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

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Alginic production and degree of polymerization were affected when the highly mucoid Pseudomonas aeruginosa 8821M was grown with growth-inhibitory concentrations of Cu²⁺ (supplied as CuCl₂; 1-5 mM). The inhibition of alginate biosynthesis was consistent with the decreased activity in Cu²⁺-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 CuCl₂ below 2 mM, the steady-state mRNA levels from algA, algC, algD and from the regulatory gene algR 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 Cu²⁺ 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 Cu²⁺-grown cells was correlated with changes in gene expression, with the inhibitory effect of Cu²⁺ on enzyme activities and with Cu²⁺-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 algR (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)
and \( \text{algD} \), encoding GDP-mannose dehydrogenase (GMD) (Deretic et al., 1987; May & Chakrabarty, 1994). The regulatory \( \text{AlgR1} \) protein mediates the activation of both \( \text{algC} \) and \( \text{algD} \) 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) \( \text{algC} \) and \( \text{algD} \) 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 \( \text{algU} \) (\( \text{algT} \)), \( \text{mucA} \) (\( \text{algS} \)) and \( \text{mucB} \) (\( \text{algN} \)). The \( \text{mucA} \) and \( \text{mucB} \) genes have a negative effect on alginate biosynthesis while the \( \text{algU} \) (\( \text{algT} \)) protein is required for transcription of the response regulator genes \( \text{algB} \) and \( \text{algR1} \) (\( \text{algR} \)) necessary for \( \text{algD} \) expression. \( \text{AlgU} \) also directly activates the \( \text{algD} \) promoter and the promoter of its own structural gene (Wozniak & Ohman, 1994; DeVries & Ohman, 1994; Deretic et al., 1994; May & Chakrabarty, 1994). Recent evidence indicates that \( \text{algU} \) encodes an alternative \( \sigma \) 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 production 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 \( \text{Mg}^{2+} \), \( \text{Mn}^{2+} \) and \( \text{Ca}^{2+} \) in \( P. \) aeruginosa (Martins et al., 1990) and by \( \text{Cu}^{2+} \) in a subset of \( P. \) syringae strains (Kidambi et al., 1995).

In this study we investigated the effect of growth-inhibitory concentrations of \( \text{Cu}^{2+} \) on the level of transcription of \( \text{algA} \), \( \text{algC} \), \( \text{algD} \) and \( \text{algR1} \) 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 \( \text{Cu}^{2+} \) 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 \( \text{Cu}^{2+} \) 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 \( \text{algA} \), \( \text{algC} \) and \( \text{algD} \), and of the regulatory gene \( \text{algR1} \), was used. Recombinant plasmids pAD4038 (containing the \( \text{algA} \) gene; Sá-Correia et al., 1987), pNZ49 (containing the \( \text{algC} \) gene; Zielinski et al., 1991), pVD211 (containing the \( \text{algD} \) gene; Deretic et al., 1987) and pVDTrac5 (containing the \( \text{algR1} \) gene; Deretic & Konyesni, 1989), hosted in \( E. \) coli HB101, were used to prepare the probes for Northern blot analysis. Bacterial growth was carried out at 30 °C with orbital agitation (220 r.p.m.) in 100 ml growth medium contained in 250 ml shake flasks (initial \( \text{OD}_{600} = 0.3 \pm 0.02 \)). Growth medium was Lennox Broth (Sigma) plus 1% (w/v) glucose and \( \text{MnCl}_2 \) and \( \text{MgCl}_2 \) (0.5 mM each) (Martins et al., 1990). Growth medium was supplemented with \( \text{CuCl}_2 \) (final concentrations ranging from 0 to 5 mM). Liquid inocula were prepared in growth medium without \( \text{CuCl}_2 \) supplementation as described elsewhere (Leitão et al., 1992).

**Effects of \( \text{Cu}^{2+} \) on \( P. \) aeruginosa 8821M growth and alginate production and properties.** Growth in the absence or presence of \( \text{Cu}^{2+} \) supplementation was monitored by measuring culture \( \text{OD}_{600} \). The specific growth rates of \( P. \) aeruginosa 8821M in media with or without \( \text{Cu}^{2+} \) 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 \( \text{CuCl}_2 \). Results are given as median values of at least two different cultures grown at each \( \text{CuCl}_2 \) concentration.

Alginate concentration was determined after ethanol precipitation from cell-free supernatants by the modified carbazole method (Knight & Jeann, 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 \( \text{Cu}^{2+} \) supplementation. The viscosity of aqueous solutions (3 g l\(^{-1}\)) of the alginites 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 \( \text{Cu}^{2+} \), was measured at 30 °C using a cone and plate Brookfield Viscometer, model L VVT, 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 CuCl₂ and harvested when the culture OD₆₆₀ reached the standardized value 1.00 ± 0.05. Cell pellets obtained by centrifugation of two independent cultures at the various concentrations of Cu²⁺ 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/cm² (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 ± SD of at least three independent Northern experiments using RNA extracts prepared from cells obtained from two independent cultures at the same CuCl₂ 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 CuCl₂ (0-5 mM) and harvested when the culture OD₆₆₀ reached the standardized value 1.50 ± 0.05. The in vitro effect of increasing concentrations of Cu²⁺ was assessed by the addition of CuCl₂ 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 Cu²⁺-grown cells, based on the Cu²⁺ 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 ± 2 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 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 CuCl₂ 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 (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 ± SD of at least three enzyme assays.

Determination of Cu²⁺ concentration in crude extracts. The concentration of Cu²⁺ in crude extracts prepared, as for the enzyme assays, from cells of P. aeruginosa 8821M grown with increasing concentrations of CuCl₂ was determined by atomic absorption spectroscopy at the Laboratório de Análises, Instituto Superior Técnico, Lisbon, Portugal. Each value is given as the mean ± 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 CuCl₂ supplementation.

**RESULTS**

Effect of Cu²⁺ on P. aeruginosa 8821M growth and on alginate production and properties

Growth medium supplementation with concentrations of CuCl₂ 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 Cu²⁺ and not to the counterion Cl⁻. The supply of Cu²⁺ as CuSO₄ had identical effects on growth and the addition of the same concentrations of MgCl₂, MnCl₂ or CaCl₂ was not inhibitory (Martins et al., 1990).

Alginate production during batch growth decreased with increasing CuCl₂ concentration (Fig. 1b). At or above 4.0 mM, no alginate production was detectable (Fig. 1b). Since Cu²⁺ 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

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**Fig. 1.** Effects of the addition of increasing concentrations of CuCl₂ to the growth medium of P. aeruginosa 8821M on (a) the specific growth rate (μ) (O) and the culture OD₆₆₀ 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 alginites (3 g l⁻¹) (O).
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 (Leitião & 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. 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 (Leitião & 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. 2a). 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

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**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* (○), *algC* (●), *algD* (■) and *algR1* (▲) 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 ± 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.
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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 CuCl₂ and harvested when the culture reached the standardized OD₆₆₀ of 1.5±0.05.

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 CuCl₂ supplementation, when increasing concentrations of CuCl₂ were added to the enzyme assay mixture.

Fig. 5. Concentration of Cu²⁺ in crude extracts prepared from cells of *P. aeruginosa* 8821M grown in media supplemented with increasing concentrations of CuCl₂. Cu²⁺ concentration was determined by atomic absorption.

Fig. 6. Specific activities (relative values) of PMI (○), PMM (●), GMP (△) and GMD (■) that would be present in crude extracts, prepared from Cu²⁺-grown cells of *P. aeruginosa* 8821M, if the inhibitory effect of the Cu²⁺ 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 Cu²⁺ concentrations. The concentrations of Cu²⁺ present in the various crude extracts were determined by atomic absorption spectrophotometry (Fig. 5). In the range of concentrations studied, Cu²⁺ 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 Cu²⁺ was eliminated (Fig. 6). Comparing these putative values (Fig. 6) with the steady-state mRNA levels from the structural genes in Cu²⁺-grown cells (Fig. 2), some discrepancies were found. After discounting the inhibitory effect that Cu²⁺ has on PMI and GMP, the activity of PMI was much more affected than the activity of GMP in Cu²⁺-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 Cu²⁺ 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 Cu²⁺ with the xanthine/xanthine oxidase superoxide-generating system (McCord & Fridovich, 1969) and comparing the residual activities. The higher sensitivity of PMI to oxidative inactivation as compared with
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 Cu²⁺ (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 Cu²⁺ (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 CuCl₂-supplemented medium is also consistent with the decrease of alginate production by Cu²⁺-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 CuCl₂ 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 7ₐ 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 Cu²⁺-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 CuCl₂ added to the growth medium was higher than 2 mM. The bell-shaped curves representing the effect of Cu²⁺ 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 Cu²⁺-stressed and unstressed cells. According to this hypothesis, the algA and algD transcripts would be the most sensitive. The specific pattern of Cu²⁺-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 Cu²⁺ 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 & Konyesnitsi, 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 Cu²⁺.

Despite the higher level of expression of algA, algC and algD in cells grown in medium supplemented with concentrations of CuCl₂ below 2 mM, the production and degree of polymerization of alginate were significantly affected. The decrease of alginate synthesis at low CuCl₂ concentrations is, however, consistent with the decreased activities of alginate enzymes determined in crude extracts from cells grown with concentrations of CuCl₂ below and above 2 mM. Experimental evidence obtained during this study indicates that the lower activity of alginate enzymes in Cu²⁺-stressed cells results from the direct inhibitory effect of Cu²⁺ together with the Cu²⁺-induced oxidative inactivation of the enzymes.
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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 Cu$^{2+}$-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 Cu$^{2+}$-stressed cells might result from the reduction of their polymerization activity. This could be due to the inhibition of the polymerization enzymes complex by Cu$^{2+}$ (May et al., 1994) and/or to its oxidative inactivation. In addition, it is also very likely that phospholipid peroxidation by Cu$^{2+}$-induced oxygen reactive species (Halliwell & Gutteridge, 1989; Storz et al., 1990) affects lipid–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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