Effects of growth-inhibitory concentrations of copper on alginate biosynthesis in highly mucoid *Pseudomonas aeruginosa*

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Alginate production and degree of polymerization were affected when the highly mucoid *Pseudomonas aeruginosa* 8821M was grown with growth-inhibitory concentrations of Cu^{2+} (supplied as CuCl_2 ; 1–5 mM). The inhibition of alginate biosynthesis was consistent with the decreased activity in Cu^{2+} -stressed cells of phosphomannose isomerase/GDP-mannose pyrophosphorylase (encoded by *algA*), phosphomannomutase (encoded by *algC*) and GDP-mannose dehydrogenase (encoded by *algD*). However, in cells grown with concentrations of CuCl_2 below 2 mM, the steady-state mRNA levels from *algA*, *algC*, *algD* and from the regulatory gene *algR1* increased moderately. This observation is consistent with the suggested linkage between the control of alginate gene expression and the global regulation involved in the oxidative stress response. At highly inhibitory concentrations the levels of the four alginate gene transcripts decreased from maximal values. The bell-shaped curves, representing the effect of Cu^{2+} concentration on mRNA levels from the four alginate genes, exhibited similar patterns but did not concur. The decrease of the specific activity of enzymes necessary for GDP-mannuronic acid synthesis in Cu^{2+} -grown cells was correlated with changes in gene expression, with the inhibitory effect of Cu^{2+} on enzyme activities and with Cu^{2+} -induced oxidative inactivation of enzymes, especially the particularly sensitive phosphomannose isomerase activity.

Keywords: *Pseudomonas aeruginosa*, copper, alginate biosynthesis, alginate enzymes, alginate gene expression

INTRODUCTION

The regulation of alginate synthesis in *Pseudomonas aeruginosa* has been intensively studied because mucoid strains chronically colonize the lungs of cystic fibrosis patients and alginate has been implicated in the persistence of *P. aeruginosa* in the cystic-fibrosis-affected lung (May *et al.*, 1991). Understanding of the molecular basis of alginate synthesis has made this an extremely useful model for the biosynthesis of exopolysaccharides of industrial or ecological significance in Gram-negative bacteria. Environmental and physiological factors such as the concentration of cations (Martins *et al.*, 1990) and of dissolved oxygen in the growth medium (Leitão & Sá-Correia, 1993), the growth temperature (Leitão *et al.*,

1992) and the phase of growth (Leitão & Sá-Correia, 1995) modulate the yield and rheological properties of the alginate synthesized by the highly mucoid strain *P. aeruginosa* 8821M (Alves *et al.*, 1991). Under non-stressing conditions, the level of alginate produced has been correlated with the activities of the four enzymes necessary for the formation of GDP-mannuronic acid, the activated precursor for alginate polymerization (Leitão *et al.*, 1992; Leitão & Sá-Correia, 1995). In addition, during batch growth, the pattern of variation of activity of these alginate enzymes correlated with the co-ordinate transcriptional regulation of the three structural genes which in turn correlated with that of the regulatory *algR1* (*algR*) gene (Leitão & Sá-Correia, 1995). The three alginate structural genes are *algA*, encoding a bifunctional protein with phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activities (Sá-Correia *et al.*, 1987), *algC*, encoding phosphomannomutase (PMM) (Zielinski *et al.*, 1991)

Abbreviations: GMD, GDP-mannose dehydrogenase; GMP, GDP-mannose pyrophosphorylase; PMI, phosphomannose isomerase; PMM, phosphomannomutase.

and *algD*, encoding GDP-mannose dehydrogenase (GMD) (Deretic *et al.*, 1987; May & Chakrabarty, 1994). The regulatory AlgR1 protein mediates the activation of both *algC* and *algD* promoters in mucoid strains and in response to high concentrations of salt (Berry *et al.*, 1989; Deretic *et al.*, 1987; Fujiwara *et al.*, 1993). Prolonged stress conditions are thought to trigger a genetic switch to mucoidy that is accompanied by the activation of (at least) *algC* and *algD* gene transcription (Deretic *et al.*, 1994; May & Chakrabarty, 1994). The regulation of alginate biosynthesis in *P. aeruginosa* is complex, involving a hierarchy of several genes (Deretic *et al.*, 1994; DeVries & Ohman, 1994; May & Chakrabarty, 1994). At the top of the hierarchy are *algU* (*algT*), *mucA* (*algS*) and *mucB* (*algN*). The *mucA* and *mucB* genes have a negative effect on alginate biosynthesis while the AlgU (AlgT) protein is required for transcription of the response regulator genes *algB* and *algR1* (*algR*) necessary for *algD* expression. AlgU also directly activates the *algD* promoter and the promoter of its own structural gene (Wozniak & Ohman, 1994; DeVries & Ohman, 1994; Deretic *et al.*, 1994; May & Chakrabarty, 1994; Yu *et al.*, 1995). Recent evidence indicates that *algU* encodes an alternative σ factor participating in the global stress response in bacteria (Martin *et al.*, 1994; Yu *et al.*, 1995).

Depending on concentration, copper, which is a cofactor in a variety of enzymes, can cause extensive damage in bacteria, leading to growth inhibition and loss of cell viability. Copper readily reacts with the superoxide anion and with hydrogen peroxide via a Fenton-like reaction to generate the highly reactive hydroxyl radical (Halliwell & Gutteridge, 1989; Storz *et al.*, 1990). Oxygen free radicals are generated as intermediates during the reduction of oxygen to water during normal cellular respiration. These reactive oxygen species lead to the oxidation of sugars, amino acids, phospholipids and nucleic acids with deleterious results (Halliwell & Gutteridge, 1989). Bacterial defence against, and adaptation to oxidative stress require the induction of the synthesis of a sub-set of proteins and therefore the activation of transcription of the structural genes. Many of these proteins act either by destroying oxidants or by repairing the resulting damage (Storz *et al.*, 1990). Cations are known to influence exopolysaccharide synthesis both qualitatively (chemical composition and properties) and quantitatively (Appanna & Preston, 1987; Martins *et al.*, 1990; Sutherland, 1990; Kidambi *et al.*, 1995). For example, the synthesis of the anionic exopolysaccharide alginate is stimulated by Mg^{2+} , Mn^{2+} and Ca^{2+} in *P. aeruginosa* (Martins *et al.*, 1990) and by Cu^{2+} in a subset of *P. syringae* strains (Kidambi *et al.*, 1995).

In this study we investigated the effect of growth-inhibitory concentrations of Cu^{2+} on the level of transcription of *algA*, *algC*, *algD* and *algR1* in the highly mucoid *P. aeruginosa* 8821M (Alves *et al.*, 1991). This study was undertaken because a linkage between the control of alginate biosynthesis and the global regulation involved in oxidative stress responses in bacteria has

been suggested recently (Deretic *et al.*, 1994; Yu *et al.*, 1995). The effects of Cu^{2+} on the activities of the four enzymes necessary for the synthesis of the alginate precursor GDP-mannuronic acid, on alginate synthesis and on the rheological properties of the product were also analysed. Although the concentrations of Cu^{2+} examined are not characteristic of the lung environment, this analysis gives some insight into the complexity of alginate regulation. It also gives some clues to the understanding of the effect of toxic metals in the biosynthesis of exopolysaccharides of ecological significance in polluted environments by Gram-negative bacteria.

METHODS

Bacterial strains, plasmids and culture conditions. The high alginate-producing *P. aeruginosa* strain 8821M (Alves *et al.*, 1991), which exhibits high levels of the four alginate biosynthetic enzymes and high levels of transcription of their structural genes *algA*, *algC* and *algD*, and of the regulatory gene *algR1*, was used. Recombinant plasmids pAD4038 (containing the *algA* gene; Sá-Correia *et al.*, 1987), pNZ49 (containing the *algC* gene; Zielinski *et al.*, 1991), pVD211 (containing the *algD* gene; Deretic *et al.*, 1987) and pVDRtac5 (containing the *algR1* gene; Deretic & Konyescni, 1989), hosted in *Escherichia coli* HB101, were used to prepare the probes for Northern blot analysis. Bacterial growth was carried out at 30 °C with orbital agitation (250 r.p.m.) in 100 ml growth medium contained in 250 ml shake flasks (initial $OD_{640} = 0.3 \pm 0.02$). Growth medium was Lennox Broth (Sigma) plus 1% (w/v) glucose and $MnCl_2$ and $MgCl_2$ (0.5 mM each) (Martins *et al.*, 1990). Growth medium was supplemented with $CuCl_2$ (final concentrations ranging from 0 to 5 mM). Liquid inocula were prepared in growth medium without $CuCl_2$ supplementation as described elsewhere (Leitão *et al.*, 1992).

Effects of Cu^{2+} on *P. aeruginosa* 8821M growth and alginate production and properties. Growth in the absence or presence of Cu^{2+} supplementation was monitored by measuring culture OD_{640} . The specific growth rates of *P. aeruginosa* 8821M in media with or without Cu^{2+} supplementation were calculated by least-square fitting to the linear part of semi-log growth plots from at least two independent cultures with the same concentration of $CuCl_2$. Results are given as median values of at least two different cultures grown at each $CuCl_2$ concentration.

Alginate concentration was determined after ethanol precipitation from cell-free supernatants by the modified carbazole method (Knutson & Jeanes, 1968) using sodium alginate from *Laminaria hyperborea* (BDH) as standard. Results are given as median values of three independent analyses of the alginate produced during at least two independent batch cultivations carried out with the same level of Cu^{2+} supplementation. The viscosity of aqueous solutions (3 g l^{-1}) of the alginates isolated from cultures during early stationary phase (less than 5 h after entering the stationary phase) of growth, carried out in the absence or presence of Cu^{2+} , was measured at 30 °C using a cone and plate Brookfield Viscometer, model LVIIT, at a shear rate of 12 s^{-1} . Results are given as median values of three determinations using at least two independently prepared alginate solutions.

Northern blot analysis. Total RNA was extracted from cells of *P. aeruginosa* 8821M grown in the presence of increasing

concentrations of CuCl_2 and harvested when the culture OD_{640} reached the standardized value 1.00 ± 0.05 . Cell pellets obtained by centrifugation of two independent cultures at the various concentrations of Cu^{2+} were immediately frozen in liquid nitrogen and processed using the method described by Schmitt *et al.* (1990). Samples containing 10 μg total RNA (estimated spectrophotometrically at 260 nm) were loaded in a 1.5% (w/v) agarose (FMC)/formaldehyde (Merck) denaturing gel and separated by electrophoresis at 2 V cm^{-1} (Sambrook *et al.*, 1989). The 16S and 23S rRNA bands were visualized after ethidium bromide staining to confirm that equal amounts of RNA had been loaded in each lane. The gels were blotted onto nylon membranes and the RNA was fixed by UV cross-linking (Sambrook *et al.*, 1989). Prehybridization, preparation of the radiolabelled specific probes for *algA*, *algC*, *algD* or *algR1* hybridization experiments, autoradiography and densitometric analysis of the hybridization signals were carried out as described previously (Leitão & Sá-Correia, 1995). Densitometric results are given as the sum of the intensities of the hybridization bands of the various transcripts corresponding to a specific gene, whenever they were detected, and are means $\pm$ SD of at least three independent Northern experiments using RNA extracts prepared from cells obtained from two independent cultures at the same CuCl_2 concentration.

Enzymes assays. PMI, PMM, GMP and GMD activities were assayed in crude cell extracts as described by Martins & Sá-Correia (1991). Crude extracts were prepared (Martins & Sá-Correia, 1991) using cells of *P. aeruginosa* 8821M grown in media with increasing concentrations of CuCl_2 (0–5 mM) and harvested when the culture OD_{640} reached the standardized value of 1.50 ± 0.05 . The *in vitro* effect of increasing concentrations of Cu^{2+} was assessed by the addition of CuCl_2 to the enzyme assay mixture (final concentrations in the range 0–40 μM) and by measuring residual activity after 3 min incubation. The range of concentrations studied included values that were predicted to be present in the enzyme assay mixtures, prepared with crude extracts from Cu^{2+} -grown cells, based on the Cu^{2+} concentrations determined in these extracts by atomic absorption spectroscopy. One unit of activity was defined as the amount of enzyme that reduced 1 μmol NAD or NADP min^{-1} under the assay conditions. Protein concentration in crude cell extracts was in the range $15 \pm 2 \text{ g l}^{-1}$ and was determined by the method of Bradford (1976) with bovine serum albumin fraction V (Merck) as standard. Specific activities of alginate enzymes are given as the means $\pm$ SD of at least three enzyme assays and three protein determinations.

Sensitivity of alginate enzymes to oxidative inactivation by the superoxide radical. To compare the sensitivity of alginate enzymes to inactivation induced by the superoxide radical, crude extracts prepared from cells grown in medium without CuCl_2 supplementation were incubated at 30°C with the xanthine/xanthine oxidase superoxide radical generating system (McCord & Fridovich, 1969), containing 0.2 U xanthine oxidase (Sigma) and 25 μg xanthine $(\text{ml extract})^{-1}$, for up to 35 min and the residual activity was immediately determined. A control assay lacking xanthine/xanthine oxidase was carried out and it was confirmed that the four enzyme activities maintained initial activities. Results are presented as means $\pm$ SD of at least three enzyme assays.

Determination of Cu^{2+} concentration in crude extracts. The concentration of Cu^{2+} in crude extracts prepared, as for the enzyme assays, from cells of *P. aeruginosa* 8821M grown with increasing concentrations of CuCl_2 was determined by atomic absorption spectroscopy at the Laboratório de Análises,

Instituto Superior Técnico, Lisbon, Portugal. Each value is given as the mean $\pm$ SD of the results of two determinations carried out in two independently prepared cell extracts from cells obtained from two independent cultures in medium with the same level of CuCl_2 supplementation.

RESULTS

Effect of Cu^{2+} on *P. aeruginosa* 8821M growth and on alginate production and properties

Growth medium supplementation with concentrations of CuCl_2 between 1 and 5 mM led to a reduction in the growth rate of *P. aeruginosa* 8821M (Fig. 1a). Growth inhibition was due to the presence of Cu^{2+} and not to the counterion Cl^- . The supply of Cu^{2+} as CuSO_4 had identical effects on growth and the addition of the same concentrations of MgCl_2 , MnCl_2 or CaCl_2 was not inhibitory (Martins *et al.*, 1990).

Alginate production during batch growth decreased with increasing CuCl_2 concentration (Fig. 1b). At or above 4.0 mM, no alginate production was detectable (Fig. 1b). Since Cu^{2+} did not significantly modify the final concentration of biomass produced (Fig. 1a), this toxic cation affected alginate biosynthesis. In addition, the molecular mass of the exopolysaccharides synthesized during growth in the presence of increasing

Fig. 1. Effects of the addition of increasing concentrations of CuCl_2 to the growth medium of *P. aeruginosa* 8821M on (a) the specific growth rate (μ) (○) and the culture OD_{640} reached during early stationary phase (●); and (b) the concentration of alginate produced during early stationary phase (●) and the viscosity (centipoise, cP; shear rate 12 s^{-1}) of aqueous solutions prepared with these alginates (3 g l^{-1}) (○).

Fig. 2. Effects of the addition of increasing concentrations of CuCl_2 to the growth medium of *P. aeruginosa* 8821M on steady-state levels of mRNA from genes *algA* ($\circ$), *algC* ($\bullet$), *algD* ($\blacksquare$) and *algR1* ($\blacktriangle$) in cells harvested during late exponential phase. (a) Bands resulting from a representative Northern hybridization analysis (10 μg total RNA per lane) to alginate genes. (b) Results of the quantification by densitometry of the intensity of the hybridization bands, expressed as relative values; 1.0 being the value for the mRNA of each of the four alginate genes in cells grown in the absence of Cu^{2+} . Values are the sum of the intensities of the hybridization bands (identified by arrows) to the various transcripts corresponding to a specific gene, whenever they were detected, and are means $\pm$ SD of at least three independent Northern blot experiments using RNA extracts prepared from cells obtained from two independent cultures at the same concentration of toxin. Each RNA extract was probed with the four alginate genes but hybridization intensities can only be compared within a blot and not between blots.

concentrations of Cu^{2+} decreased, as suggested by the values of viscosity of aqueous solutions (3 g l^{-1}) prepared with the isolated alginates. This was confirmed by gel permeation chromatography (data not shown).

Cu^{2+} -dependent transcription of alginate genes

The effect of Cu^{2+} on the steady-state mRNA levels from the alginate genes *algA*, *algC*, *algD* and *algR1* in the highly mucoid *P. aeruginosa* 8821M was investigated. Confirming previous observations of Chitnis & Ohman (1993) and Leitão & Sá-Correia (1995), using non-stressing growth conditions, we were unable to detect a large polycistronic transcript complementary to *algA* and *algD* (Fig. 2a). Various bands of hybridization to *algD* and *algA* probes (Chitnis & Ohman, 1993; Leitão & Sá-Correia, 1995) were observed (Fig. 2a) and exhibited an identical pattern in Cu^{2+} -stressed and unstressed cells (Fig. 2a). Cells used to quantify mRNA levels from the four alginate genes were harvested at the mid-exponential phase of growth, where transcription is maximal (Leitão & Sá-Correia, 1995). The level of transcripts from *algR1* and from the three structural genes *algA*, *algC* and *algD* reached maximal values in cells grown at concentrations of CuCl_2 within the range 1–2 mM (Fig. 2). At higher concentrations, the level of transcripts decreased from maximal levels and, near the maximal concentration for growth, the mRNA from *algR1*, *algA* and *algD* reached values below those in unstressed cells (Fig. 2). The bell-shaped patterns representing the effect of increasing concentrations of Cu^{2+} on mRNA levels from the transcription of the four alginate genes were, in general, similar, but differed quantitatively. The greatest stimulation by Cu^{2+} was detected for *algC* transcription, which was higher than in unstressed cells even in cells grown at the highest Cu^{2+} concentration that permitted growth (Fig. 2). *algA* and *algD* transcription was much less sensitive to Cu^{2+} than *algC* or *algR1* transcription (Fig. 2).

Activity of alginate enzymes in Cu^{2+} -grown cells

Although the mRNA levels from *algA* and *algD* decreased to values below those in unstressed cells only above 2 mM Cu^{2+} , the activities of the encoded enzymes PMI/GMP (*algA*) and GMD (*algD*), assayed in crude extracts, were significantly affected in cells grown at lower concentrations (Fig. 3). Additionally, Cu^{2+} -grown cells also exhibited decreased PMM activity, despite the higher levels of *algC* transcripts in Cu^{2+} -grown cells as compared with unstressed cells (Figs 2 and 3). Consistent with the patterns of Cu^{2+} -dependent transcription of the three structural genes, the percentage reduction of PMM activity in Cu^{2+} -grown cells was below that of PMI/GMP and GMD activities.

The *in vitro* effect of Cu^{2+} on the activity of alginate enzymes was examined by the addition of increasing concentrations of CuCl_2 to the enzyme assay mixture using an extract prepared from cells grown with no Cu^{2+} supplementation (Fig. 4). The concentrations of CuCl_2 studied were in the range of those thought to be

Fig. 3. Specific activity (relative values) of PMI (○), PMM (●), GMP (△) and GMD (■), assayed in crude extracts prepared from cells of *P. aeruginosa* 8821M grown at 30 °C, in the presence of increasing concentrations of CuCl_2 and harvested when the culture reached the standardized OD_{640} of 1.5 ± 0.05 .

Fig. 5. Concentration of Cu^{2+} in crude extracts prepared from cells of *P. aeruginosa* 8821M grown in media supplemented with increasing concentrations of CuCl_2 . Cu^{2+} concentration was determined by atomic absorption.

Fig. 4. Residual activity of PMI (○), PMM (●), GMP (△) and GMD (■) in crude extracts, prepared from cells of *P. aeruginosa* 8821M grown in the absence of CuCl_2 supplementation, when increasing concentrations of CuCl_2 were added to the enzyme assay mixture.

Fig. 6. Specific activities (relative values) of PMI (○), PMM (●), GMP (△) and GMD (■) that would be present in crude extracts, prepared from Cu^{2+} -grown cells of *P. aeruginosa* 8821M, if the inhibitory effect of the Cu^{2+} concentrations present in the crude extracts was eliminated. Values were estimated using experimental values in Figs 4 and 5.

introduced into the enzyme assay mixtures by the addition of crude extracts prepared from cells grown at different Cu^{2+} concentrations. The concentrations of Cu^{2+} present in the various crude extracts were determined by atomic absorption spectrophotometry (Fig. 5). In the range of concentrations studied, Cu^{2+} did not inhibit GMD activity but inhibited, in a similar way, the activities of PMI, PMM and GMP (Fig. 4). Based on these results we have calculated the relative values of the specific activities of the four alginate enzymes that would be assayed in the crude extracts if the inhibitory effect of Cu^{2+} was eliminated (Fig. 6). Comparing these putative values (Fig. 6) with the steady-state mRNA levels from the structural genes in Cu^{2+} -grown cells (Fig. 2), some discrepancies were found. After discounting the inhibitory effect that Cu^{2+} has on PMI and GMP, the activity of PMI was much more affected than the activity

of GMP in Cu^{2+} -stressed cells (Fig. 6). A similar discrepancy was reported previously for cells grown at high concentrations of dissolved oxygen (Leitão & Sá-Correia, 1993), despite PMI and GMP being activities of the same bifunctional protein (Sá-Correia *et al.*, 1987; May *et al.*, 1994). This suggested that an additional mechanism underlies the decrease of the activity of alginate enzymes in cells of *P. aeruginosa* 8821M grown under Cu^{2+} stress. We therefore compared the sensitivity of PMI and GMP and the other two enzymes necessary for GDP-mannuronic acid synthesis to oxidative inactivation. This was assessed by incubating a crude extract prepared from cells grown in the absence of Cu^{2+} with the xanthine/xanthine oxidase superoxide-radical-generating system (McCord & Fridovich, 1969) and comparing the residual activities. The higher sensitivity of PMI to oxidative inactivation as compared with

Fig. 7. Residual activities (relative values) of PMI (○), PMM (●), GMP (△) and GMD (■) in a crude extract prepared from cells of *P. aeruginosa* 8821M grown in the absence of Cu^{2+} , after incubation at 30 °C with the xanthine/xanthine oxidase superoxide-radical-generating system for different times up to 35 min.

GMP, previously suggested by its sensitivity to hydrogen peroxide (Leitão & Sá-Correia, 1993), was confirmed (Fig. 7).

In summary, differences found in the relative specific activities of the four alginate enzymes in crude extracts prepared from cells grown with increasing concentrations of Cu^{2+} (Fig. 3) are consistent with the level of expression of the structural genes (Fig. 2), if we take into consideration the inhibition of the alginate enzymes by Cu^{2+} (Fig. 4) and their distinct sensitivity to oxidative inactivation (Fig. 7). The decrease of the activity of all the four enzymes necessary for GDP-mannuronic acid synthesis in cells grown in CuCl_2 -supplemented medium is also consistent with the decrease of alginate production by Cu^{2+} -stressed cells (Figs 1 and 3).

DISCUSSION

Environmental stress-inducing conditions such as high osmolarity, presence of ethanol and nutrient (nitrogen source or phosphate) deprivation have been reported to activate the *algD* and *algC* promoters (Berry *et al.*, 1989; Fujiwara *et al.*, 1993; May & Chakrabarty, 1994). In this study we have shown that growth-inhibitory concentrations of CuCl_2 below 2 mM also moderately activate the transcription of the alginate structural genes *algA*, *algC* and *algD* and of the regulatory gene *algR1*. This observation is consistent with the suggested link between the regulation of alginate synthesis and the oxidative stress response in bacteria (Deretic *et al.*, 1994; Yu *et al.*, 1995). The AlgU (AlgT) protein is thought to be a global regulator participating in the oxidative stress response in bacteria and in the activation of some *alg* promoters (Deretic *et al.*, 1994; Yu *et al.*, 1995). The AlgU (AlgT) protein directly activates the *algD* and *algR1* promoters as well as its own structural gene promoter. All these promoters display a strong

conservation of the -35 and -10 regions and share a high level of similarity with the consensus sequence for promoters transcribed by the σ^E RNA polymerase holoenzyme (Martin *et al.*, 1994). It is also expected that AlgU indirectly activates the transcription of *algA* that is located in the alginate biosynthetic operon controlled from the *algD* promoter (Chitnis & Ohman, 1993). Therefore, the up-regulation of alginate structural genes under study by mild copper stress may depend on fluctuations in the concentration of the AlgR1 protein (also AlgU-dependent) that activates the *algC* and *algD* promoters (May & Chakrabarty, 1994) and, indirectly, *algA* transcription. Interestingly, the pattern of Cu^{2+} -dependent transcription of *algR1* showed some similarity to that of the three structural genes, particularly *algC*. The steady-state mRNA levels from the four alginate genes decreased when the concentration of CuCl_2 added to the growth medium was higher than 2 mM. The bell-shaped curves representing the effect of Cu^{2+} on mRNA levels from the four genes, although similar, did not concur. This contrasted with the patterns of their growth-phase-dependent transcription under non-stressing conditions, which were coincident (Leitão & Sá-Correia, 1995), and may result, at least partially, from the relative sensitivity of the various transcripts to mRNA degradation (Higgins *et al.*, 1993) in Cu^{2+} -stressed and unstressed cells. According to this hypothesis, the *algA* and *algD* transcripts would be the most sensitive. The specific pattern of Cu^{2+} -dependence of the steady-state mRNA level of each one of the four alginate genes might result from several mechanisms participating differently depending on the Cu^{2+} concentration. As well as the fluctuations in AlgU (AlgT) protein concentration, it may be influenced by the level of phosphorylation of AlgR1 (Deretic *et al.*, 1992, 1989; Fujiwara *et al.*, 1993), which appears to be a component of a two-component responsive system (Stock *et al.*, 1989), the energetic state of the cell (Schlichtman *et al.*, 1994), and also by fluctuations in the level of other regulatory proteins such as AlgR2 (AlgQ) and AlgR3 (AlgP) that also mediate the activation of *algC* and *algD* promoters (Deretic & Konyescni, 1989; Deretic *et al.*, 1994; May & Chakrabarty, 1994). It is also possible that structural modifications of the various alginate regulatory proteins, due to the action of reactive oxygen species, might additionally affect alginate biosynthesis in the presence of Cu^{2+} .

Despite the higher level of expression of *algA*, *algC* and *algD* in cells grown in medium supplemented with concentrations of CuCl_2 below 2 mM, the production and degree of polymerization of alginate were significantly affected. The decrease of alginate synthesis at low CuCl_2 concentrations is, however, consistent with the decreased activities of alginate enzymes determined in crude extracts from cells grown with concentrations of CuCl_2 below and above 2 mM. Experimental evidence obtained during this study indicates that the lower activity of alginate enzymes in Cu^{2+} -stressed cells results from the direct inhibitory effect of Cu^{2+} together with the Cu^{2+} -induced oxidative inactivation of the enzymes,

especially of the particularly sensitive PMI activity. This last hypothesis is consistent with the distinct level of reduction of PMI and GMP activities in crude extracts of Cu²⁺-grown cells. These two activities correspond to two independent domains of the AlgA protein and have different sensitivities to hydrogen peroxide (Leitão & Sá-Correia, 1993) and superoxide anion (this work). The decreased molecular mass of the alginates produced by Cu²⁺-stressed cells might result from the reduction of their polymerization activity. This could be due to the inhibition of the polymerization enzymes complex by Cu²⁺ (May *et al.*, 1994) and/or to its oxidative inactivation. In addition, it is also very likely that phospholipid peroxidation by Cu²⁺-induced oxygen reactive species (Halliwell & Gutteridge, 1989; Storz *et al.*, 1990) affects lipid-lipid and lipid-protein interactions and, therefore, the activity of membrane-embedded polymerization enzymes.

In summary, the up-regulation of alginate gene promoters in cells of mucoid *P. aeruginosa* exposed to mild oxidative stress conditions does not necessarily result in the stimulation of alginate biosynthesis. Cell response might depend upon the deleterious effects of stress on the activity of enzymes involved in the alginate pathway as suggested by results reported in this study. Alginate synthesis in stressed cells is certainly also influenced by the availability of alginate precursors and the energetic state of the cell.

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