass during the culture period.

In both control and $10^{-4}$M Cu-cultured cells, bacterial dry mass initially showed a significant increase during the lag phase, reaching a maximum level at about 3h after inoculation of the medium. This was followed by a sharp and significant fall during the early part of the log phase (to 9h), continuing at a low level for the rest of the culture period. Cells cultured in $10^{-4}$M Cu showed a further significant decrease in the 24h sample.

At $10^{-3}$M Cu, the high degree of variability in continuum level within samples made assessment of general trends difficult. The low level at 9h after inoculation was statistically significant, however, indicating that bacterial mean dry mass reached a minimum value at this time.

Elemental correlations

Correlations between elemental levels within the 15 individual analyses from each sample provide useful information on...
elemental associations within the bacterial cells, and are shown for control, $10^{-4}$M Cu and $10^{-3}$M Cu media in Table 2.

The majority of the correlations seen in Table 2 are positive. The results obtained for control and $10^{-4}$M Cu media are broadly similar and reveal major correlations between the presence of certain cations and anions (Na-Cl, K-P, and Na-P) but not others (eg. K-Cl). Correlations also occur between cations (Na-Ca, Na-K) and anions (P-Cl). Phosphorus is assumed to occur mainly as the anion $PO_4^{3-}$.

Cells grown at toxic levels of copper differed in showing no correlations between Na-Cl, and correlation between Na-P and K-P only occurred in the first two samples. Positive correlations between

Table 2. Main elemental correlations for bacteria grown in control and Cu-supplemented media.

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<th>Na-P</th>
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<th>K-P</th>
<th>Ca-P</th>
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<th>Ca-S</th>
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<td><strong>$10^{-3}$M Cu</strong></td>
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</tr>
</tbody>
</table>

Correlations are indicated (+ or -) where the correlation coefficient is 0.50-1.00.

Other correlations not included in the table were:
Control: 0h Na-S(-); P-S(+); Cl-S(-); K-Ca(-) 9h K-Cl(-) 
$10^{-4}$M Cu: 12h P-S(+); 24h Na-S(+), K-Cl(+) 
$10^{-3}$M Cu: 6h K-Ca(+), S-Cl(+); 9h Cu-Cl(+) 

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Ca-P and Cu-Cl occurred over the 9h-24h period.

Discussion

The results obtained in this study provide information on in vitro aspects of Cu toxicity in Erwinia amylovora, with specific reference to the effects of the cation on bacterial growth and elemental composition.

Bacterial growth.

The toxic effect of Cu²⁺ ions on both prokaryote and eukaryote cells has been widely documented. In the case of bacterial cells, it has been demonstrated for organisms as diverse as soil microflora (Maliszewska et al., 1985), halobacteria (Nieto et al., 1987) and aerial plant pathogens (Thind et al., 1984). In this study, the toxic effects of Cu²⁺ ions (as CuSO₄ or CuCl₂) on bacterial growth is shown by their effect on population increase and also by changes in bacterial size and dry mass.

Population changes. Addition of Cu²⁺ caused complete inhibition of population increase at levels of 10⁻³M and above, partial inhibition at 10⁻⁴M Cu and no inhibition at all at 10⁻⁵M Cu over a 24h period. These results parallel previous studies (Sigee & Al-Rabae, 1986) on the toxic effect of Ni²⁺ on Pseudomonas syringae pv tabaci — where partial inhibition also occurred at the 10⁻³M cation level. Although the use of nutrient broth as growth medium has the disadvantage that a substantial part of the added Cu²⁺ will be complexed with organic constituents (Zevenhuizen et al., 1979), this medium was chosen since it supports active growth of the bacterium and organic (complexing) compounds would normally be present if cells were treated with Cu-containing bactericide in the field. The use of 10⁻³M Cu/nutrient broth as the growth-limiting medium does not therefore reflect the full toxic potency of Cu²⁺ cations, but does allow a study of the effects of Cu²⁺ under active growth conditions at the borderline of toxicity.

Bacterial size and dry mass. The different phases of population change reflect differences in the balance between synthesis of cell constituents and cell division — as indicated by changes in the mean size and dry mass of bacterial cells during batch culture.

Changes in these parameters in control and 10⁻³M Cu cultures were closely similar, and resemble the changes noted previously in another phytopathogen - Pseudomonas syringae pv tabaci (El-Masry & Sigee, 1989; Sigee & El-Masry, 1989). In both cases, the lag phase - which is a period of increasing synthetic activity without cell division - involves an increase in mean size and dry mass. During the subsequent log phase, cell division outpaces synthetic activity, and mean bacterial size and mass show a sharp decline. The stationary phase is characterised by an equilibrium between synthesis and cell division, with little change in either mean size or dry mass of the cells.

At 10⁻³M Cu, the limited population increase up to 9h after inoculation is accompanied by completely opposite changes in cell size and dry mass — with cells becoming very large but having a minimal continuum level. These opposing changes are difficult to explain, but might result, for example, under conditions of limited cell division if there was an inhibition by Cu²⁺ of macromolecule synthesis (decreased dry mass) with a change in osmotic balance (leading to increased cell size). The latter might arise due to increased osmotic potential (though the level of K⁺ is low in these cells) or to a change in cell wall properties, and will be the subject of future study.

Elemental composition of cryosections

Analysis of cryosections provides useful information on the general partitioning of elements within control cells, confirming that cations were present mainly in the main body of the cell, with only low (non-detectable) levels in the extracellular polysaccharide. The detection of K⁺ in the protoplasm but not the cell wall complex suggests that this cation is largely present internal to the cell surface inner membrane, damage to which would cause leakage of the cation out of the cell.

Elemental composition of whole cells.

X-ray microanalysis of whole cells provides quantitative information on the specific uptake of Cu and also the wider elemental changes that occur in copper-treated cells. These events are considered in terms of alterations in elemental mass fractions and correlation coefficients.

Elemental mass fractions. The effect of toxic levels of Cu on the elemental composition of the bacterial cells is complex, and is superimposed on endogenous changes that occur as a natural part of the transition from one growth phase to the next during batch culture.

During the first 6h of culture, 10⁻³M Cu appears to have little overall effect on elemental mass fractions. The initial increase of phosphorus, for example, occurs in all treatments and is consistent with a major synthesis of nucleic acids, of which this element is a major structural component. A similar rise occurs in the level of sulphur, possibly relating to an increase in the level of sulphur-containing proteins.

The major soluble cations, sodium and potassium, exhibit very distinct patterns.
of change. In control cells, there is an immediate increase in the level of sodium, followed (6h later) by a rise in the level of potassium. These two waves of activity reflect major influxes of the cations at different stages of the batch culture, and are partially balanced by an influx of chloride over the initial 12h period. Treatment of the bacterial cells with 10^-3M Cu has no effect on the initial uptake of sodium, but the uptake and/or retention of potassium is completely inhibited. The results suggest that the Cu^2+ cations either block the active uptake of potassium, or cause a large scale leakage from the cell, or both. A Cu^2+-induced leakage of K^+ by damage to the cell membrane would parallel the activity of other agents known to have this mode of action, including phenol (Kroll & Anagnostopoulos, 1981), phenethyl alcohol (Silva et al., 1976), chlorhexidine (Hugo & Longworth, 1966) and local anaesthetics (Silva et al., 1979).

Further indication that Cu^2+ may be acting primarily at the cell surface rather than as a result of internal accumulation is provided by the Cu mass fraction data, since Cu was not detected at significant levels until 9h after inoculation - when its effect on bacterial growth were already apparent.

The high internal levels of Cu from 9h onwards may result due to passive influx through a damaged (leaky) surface membrane, or from an increased rate in a specific transport mechanism. Studies by Mokhele et al. (1987) have shown the presence of a surface protein (NosA) in Pseudomonas putrefaciens which is implicated in Cu transport, and a similar situation may occur in Erwinia amylovora. Uptake of Cu under conditions of copper-toxicity has also been reported in the filamentous bacterium Sphaerotilus natans (Slowik, 1981), where the bioaccumulation of the metal depended both on the external concentrations and the chemical associations of the cation. The high levels of Fe and Ca detected in parallel with Cu accumulation in Erwinia amylovora suggest an additional influx of nontoxic divalent cations - possibly by a similar route.

Elemental correlations. Although some information on elemental distribution can be readily obtained from the cryosection specimens, this is not the case for whole cell preparations, where the cellular location of elements is not directly determined. Some indication of the location and spatial associations of elements in these preparations may, however, be obtained by determination of correlations in occurrence. Positive correlations would be expected to occur either if the elements concerned are chemically associated, or if the elements occupy a similar site in a non-competitive manner. Negative correlations would be expected where single-site occupation is competitive.

The results obtained show broadly similar types of correlation in the control and 10^-4M Cu treatments, indicating that Cu^2+ has little effect on elemental associations at the 10^-4M level. Anion/cation associations appear to fall into three major groups -

(1) K-P (and to a lesser extent Na-P). Since phosphorus in bacterial cells is largely present in nucleic acids, this is consistent with K^+ being the major cation associated with these macromolecules, and is in agreement with previous X-ray microanalytical studies on isolated bacterial DNA (Sigee & El-Masry, 1988).

(2) Na-Cl. Probably associated within the cell in free ionic form, and present at high level in the external medium.

(3) Calcium associations - mainly as Ca-Cl and Ca-S (probably with sulphur containing proteins).

The elemental associations found in Erwinia amylovora are similar to those of Pseudomonas syringae (El-Masry & Sigee, 1989). It is interesting that in both cases, certain correlations, such as K-Ca, hardly ever occur.

Treatment with 10^-3M Cu affects ion associations partly by altering their presence/absence within the cell, with increased levels of Ca, Fe, Cu and decreased levels of K. It may also affect their interactions within the cell, and although Na and Cl are present at high overall levels in early stages of batch culture (similar to control and 10^-4M Cu cells) there is no correlation between the two.

General Conclusions

The in vitro studies reported here on Erwinia amylovora show that, in the presence of nutrient growth medium, Cu^2+ is at the limits of toxicity at a concentration of about 10^-3M. Treatment of bacterial cells with this level of cation leads to early changes in cell division and synthetic activity, plus subsequent wide-ranging effects on bacterial elemental composition - including reduced levels of K, and increased uptake of Ca, Fe and Cu.

References


El-Masry MH, Sigee DC (1989). Electron probe X-ray microanalysis of Pseudomonas syringae pv. tabaci isolated...
Copper toxicity in *Erwinia amylovora*


Discussion with Reviewers

J.Mansfield: Have the authors attempted to obtain Cu resistant mutants of *E. amylovora*?
Authors: Yes, we have attempted to isolate colonies growing long term on limiting concentrations of Cu, but so far without success.

J.Mansfield: Is the microanalytical technique sufficiently sensitive to detect the very low levels of intracellular Cu2+ that may have general toxicity?
Authors: The microanalytical technique will detect elemental levels down to about 0.1% on a mass fraction basis, and is therefore not sufficiently sensitive to detect the low levels of Cu2+ in whole cells that may be important during the early stages of Cu toxicity. The effective sensitivity of the technique may, however, be increased by analysis of extracted target molecules - such as protein and nucleic acids (see Sigee & El-Masry, 1988).

L.Sicko-Goad: What is the effective concentration of Cu from copper-containing compounds used to treat fireblight disease? Are these concentrations comparable to the range you used in your experiments?
Authors: The effective range of compounds used in the treatment of fireblight in the field would certainly range from complete toxicity (direct application of control compound) to subtoxicity (dispersed application and rain dilution), and would therefore parallel the range of...
concentrations used in these experiments.

L. Sicko-Goad: Do you have evidence from thin-sectioned material of partially-formed walls or unusual divisions (which would suggest that intracellular sites are involved in the toxicity)?

Authors: There is no evidence of either of these features in cryosectioned cells, and we have not examined ultrathin sections of fixed material.

J.A. Nott: Would an interpretation of the effects of copper noted in this paper be assisted by an analysis of the liquid nutrient broth to determine the speciation of copper at the different concentrations?

Authors: Determination of Cu speciation and complexing with organic compounds in the growth medium would certainly provide useful information on the external parameters involved in toxicity, and might also provide a valuable insight on how the cation is interacting with the bacterium at the cell surface.

A.G. Sangster: Specimens were centrifuged, washed in water and air-dried on the carbon-formvar grids. The latter technique especially could contribute to the redistribution of free ionic forms and possibly to the loss of some fraction of these from the cells. What bearing would this migration have upon the elemental associations of mobile forms and upon their original presence/absence within the cells as discussed under elemental correlations?

Authors: Any cell preparation technique is liable to cause some cation migration, and this should always be taken into account in considering the results obtained. In the case of air-dry preparations, it seems likely that this effect will be minimal, since the procedure is sufficiently brief to avoid any chronic alteration in cell physiology (that might lead to ion leakage), and no direct damage is caused to the cell wall/cell membranes while free water is present. Analysis of areas of formvar close to bacterial cells do not give any indication of the presence of cations, suggesting that any cation loss that might occur is minimal.

A.G. Sangster: It is stated in the Introduction that little is known regarding the in vivo action of Cu²⁺ in controlling fire blight, whether at the surface or within the host plant tissues. Could the authors comment on to how the results of their in vitro study might contribute to the elucidation of the inhibition mechanisms occurring under field conditions?

Authors: The growth (and inhibition) of bacteria in the field occurs on general plant aerial surfaces and also at specific sites of infection, particularly the flower stigmatic and nectary surfaces. The latter are typically wet, with high levels of nutrient, and it is these sites in particular which will most closely relate to the in vitro laboratory experiments and the observed levels of Cu toxicity.

A.R. Spurr: Since the electron beam had to be diverged in order to match the increased size of the bacteria when grown at 10⁻³ M Cu it doesn't seem as though the X-ray counts for Cu would be strictly comparable with the determinations made on the control and those cells grown at 10⁻³ M Cu. Also, how could a rounded diverged beam match the shape of an oval or elongated bacterial cell? The readings would seem more comparable if the beam size and setup conditions were held constant and several spot readings at comparable sites had been taken.

Authors: The X-ray counts relate to the mass of the specimen rather than the size of the probe area and will not in any case affect the calculation of elemental mass fractions since this depends on the peak/continuum ratio. Differences in probe size at different cell sizes will not therefore affect the assessment of Cu levels.

A rounded diverged beam can be adjusted to match the shape of a single bacterial cell quite well. Although this is never an exact match, lateral sensitisation of the specimen beyond the immediate probe area will ensure that all of the bacterial cell is involved in the emission of X-rays and that the whole cell contributes to the emission spectrum.