Plasmolysis induced by very low concentrations of Cu^{2+} in *Pseudomonas syringae* ATCC 12271, and its relation with cation fluxes

JOÃO P. S. CABRAL

INIC, Centro de Citologia Experimental da Universidade do Porto, Rua do Campo Alegre 823, 4100 Porto, Portugal

(Received 17 April 1990; revised 2 August 1990; accepted 13 August 1990)

Very low concentrations of CuSO_4 induced a massive leakage of K^+ from *Pseudomonas syringae* ATCC 12271 cells suspended in distilled water, Ca^{2+} /PIPES or Na^+ /PIPES. Cell suspensions in distilled water, Ca^{2+} / or Mg^{2+} /PIPES, treated with Cu^{2+} , showed appreciable increases in optical density, whereas suspensions in Na^+ /PIPES were unaffected. The addition of monovalent cations to suspensions in distilled water, Ca^{2+} / or Mg^{2+} /PIPES prevented the optical density increase induced by Cu^{2+} , whereas the addition of Ca^{2+} or Mg^{2+} to suspensions in distilled water did not have this effect. Cells suspended in Na^+ /PIPES and treated with Cu^{2+} showed no major ultrastructural alterations, but cells treated with Cu^{2+} in distilled water showed pronounced plasmolysis. At all Cu^{2+} concentrations, two types of cells were observed, normal and heavily plasmolysed. An increase in Cu^{2+} concentration resulted in an increase in the percentage, but not in the degree of plasmolysis, of the plasmolysed cells. Cells suspended in distilled water or Na^+ /PIPES bound significant amounts of copper. Cu^{2+} concentrations that induced leakage of most of the unbound K^+ did not saturate the copper-binding sites in the cells. These results indicated that plasmolysis is a direct consequence of the massive K^+ leakage from the cells, in agreement with the notion that K^+ is the main osmolyte in bacteria grown under normal conditions. Monovalent (but not divalent) cations prevented plasmolysis induced by copper by entry into the cells after the release of K^+ ions. Na^+ and Li^+ probably replaced resident K^+ ions in the neutralization of negative charges of cytoplasmic constituents. K^+ efflux and plasmolysis, induced by Cu^{2+} , appeared to be essentially 'all-or-nothing' effects.

Introduction

Pseudomonas syringae van Hall is one of the most important plant pathogens, having a very wide host range, and causing spots and necrosis of leaves, twigs and fruits, and stem cankers (Bradbury, 1986). Copper compounds have been used for centuries in the control of diseases of plants (Horsfall, 1956), and are effective in the control of several diseases caused by *P. syringae*, namely, the halo blight of beans (Tamiotti & Garibaldi, 1984) and the bacterial speck of tomato (Colin & Chafik, 1986; Colin & McCarter, 1983). The mechanism of action of Cu^{2+} as an antibacterial agent is not fully understood. Cu^{2+} ions can inhibit several enzymes and metabolic pathways in bacterial cells (Kleiner, 1978; Green *et al.*, 1985; Domek *et al.*, 1987; Lowe *et al.*, 1987), and in *P. syringae* they can induce leakage of K^+ and low-molecular-mass metabolites, and cause cell death (Cabral, 1989, 1990).

K^+ is the dominant free cation in the cytoplasm of bacterial cells. In non-halophilic bacteria, the intracellular K^+ concentration usually ranges from 0.2 to 0.6 M

(Christian & Waltho, 1962; Dinnbier *et al.*, 1988). K^+ ions are required for normal protein synthesis (Lubin & Ennis, 1964), optimum function of several enzyme systems (Klenow & Henningsen, 1969), and regulation of cytoplasmic pH (Booth, 1985). K^+ ions also play a key role in the osmoregulation of bacterial cells. For example, when grown in the presence of sodium chloride, non-halophilic bacteria counteract the osmotic pressure of the medium by accumulating substantial amounts of K^+ (Christian & Waltho, 1961). Halophiles show massive K^+ accumulation, reaching intracellular concentrations as high as 4.6 M (Christian & Waltho, 1962). In osmotically-stressed Gram-negative cells, the internal K^+ concentration is directly proportional to the osmolarity of the medium (Meury *et al.*, 1985), and Gram-negatives, stressed by osmotic shock, can accumulate high concentrations of glutamic acid. This process is intimately related to the uptake of K^+ since the enzyme generally involved in the synthesis of this amino acid is stimulated up to tenfold by high concentrations of K^+ (Measures, 1975). However, since very high concentrations of K^+ are potentially deleterious to enzyme function

(except in halobacteria), bacteria that are under osmotic stress accumulate other 'compatible solutes' such as proline, betaine and trehalose (Measures, 1975; Yancey *et al.*, 1982; Larsen *et al.*, 1987; Dinnbier *et al.*, 1988).

Since K^+ is generally the major osmoactive ion, its rapid depletion from cells should be followed by a contraction of the cytoplasmic membrane and plasmolysis. This phenomenon was reported by Thompson *et al.* (1970) in a marine pseudomonad suspended in a diluted solution of magnesium sulphate. In the present work, which was aimed at obtaining a better understanding of the mode of action of Cu^{2+} as an antibacterial agent, and of the roles of K^+ in bacterial physiology, the same phenomenon was shown to occur when *P. syringae* cells are treated with low concentrations of copper sulphate. A preliminary report of this work has been presented (Cabral & Silva, 1984).

Methods

Culture and cell suspensions. *Pseudomonas syringae* ATCC 12271 was grown in a medium containing (per litre of distilled water): 0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4 , 0.5 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract (Difco) and 5 g glycerol (pH 6.2). Cultures (100 ml) in 250 ml Erlenmeyer flasks were incubated with shaking at 27 °C. At the end of the exponential phase (corresponding to $1-3 \times 10^9$ c.f.u. ml⁻¹), cells were harvested by centrifugation, and washed and suspended in one of the following media: distilled water (pH 6.5); 2 mM- or 10 mM- Na^+ , Ca^{2+} or Mg^{2+} /PIPES buffer (pH adjusted to 6.5 with NaOH, $Ca(OH)_2$ or MgO, respectively). The concentration of Na^+ , Ca^{2+} and Mg^{2+} in 2 mM-PIPES buffer was, respectively, 2.7, 1.8 and 1.7 mM. The OD_{568} was adjusted to 1.06, using a Lambda 3 Perkin-Elmer or a Shimadzu double-beam spectrophotometer with 1 cm light-path cuvettes. In all experiments this cell suspension was diluted 9:10, and the final suspensions had a concentration of approximately 0.28 mg dry weight of cells ml⁻¹ ($\approx 10^9$ c.f.u. ml⁻¹).

Electron microscopy. Cell suspensions in distilled water or in 10 mM- Na^+ /PIPES buffer were treated as described below. At intervals, samples were withdrawn and fixed in glutaraldehyde (TAAB, London, UK; final concentration 2.5%, v/v) for 1 d. Dehydration was performed by washing the samples in increasingly concentrated aqueous ethanol, followed by four washings in absolute ethanol. Samples were embedded in Epon resin (TAAB) with infiltration in propylene oxide. Ultrathin sections were cut, and stained with uranyl acetate followed by lead citrate. Observations were carried out in a Siemens Elmiskop IA electron microscope, working at 80 kV. To quantify cell plasmolysis, the following procedure was used: cell suspensions were treated with $CuSO_4$ concentrations ranging from 5 to 40 μM , at room temperature (22–23 °C). After 1.5 h, samples were withdrawn and processed for electron microscopy as described above. Micrographs taken at a magnification 8000 $\times$ were enlarged 3 $\times$, and the percentage of plasmolysed cells for each copper concentration was calculated after counting 500–2000 cells. For each copper concentration, 50 plasmolysed cells were chosen at random, and for each cell, a plasmolysis index was determined planimetrically as the ratio of the weight of the photomicrograph bearing the image of the plasmolysed region to the weight of paper bearing the image of the whole cell. The mean plasmolysis index was calculated by averaging the plasmolysis index of 50 cells examined.

Spectrophotometry. Plasmolysis of the cells was assessed by measuring the change in OD_{568} of suspensions treated with $CuSO_4$ (5–50 μM), at room temperature, using a Lambda 3 Perkin-Elmer or a Shimadzu double-beam spectrophotometer. Cell suspension (3 ml) was placed in each of two glass cuvettes (1 cm light path). $CuSO_4$ solution was rapidly mixed with one of the suspensions and after allowing 15 s for equilibration, the OD_{568} of the treated suspension was read, at intervals, against the untreated suspension.

K^+ leakage experiments. Cell suspensions were treated with $CuSO_4$ concentrations ranging from 2.5 to 40 μM at room temperature. At intervals, the suspensions were filtered through a 0.45 μm pore filter (Sartorius), and potassium was assayed in the filtrate by flame photometry using a Corning flame photometer.

Copper binding experiments. Cell suspensions in distilled water and in 10 mM- Na^+ /PIPES buffer were treated with $CuSO_4$ concentrations ranging from 10 to 100 μM at room temperature. After 15 min, cells were harvested by centrifugation, washed with the appropriate suspending medium, and re-centrifuged. The pellet was treated with diluted HNO_3 (20%, v/v) and autoclaved at 121 °C for 20 min. Copper was assayed by atomic absorption spectrophotometry, using a Varian 1250 spectrophotometer with an air-acetylene flame, working at 324.7 nm.

Results

K^+ efflux

In distilled water, or 2 mM- Ca^{2+} or Na^+ /PIPES buffer, very low concentrations of $CuSO_4$ induced a massive leakage of K^+ from the cells. In these suspension media, the course of K^+ efflux and the amount of K^+ released after 15 min of copper treatment followed a sigmoidal pattern (Fig. 1) similar to that in 10 mM- Na^+ /PIPES recently described (Cabral, 1989, 1990).

The total concentration of 'pool' K^+ in untreated cell suspensions was estimated to be about 80 μM [0.286 μmol K^+ (mg dry weight)⁻¹ (Cabral, 1990)]. Therefore, treatment of the cells suspended in various media with 30–40 μM - Cu^{2+} for 15 min resulted in the release of most or all the intracellular unbound K^+ .

The release of K^+ from the cells after addition of $CuSO_4$ was considered to be a direct consequence of the action of added Cu^{2+} ions, since the background concentration of copper in the suspending media was negligible [below the detection limit of the Varian atomic absorption spectrophotometer (0.5 μM)] and treatment of the cells with various metal sulphates did not cause K^+ efflux (Cabral, 1989).

Spectrophotometry

Since K^+ is generally the major osmoactive ion in bacteria, its rapid depletion from the cells was expected to be followed by a contraction of the cytoplasm, with a concomitant increase in the optical density of the treated suspension.

Fig. 1. K^+ released after 15 min of Cu^{2+} treatment, plotted against Cu^{2+} concentration. K^+ was assayed by flame photometry in the supernatant of treated suspensions. Cells were suspended in 2 mM- Na^+ /PIPES (■), 2 mM- Ca^{2+} /PIPES (●), or distilled water (○). Mean number of determinations per copper concentration and mean coefficient of variation: 2 mM- Na^+ /PIPES 2.0, 14.3%; 2 mM- Ca^{2+} /PIPES 1.6, 6.0%; distilled water 1.9, 16.6%.

Fig. 2. Time course of increase in OD_{568} in cell suspensions treated with $50 \mu\text{M-Cu}^{2+}$. The OD_{568} was measured against an untreated suspension. Cells were suspended in 10 mM- Na^+ /PIPES (□), 2 mM- Na^+ /PIPES (■), 2 mM- Ca^{2+} /PIPES (●), 2 mM- Mg^{2+} /PIPES (△), or distilled water (○). Results are from one experiment.

To check this hypothesis, cell suspensions were treated with CuSO_4 and the OD_{568} monitored as described in Methods. Cu^{2+} treatment of cells suspended in distilled water, Ca^{2+} / or Mg^{2+} /PIPES buffer indeed resulted in an increase in OD_{568} of the suspension (Fig. 2). With $50 \mu\text{M-Cu}^{2+}$, the increase in OD_{568} was essentially complete about 20 min after the addition of Cu^{2+} ions (Fig. 2), and in a fixed-time assay at 15 min, an increase in Cu^{2+} concentration was seen to result in an increase in OD_{568} (Fig. 3).

Fig. 3. Increase in OD_{568} after 15 min of Cu^{2+} treatment, plotted against Cu^{2+} concentration. The OD_{568} was measured against an untreated suspension. Cells were suspended in 10 mM- Na^+ /PIPES (□), 2 mM- Na^+ /PIPES (■), 2 mM- Ca^{2+} /PIPES (●), or distilled water (○). Mean number of determinations per copper concentration and mean coefficient of variation: 2 mM- Ca^{2+} /PIPES 3, 8.6%; distilled water 1.6, 7.5%. For Na^+ /PIPES, the results are from one experiment.

In contrast, cell suspensions in Na^+ /PIPES buffer did not show a significant increase in OD_{568} after addition of similar CuSO_4 concentrations (Figs 2 and 3), although pronounced K^+ leakage was observed. Since all treatments were carried out at the same pH, it seemed possible that the presence of Na^+ ions prevented the increase in OD_{568} induced by Cu^{2+} . To test this hypothesis, suspensions in distilled water, Ca^{2+} / and Mg^{2+} /PIPES buffer were pre-treated with NaCl . After 2 min, CuSO_4 was added, and the OD_{568} was monitored. The addition of Na^+ to suspensions in these media effectively prevented the increase in OD_{568} induced by Cu^{2+} (Fig. 4a, b). Furthermore, in 2 mM- Na^+ /PIPES buffer, the presence of additional NaCl prevented the small increase in OD_{568} induced by Cu^{2+} (Fig. 4a). This effect increased with increasing NaCl concentration (Fig. 4a). In contrast, the presence of Ca^{2+} or Mg^{2+} in suspensions in distilled water did not prevent the OD_{568} increase induced by Cu^{2+} (data not shown). Other cations were tested for their ability to prevent the Cu^{2+} -induced increase in OD_{568} . Of these, K^+ and Li^+ were also effective with cells suspended in distilled water or in Ca^{2+} / or Mg^{2+} /PIPES buffer (Fig. 4a, b, and further data not shown). In cells suspended in distilled water, the presence of monovalent cations (Na^+ , K^+ or Li^+) in the suspending medium also resulted in a reversion of the initial small increase in OD_{568} caused by treatment with $40 \mu\text{M-Cu}^{2+}$ (Fig. 4b).

Electron microscopy

The spectrophotometric data strongly suggested that treatment of the cells with very low concentrations of

Fig. 4. (a) Effect of Na^+ or K^+ concentration on the increase in OD_{568} after 15 min treatment with $50 \mu M$ - Cu^{2+} . The OD_{568} was measured against an untreated suspension. The monovalent cations were added 2 min before addition of Cu^{2+} . Cells were suspended in 2 mM- Na^+ /PIPES + $NaCl$ (■), 2 mM- Ca^{2+} /PIPES + $NaCl$ (▼), 2 mM- Ca^{2+} /PIPES + KCl (▽), 2 mM- Mg^{2+} /PIPES + $NaCl$ (▲), 2 mM- Mg^{2+} /PIPES + KCl (△) or distilled water + $NaCl$ (●). In distilled water, the mean number of determinations per copper concentration and mean coefficient of variation was 1.8 and 15.8%, respectively. For the other media, the results are from one experiment. (b) Effect of the presence of monovalent cations (5 mM) on the time course of increase in OD_{568} in cell suspensions in distilled water treated with $40 \mu M$ - Cu^{2+} . The OD_{568} was measured against an untreated suspension. ○, No monovalent cation added; ▽, $NaCl$; △, KCl ; □, $LiCl$. The results are from one experiment.

$CuSO_4$ resulted in plasmolysis unless monovalent cations were present in the supernatants of the suspensions.

To test this hypothesis, cells suspended in distilled water or Na^+ /PIPES were treated with $CuSO_4$ and examined by electron microscopy. The cells suspended in 10 mM- Na^+ /PIPES buffer and treated with 10–50 μM - Cu^{2+} for 1 h showed no major ultrastructural alterations, but those suspended in distilled water displayed a pronounced plasmolysis that was not an artefact due to the fixation process, since it could be observed by light microscopy in unfixed cells.

Table 1. Quantification of cell plasmolysis induced by Cu^{2+} in *P. syringae* cells suspended in distilled water (pH 6.5), 1.5 h after addition of Cu^{2+}

Pooled results from three experiments are shown.

Cu^{2+} concn (μM)	No. of cells examined	Percentage of plasmolysed cells	Mean plasmolysis index*
5	1186	2.53	0.347†
10	2045	15.9	0.319
15	2137	32.8	0.383
20	543	56.0	0.447
25	941	78.9	0.398
30	914	89.1	0.387
40	1366	93.8	0.394

* Calculated as the mean of the plasmolysis index of 50 random plasmolysed cells. The plasmolysis index was determined as described in Methods.

† Due to the very low percentage of plasmolysed cells, for this sample only 20 plasmolysed cells were examined.

Table 2. Binding of Cu^{2+} by cell suspensions in 10 mM- Na^+ /PIPES or distilled water

Copper was assayed by atomic absorption spectrophotometry after digestion of cells with HNO_3 . Results are means from four to nine determinations, $\pm$ SD.

Initial Cu^{2+} concn		Bound Cu^{2+} [μmol (mg dry wt) $^{-1}$] in cells suspended in:	
(μM)	[μmol (mg dry wt) $^{-1}$]	Na^+ /PIPES	Distilled water
10	0.035	0.009 $\pm$ 0.004	—
20	0.071	0.033 $\pm$ 0.003	—
25	0.089	—	0.060 $\pm$ 0.004
30	0.107	0.055 $\pm$ 0.006	—
40	0.143	0.078 $\pm$ 0.005	—
50	0.178	0.110 $\pm$ 0.004	0.141 $\pm$ 0.002
60	0.214	0.138 $\pm$ 0.011	—
70	0.250	0.159 $\pm$ 0.008	—
75	0.268	—	0.195 $\pm$ 0.013
80	0.286	0.177 $\pm$ 0.013	—
90	0.321	0.202 $\pm$ 0.010	—
100	0.357	0.224 $\pm$ 0.016	0.291 $\pm$ 0.007

This phenomenon was studied in suspensions in distilled water treated with Cu^{2+} concentrations ranging from 5 to 40 μM , and for times of exposure up to several hours. After the addition of Cu^{2+} , plasmolysis progressively increased with time until a maximum was achieved. At all copper concentrations, two subpopulations could be differentiated: normal cells and heavily plasmolysed cells (Fig. 5). The degree of plasmolysis of individual cells was assessed by a plasmolysis index as described in Methods. The mean plasmolysis index was calculated by averaging the plasmolysis index of 50 plasmolysed cells.

Fig. 5. (a) Control cells of *P. syringae* suspended in distilled water. (b) Plasmolysis induced by $25 \mu\text{M-Cu}^{2+}$ in *P. syringae* suspended in distilled water (pH 6.5; 1 h treatment). Fixation with 2.5% glutaraldehyde. Bars, 1 μm .

Fig. 6. Distribution of the plasmolysis index in cells of *P. syringae* treated with $10\text{--}40 \mu\text{M-Cu}^{2+}$ for 1.5 h. The plasmolysis index as described in Methods was determined for each of 50 random plasmolysed cells at each copper concentration. Ten equally spaced frequency classes were considered, and for each, the relative frequency was determined by dividing the number of cells belonging to the class by 50. From left to right: upper row: 10, 15, $20 \mu\text{M-Cu}^{2+}$; lower row: 25, 30, $40 \mu\text{M-Cu}^{2+}$.

This parameter was therefore a measure of the degree of plasmolysis in the population of cells that were plasmolysed. In the range 5 to $40 \mu\text{M-Cu}^{2+}$, an increase in Cu^{2+} concentration resulted in an increase in the percentage of plasmolysed cells (Table 1). In contrast, the mean

plasmolysis index and the distribution of the plasmolysis index of the cells were similar for all copper concentrations (Table 1, Fig. 6).

Binding experiments

Cells suspended in distilled water or Na^+ /PIPES buffer and treated with Cu^{2+} at concentrations up to $100 \mu\text{M}$ bound a significant amount of the Cu^{2+} ions present in solution (Table 2). For a given initial Cu^{2+} concentration, cells suspended in Na^+ /PIPES buffer bound less copper than cells suspended in water (Table 2).

Discussion

It has recently been reported that very low concentrations of CuSO_4 induced massive K^+ efflux in *P. syringae* cells suspended in 10 mM-Na^+ /PIPES buffer (Cabral, 1989, 1990). The present work has shown that Cu^{2+} also induces significant K^+ loss in cells suspended in distilled water, or 2 mM-Ca^{2+} / or 2 mM-Na^+ /PIPES buffer. K^+ efflux in Ca^{2+} /PIPES buffer occurred at Cu^{2+} concentrations lower than in Na^+ /PIPES buffer or distilled water. These observations are in accordance with those reported earlier (Cabral, 1989) in that in Na^+ /PIPES

buffer, pre-treatment of the cells with divalent cations (e.g. Ca^{2+}) increased both the rate of K^+ efflux and the amount of K^+ released after copper treatment.

In Na^+ /PIPES buffer and distilled water, $40\text{ }\mu\text{M}$ - Cu^{2+} induced leakage of most of the unbound cellular K^+ , but did not saturate the binding sites for Cu^{2+} ions. Therefore, besides the Cu^{2+} -binding sites involved in the process of K^+ efflux, *P. syringae* cells have other sites available for Cu^{2+} binding.

Since K^+ is the main osmolyte in bacteria grown under normal conditions and the principal species responsible for maintenance of turgor pressure, its rapid depletion from the cells was expected to cause plasmolysis, which should be detected by an increase in the optical density of the treated suspension. For cells suspended in distilled water and Ca^{2+} / or Mg^{2+} /PIPES buffer this was indeed observed. Thompson *et al.* (1970) reported that when a marine pseudomonad was suspended in a 0.05 M - MgSO_4 solution, cells plasmolysed. These authors concluded that under these conditions, cells showed extensive plasmolysis due to massive leakage of K^+ . In yeast cells, K^+ efflux caused by inhibitors of the plasma membrane ATPase has been reported to cause a pronounced decrease in cell volume (Borst-Pauwels *et al.*, 1983). Ohsumi *et al.* (1988) reported that Cu^{2+} induced K^+ loss from yeast cells with a concomitant increase in the turbidity of the suspension due to shrinkage of the cells. However, these authors did not interpret the shrinkage of the cells as being due to K^+ leakage.

In Na^+ /PIPES buffer, K^+ leakage was not followed by cell plasmolysis. In distilled water, or Ca^{2+} / or Mg^{2+} /PIPES buffer, plasmolysis induced by Cu^{2+} could be significantly reduced if cells were pre-incubated with monovalent (but not with divalent) cations. It would appear that externally added Na^+ , Li^+ and K^+ effectively antagonized plasmolysis induced by Cu^{2+} , by entering the cells soon after the release of the resident K^+ ions. Results different from these were reported by Thompson *et al.* (1970): in their marine pseudomonad plasmolysed by washing in MgSO_4 , K^+ , but not Li^+ , was effective in deplasmolysing the cells.

The results presented in this paper therefore agree with the interpretation that in bacteria grown in normal conditions, K^+ is the main osmoactive solute. Our results also suggest that, under conditions of severe K^+ leakage, Na^+ and Li^+ could enter the cells and probably replace resident K^+ ions in the neutralization of negative charges of cytoplasmic constituents. It has been pointed out by several authors that in non-halophilic bacteria, Na^+ and Li^+ can not (or poorly) substitute for K^+ in metabolism. K^+ -dependent enzymes are not activated (and can be inhibited) by Na^+ or Li^+ (Evans & Sorger, 1966; Strickland & Miller, 1968; Klenow & Henningsen, 1969; Bishop & Gill, 1971). Replacement of intracellular K^+ by

Fig. 7. Probit plot of the time course of K^+ efflux induced by $25\text{ }\mu\text{M}$ - Cu^{2+} in cells suspended in 10 mM - Na^+ /PIPES (results taken from Cabral, 1989). K^+ was assayed using a K^+ -sensitive electrode. The values of K^+ concentration were converted into percentages of the maximum K^+ released ($67\text{ }\mu\text{M}$), and these were converted to probits by the formula of Davies (1971). Inset: probit plot of maximum K^+ released from the cells in 10 mM - Na^+ /PIPES as a function of Cu^{2+} concentration (results taken from Cabral, 1990). K^+ was assayed by flame photometry. The values of K^+ concentration were converted into percentages of the maximum K^+ released ($86\text{ }\mu\text{M}$), and these were converted to probits, as described above.

Na^+ has been reported to cause a severe decrease in protein synthesis and in the composition of rRNA (Ennis & Lubin, 1965). *In vitro*, several activities of the sub-units of the bacterial ribosome are strongly dependent on high K^+ concentrations, but are not influenced by Na^+ or Li^+ (Zamir *et al.*, 1974). Therefore, uptaken Na^+ and Li^+ probably re-equilibrated turgor pressure but did not restore normal metabolism in Cu^{2+} -treated *P. syringae* cells.

If plasmolysis was mainly caused by K^+ leakage, then the loss of K^+ from individual cells could be assessed by examining their morphology. As reported above, at all Cu^{2+} concentrations, two sub-populations could be distinguished: normal cells and heavily plasmolysed cells. The mean plasmolysis index and the distribution of the plasmolysis index of the cells were similar for all Cu^{2+} concentrations, which indicated that probably K^+ efflux and plasmolysis were essentially 'all-or-nothing' reactions. If K^+ loss and plasmolysis were graded responses, an increase in Cu^{2+} concentration would result in an increase in the mean plasmolysis index, and a shift in the distribution of the plasmolysis index to higher values. Further support of this interpretation came from an analysis of the process of K^+ efflux itself. In a 'quantal' ('all-or-nothing') phenomenon (e.g. death by treatment

with a chemical agent), when the susceptibility of the members of the population is normally distributed, the percentage of response (mortality, in the above example) is a sigmoidal function of the dose or time. This curve can be converted into a straight line by probit transformation (Finney, 1952; Hewlett & Plackett, 1979). When the values of the time course of K^+ efflux (studied using a K^+ -sensitive electrode and taken from Cabral, 1989) were plotted as probits of the percentages of K^+ released against log time, and the probits of the percentages of maximum K^+ released were plotted against log copper concentration, linear relationships were indeed found (Fig. 7). K^+ loss due to an 'all-or-nothing' reaction has been reported also in yeast cells treated with mercury and several inhibitors of the plasma-membrane ATPase (Passow & Rothstein, 1959; Borst-Pauwels *et al.*, 1983).

I am especially indebted to Dr M. T. Silva (Centro de Citologia Experimental da Universidade do Porto) and Dr A. R. W. Smith (Thames Polytechnic, London, UK) for guidance and helpful discussions, and to Mrs Teresa Serra for excellent technical assistance in the atomic absorption spectrophotometry. This work was supported by INIC (grants PB/1 and 83-CEN-12).

References

- BISHOP, C. C. & GILL, J. E. (1971). Inhibition of *Escherichia coli* DNA polymerase by monovalent cations. *Biochimica et Biophysica Acta* **227**, 97–105.
- BOOTH, I. R. (1985). Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews* **49**, 359–378.
- BORST-PAUWELS, G. W. F. H., THEUVENET, A. P. R. & STOLS, A. L. H. (1983). All-or-none interactions of inhibitors of the plasma membrane ATPase with *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **732**, 186–192.
- BRADBURY, J. F. (1986). *Guide to Plant Pathogenic Bacteria*. Kew: CAB International Mycological Institute.
- CABRAL, J. P. S. (1989). Induction of potassium efflux by cupric ions in *Pseudomonas syringae* ATCC 12271 and its correlation with cell viability. *Microbios* **60**, 141–150.
- CABRAL, J. P. S. (1990). Cupric ions induce both an efflux of potassium and low molecular mass metabolites in *Pseudomonas syringae*. *FEMS Microbiology Letters* (in the Press).
- CABRAL, J. P. S. & SILVA, M. T. (1984). Binding of copper to *Pseudomonas syringae*: a chemical and morphological study. *Proceedings of the 2nd Working Group on Pseudomonas syringae Pathovars*, pp. 43–44. Athens: Hellenic Phytopathological Society.
- CHRISTIAN, J. H. B. & WALTHO, J. A. (1961). The sodium and potassium content of non-halophilic bacteria in relation to salt tolerance. *Journal of General Microbiology* **25**, 97–102.
- CHRISTIAN, J. H. B. & WALTHO, J. A. (1962). Solute concentrations within cells of halophilic and non-halophilic bacteria. *Biochimica et Biophysica Acta* **65**, 506–508.
- COLIN, J. E. & CHAFIK, Z. (1986). Comparison of biological and chemical treatments for control of bacterial speck of tomato under field conditions in Morocco. *Plant Disease* **70**, 1048–1050.
- COLIN, K. C. & MCCARTER, S. M. (1983). Effectiveness of selected chemicals in inhibiting *Pseudomonas syringae* pv. *tomato* in vitro and in controlling bacterial speck. *Plant Disease* **67**, 639–644.
- DAVIES, R. G. (1971). *Computer Programming in Quantitative Biology*, chapter 14. New York & London: Academic Press.
- DINNBIER, U., LIMPSEL, E., SCHMID, R. & BAKKER, E. P. (1988). Transient accumulation of potassium glutamate and its replacement by trehalose during adaption of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. *Archives of Microbiology* **150**, 348–357.
- DOMEK, M. J., ROBBINS, J. R., ANDERSON, M. E. & MCFETERS, G. A. (1987). Metabolism of *Escherichia coli* injured by copper. *Canadian Journal of Microbiology* **33**, 57–62.
- ENNIS, H. L. & LUBIN, M. (1965). Pre-ribosomal particles formed in potassium-depleted cells. Studies on degradation and stabilization. *Biochimica et Biophysica Acta* **95**, 605–623.
- EVANS, H. J. & SORGER, G. J. (1966). Role of mineral elements with emphasis on the univalent cations. *Annual Review of Plant Physiology* **17**, 47–76.
- FINNEY, D. J. (1952). *Probit Analysis*, 2nd edn. Cambridge: Cambridge University Press.
- GREEN, J., PRIOR, S. D. & DALTON, H. (1985). Copper ions as inhibitors of protein C of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). *European Journal of Biochemistry* **153**, 137–144.
- HEWLETT, P. S. & PLACKETT, R. L. (1979). *The Interpretation of Quantal Responses in Biology*. London: Edward Arnold.
- HORSFALL, J. G. (1956). *Principles of Fungicidal Action*, chapter I. Waltham: Chronica Botanica Company.
- KLEINER, D. (1978). Inhibition of the respiratory system in *Azotobacter vinelandii* by divalent transition metal ions. *FEBS Letters* **96**, 364–366.
- KLENOW, H. & HENNINGSSEN, I. (1969). Effect of monovalent cations on the activity of the DNA polymerase of *Escherichia coli* B. *European Journal of Biochemistry* **9**, 133–141.
- LARSEN, P. I., SYDNES, L. K., LANDFALD, B. & STRØM, A. R. (1987). Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaine, glutamic acid, and trehalose. *Archives of Microbiology* **147**, 1–7.
- LOWE, J. A., CHAN, Y. M. & TSANG, J. C. (1987). Investigation of biosynthesis of prodigiosin and its condensing enzyme in *Serratia marcescens*. *Microbios* **51**, 71–80.
- LUBIN, M. & ENNIS, H. L. (1964). On the role of intracellular potassium in protein synthesis. *Biochimica et Biophysica Acta* **80**, 614–631.
- MEASURES, J. C. (1975). Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature, London* **257**, 398–400.
- MEURY, J., ROBIN, A. & MONNIER-CHAMPEIX, P. (1985). Turgor-controlled K^+ fluxes and their pathways in *Escherichia coli*. *European Journal of Biochemistry* **151**, 613–619.
- OHSUMI, Y., KITAMOTO, K. & ANRAKU, Y. (1988). Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *Journal of Bacteriology* **170**, 2676–2682.
- PASSOW, H. & ROTHSTEIN, A. (1960). The binding of mercury by the yeast cell in relation to changes in permeability. *Journal of General Physiology* **43**, 621–633.
- STRICKLAND, J. E. & MILLER, O. N. (1968). Inhibition of glycerol dehydrogenase from *Aerobacter aerogenes* by dihydroxyacetone, high ionic strength, and monovalent cations. *Biochimica et Biophysica Acta* **159**, 221–226.
- TAMIETTI, G. & GARIBALDI, A. (1984). Eradication of *Pseudomonas syringae* pv. *phaseolicola* in bean seeds in Italy. *Phytopathology and Plant Bacterial Diseases of Quarantine Significance* (Italian-US Workshop/Seminar 'Plant Pathology and Quarantine', Rome), pp. 69–76.
- THOMPSON, J., COSTERTON, J. W. & MACLEOD, R. A. (1970). K^+ -dependent deplasmolysis of a marine pseudomonad plasmolysed in a hypotonic solution. *Journal of Bacteriology* **102**, 843–854.
- YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMER, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.
- ZAMIR, A., MISKIN, R., VOGEL, Z. & ELSON, D. (1974). The inactivation and reactivation of *Escherichia coli* ribosomes. *Methods in Enzymology* **30**, 406–426.