Effects of growth temperature on alginate synthesis and enzymes in *Pseudomonas aeruginosa* variants

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Spontaneous variation of the level of alginate synthesis in *Pseudomonas aeruginosa* was associated with changes in the activity of all four enzymes leading to synthesis of GDP-mannuronic acid, the activated precursor for polymerization. For the high-alginate-producing variant 8821M, alginate yield and properties, as well as the levels of alginate enzymes, were dependent on growth temperature. In contrast, levels of alginate and enzymes in the mucoid parent strain 8821 were very low and near temperature-independent. The difference in the specific activity of GDP-mannose dehydrogenase (GMD), encoded by the *algD* gene, between the two strains was associated with the alginate biosynthetic ability and with the degree of activation of the *algD* promoter, measured using the *algD*-*xyIE* transcription fusion on plasmid pVD2X. Maximal activity of the four enzymes was observed in strain 8821M grown at 30 °C, a temperature below the optimum for growth (35 °C). The effect of temperature on GMD activity could not be explained by the regulation of the *algD* promoter by temperature, since expression of pVDZX appeared to be more active at 35 °C, when the decrease of pVD2X copy number with increasing temperature was taken into account. The involvement of enzymes that catalyse steps downstream from the formation of the activated precursor should also be considered, as suggested by differences in the molecular mass of alginates synthesized by the two strains at various temperatures. Acetyl content of alginates increased as temperature decreased and strain 8821M produced the highest levels of acetylated polymers. The degree of acetylation appeared to be related to growth rate and could reflect acetyl-CoA availability.

Introduction

During synthesis of exopolysaccharides at the cell membrane, there is a requirement for activated precursors formed in the cytoplasm (Sutherland, 1990). For alginate synthesis in *Pseudomonas aeruginosa*, GDP-mannuronic acid is thought to be the precursor (DeVault *et al.*, 1989). Transcriptional activation of the *algD* gene, encoding GDP-mannose dehydrogenase (GMD) which catalyses the irreversible oxidation of GDP-mannose to GDP-mannuronic acid, has been shown to be involved in the expression of the mucoid phenotype in *P. aeruginosa* (Deretic *et al.*, 1991; DeVault *et al.*, 1989). The very low activities of the enzymes leading to GDP-mannuronic acid in *P. aeruginosa* are difficult to assay (Sá-Correia *et al.*, 1987). Nevertheless, comparison of the

specific activity of GMD in the mucoid cystic fibrosis isolate *P. aeruginosa* 8821 with the activities in non-mucoid (8821N or 8822) and highly mucoid (8821M) spontaneous variants (Sá-Correia *et al.*, 1987; Martins *et al.*, 1990; Alves *et al.*, 1991) supports the role of *algD* in regulation of alginate production. The involvement of the other GDP-mannuronic acid biosynthetic enzymes in the expression of the mucoid phenotype has not been fully proved. These enzymes catalyse the conversion of fructose 6-phosphate to mannose 6-phosphate (phosphomannose isomerase, PMI), mannose 6-phosphate to mannose 1-phosphate (phosphomannomutase, PMM) and mannose 1-phosphate to GDP-mannose (GDP-mannose pyrophosphorylase, GMP). They were almost undetectable in mucoid (8821) and non-mucoid (8822) strains (Sá-Correia *et al.*, 1987).

Recently we found that mucoid strain 8821 spontaneously produced not only the non-mucoid variants already reported (DeVault *et al.*, 1989; Deretic *et al.*, 1991), but also variants producing exceptionally large amounts of alginate (Alves *et al.*, 1991). Among them, strain 8821M exhibited the highest GMD specific

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Abbreviations: CDO, catechol 2,3-dioxygenase; GMD, GDP-mannose dehydrogenase; GMP, GDP-mannose pyrophosphorylase; PMI, phosphomannose isomerase; PMM, phosphomannomutase.

activity while the lowest activity was detected in the non-mucoid variant 8821N (Alves *et al.*, 1991). Temperature strongly influenced alginate production in these strains (Alves *et al.*, 1991). In the present work, the specific activities of the four enzymes involved in GDP-mannuronic acid synthesis were compared in the two mucoid strains, 8821 and 8821M, grown at different temperatures. Differences in the activation of the *algD* promoter at the various temperatures were studied by using the *algD*-*xylE* transcription fusion (Deretic *et al.*, 1987). The yield and characteristics of the alginates produced by both strains at the different temperatures were compared.

Methods

Bacterial strains, plasmids, media and inocula. The alginate-producing *P. aeruginosa* strains 8821 and 8821M, and their recombinants harbouring plasmid pVD2X (with the *algD*-*xylE* transcription fusion) were used. Mobilization of pVD2X from *E. coli* into *P. aeruginosa* was performed by triparental filter matings (Martins & Sá-Correia, 1991) using *E. coli* HB101(pRK2013) as the helper strain (Figurski & Helinski, 1979). Transconjugants were selected on PIA (*Pseudomonas* Isolation Agar, Difco) plates with tetracycline (300 mg l⁻¹, Sigma). Phenotypically well-defined colonies grown on PIA plates (supplemented with tetracycline in the case of recombinant cells) were used to inoculate LB (Gibco) medium (supplemented with 50 mg tetracycline l⁻¹ for recombinant cells) and the cultures were grown overnight with agitation. Bacteria were recovered by centrifugation and were resuspended in 100 ml growth medium, in a 250 ml Erlenmeyer flask to an initial OD₆₄₀ of 0.30 ± 0.02. Growth medium consisted of LB with the addition of 1% (w/v) glucose, MgCl₂ and MnCl₂ (0.5 mM each) (Martins *et al.*, 1990).

Growth and alginate production. Growth was monitored by measuring the OD₆₄₀ of the culture. Specific growth rates were calculated by least-square fitting to the linear part of semi-log growth plots. Alginate was measured in stationary-phase cultures after ethanol precipitation from cell-free supernatants, by the modified carbazole method (Knutson & Jeanes, 1968) using sodium alginate from *Laminaria hyperborea* (BDH-Merck) as standard. Results are means of three independent analyses.

Characterization of alginates. The viscosity of aqueous solutions (3 g l⁻¹) of alginate, isolated from cultures in which alginate production had just reached the maximal value, was measured at 30 °C using a cone and plate Brookfield Viscometer, model LVIIT, at a shear rate of 24 s⁻¹. Results are median values of determinations using two or three independently prepared alginate solutions. The acetyl content of the various alginates, expressed as acetate content, was determined as described by McComb & McCready (1957) using β-D-glucose pentaacetate (Sigma) as standard. Results are median values of at least two independent determinations.

Enzyme assays. Alginate enzymes. PMI, PMM, GMP and GMD were assayed in crude cell extracts by the methods of Sá-Correia *et al.* (1987) as modified by Martins *et al.* (1990). Crude extracts were prepared from bacteria grown as for alginate production and harvested at an OD₆₄₀ of 1.55 ± 0.05 (late exponential phase), by the method of Sá-Correia *et al.* (1987) as modified by Martins *et al.* (1990). One unit of activity was defined as the amount of enzyme that reduced 1 μmol NAD or NADP per min under the assay conditions.

Catechol 2,3-dioxygenase (CDO). CDO was assayed as described by Nozaki (1970) at 24 °C in extracts prepared from cells of strains 8821 and 8821M harbouring pVD2X grown in the presence of tetracycline and harvested at an OD₆₄₀ of 1.55 ± 0.05. One unit of activity was defined as the amount of enzyme that converted 1 μmol catechol into 2-hydroxymuconic semialdehyde per min.

Quoted specific activities are median values of at least three enzyme assays and three protein determinations. Protein concentration in crude cell extracts was estimated by the method of Bradford (1976) with BSA Fraction V (Merck) as standard.

pVD2X copy number. The copy number of pVD2X in cells of strain 8821M grown at different temperatures was determined by a modification of the method of Koizumi *et al.* (1985). Whole-cell lysates were subjected to agarose gel electrophoresis and ethidium-bromide-stained gels were analysed by fluorescence densitometry. The plasmid copy number (N_p) per chromosome equivalent was calculated from the equation $N_p = M_c K_p / M_p K_c$, where K_p and K_c are the proportionality constants for the linear relationship between peak area and dilution of the whole-cell lysates for the plasmid and the chromosome, respectively, M_c is the molecular mass of the chromosome [taken as 3624 kb (Pemberton, 1974)] and M_p is the molecular mass of the plasmid [32.2 kb (Deretic *et al.*, 1987)].

Whole-cell lysates for electrophoretic analysis were prepared by a modification of the method of Goldberg & Ohman (1984). Bacteria from 20 ml of culture were harvested at an OD₆₄₀ of 1.55 ± 0.05. The DNA concentration of lysates averaged 400 ± 50 μg ml⁻¹, as determined by A₂₆₀. Dilutions of these lysates were carried out in order to obtain between 0.3 and 1.25 μg or between 2 and 10 μg DNA in 50 μl in order to calculate K_c and K_p , respectively. Electrophoresis in agarose gels (0.4%, w/v) was carried out at room temperature for 14–16 h at 25 mA in TAE buffer (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide (1 μg ml⁻¹ in TAE) for 30 min and destained in TAE buffer for an additional 30 min. The fluorescent bands were photographed using Polaroid type 665 film. The negatives were scanned along the lane axis for plasmid and chromosome bands using a densitometer (Hoeffer Scientific Instruments, GS300) and peak areas were integrated using an integrator (Hewlett Packard HP3394A) connected to the densitometer.

Results

Effects of temperature on the yield and characteristics of the alginates synthesized by variants

The temperature dependence of growth and alginate production by the mucoid *P. aeruginosa* 8821 and its spontaneous highly mucoid variant 8821M (Fig. 1) was similar to that reported by Alves *et al.* (1991) who had used a production medium lacking the Mg²⁺ and Mn²⁺ supplementation necessary for higher levels of production (Martins *et al.*, 1990). At all temperatures studied, the specific growth rate of strain 8821M was lower than that of 8821, suggesting competition for precursors and energy between alginate synthesis and growth. As reported by Alves *et al.* (1991), the optimal temperature for alginate production (20–25 °C) was lower than the optimal temperature for growth (35 °C) in the case of strain 8821M, but not for 8821 (Fig. 1). Cultures of strain 8821M grown at 30 °C produced alginate with the

Fig. 1. Temperature profiles of (a) specific growth rates (μ) (●, ○) and alginate production (■, □) of strains 8821 (●, ■) and 8821M (○, □), (b) viscosity (centipoise, cP) of solutions of alginates (3 g l^{-1}) synthesized by strains 8821 (●) and 8821M (○), and (c) acetyl content of strains 8821 (●) and 8821M (○).

Fig. 2. Acetyl content of the alginates synthesized by *P. aeruginosa* 8821 (●) and 8821M (○) as a function of their specific growth rates (μ) calculated at different growth temperatures.

highest viscosity in aqueous solution (3 g l^{-1}). Strain 8821 produced, over the range of temperatures, lower concentrations of alginate, and the polymer synthesized had a lower viscosity, than strain 8821M (Fig. 1). For this low-alginate producer, the optimal temperatures for production, growth and high viscosity (displayed by solutions of isolated alginate) were coincident (35°C).

The acetyl content of the alginates increased as growth temperature decreased and alginate from strain 8821M had the higher acetyl content (Fig. 1). The acetyl content showed an inverse linear relationship with the specific growth rate over the range $20\text{--}35^{\circ}\text{C}$ (Fig. 2). The only exception was observed at 40°C , above the optimal temperature (Alves *et al.*, 1991). The relationship between acetylation and growth rate was similar for the

Fig. 3. Specific activities of GDP-mannuronic-acid-forming enzymes in *P. aeruginosa* 8821 and 8821M grown at different temperatures and assayed at these same temperatures. ○, PMI; ●, PMM; □, GMP; ■, GMD.

two mucoid strains, but the acetyl content exhibited for identical growth rates was lower in alginate synthesized by the low-alginate-producing strain, 8821 (Figs 1 and 2). Although other explanations cannot at present be eliminated, this discrepancy could be based on the methodology used to calculate the specific growth rates. This growth parameter was calculated during the first 3–4 h of batch growth and most of the alginate, produced in large amounts by strain 8821M, was synthesized after this period. It is therefore expected that the specific growth rate of the cells that synthesize most of the alginate during the batch growth was lower than calculated, due to the increase of medium viscosity and consequent decrease of the oxygen transfer rate. No relationship was found between the degree of acetylation of the alginates and the viscosity exhibited by their aqueous solutions (Fig. 1).

Relationship between growth temperature and the activity of alginate enzymes

Enzymes that led to GDP-mannuronic acid synthesis were assayed in crude extracts prepared from cells of strains 8821 and 8821M grown at various temperatures. The specific activities of all the enzymes were higher in the 8821M highly mucoid strain than in 8821 (Fig. 2). In bacteria grown at 37°C (Sá-Correia *et al.*, 1987; Martins *et al.*, 1990) the specific activities are very low, even in cells of the high-alginate-producing strain 8821M (Fig. 3 and Martins *et al.* 1990). Growth at 30°C significantly increased the levels of the four alginate enzymes in cells of strain 8821M but not in 8821 (Fig. 3), which exhibited very low levels of alginate enzyme activity, almost independent of the growth temperature. In order to correlate the level of alginate enzyme activities with the alginate yield at various temperatures, the specific activities of the alginate enzymes were determined at the same temperatures as those used for growth (Fig. 3). Maximal activity of all four enzymes in strain 8821M

Fig. 4. Specific activities in crude cell extracts of (a) GMD and (b) CDO in recombinants harbouring pVD2X of strains 8821 (■, □) or 8821M (●, ○). Bacteria were grown at different temperatures and assayed at 30 °C for GMD or 24 °C for CDO. Δ — Δ represents the value of CDO specific activity, in extracts prepared from cells of strain 8821M(pVD2X), divided by the copy number of the plasmid.

was also observed in cells grown at 30 °C when enzymes were assayed at the standard temperature of 30 °C. The optimal temperature for GMD activity is 50 °C (Roychoudhury *et al.*, 1989), while for PMI and GMP it is in the range 35–40 °C (J. H. Leitão, unpublished). It was also at 30 °C that 8821M synthesized alginate leading to solutions with the highest viscosity, although the maximal alginate yield was observed for lower temperatures (20–25 °C). Both the yield and viscosity of the alginates synthesized by the low-alginate-producing strain, 8821, slightly increased at 35 °C and this was correlated with enzyme activities (Figs 1 and 3).

Activation of the *algD* promoter at different growth temperatures

The level of expression of the *algD*–*xylE* transcription fusion, measured as the activity of CDO, was associated with the ability of strains 8821 and 8821M to produce alginate. A correlation was observed between the transcriptional activation of *algD*–*xylE* in 8821 or 8821M and the specific activity of GDP-mannose dehydrogenase in the two backgrounds (Fig. 4).

However, when the copy number of pVD2X carrying the *algD*–*xylE* gene fusion was taken into account, the results suggested that the effect of growth temperature on the specific activity of GMD in strain 8821M could not be explained by the transcriptional regulation of the *algD* promoter by temperature. Since copy number is strongly influenced by growth conditions (Zabriskie & Arcuri, 1986), the number of copies of pVD2X per cell of strain 8821M grown at the various temperatures was measured. There were four copies at 25 °C, three at 30 °C and one at 35 °C. The specific activities of CDO at different temperatures were therefore divided by the respective copy number in order to take into consideration the

different number of copies of the gene coding for CDO present in cells grown at the various temperatures (Fig. 4). The values calculated suggested that *algD* transcription was greatest at 35 °C, although the maximal specific activity of GMD was observed in cells grown at 30 °C.

Discussion

Variation of the level of alginate synthesis in *P. aeruginosa* was associated with changes in the activity of all four enzymes that led to GDP-mannuronic acid formation and not only of GMD (DeVault *et al.*, 1989; Deretic *et al.*, 1991). The model of transcriptional activation of *algD* in the mucoid versus the non-mucoid variants (Deretic *et al.*, 1987) was here extended to mucoid variants capable of producing different yields of alginate.

Although critical for alginate synthesis, the formation of GDP-mannuronic acid cannot be considered the only rate-limiting step since the levels of the enzymes assayed only partially explained differences observed in alginate synthesis by both variants at the various temperatures. The highest viscosities were observed with the alginates produced by strain 8821M, particularly at 30 °C. This combination of strain and temperature led to the highest levels of the enzymes involved in GDP-mannuronic acid synthesis, suggesting that polymerization activity might possibly be under a similar control and/or dependent on the GDP-mannuronic acid pool. Although no information is available about the molecular mass of the alginates produced, the biopolymers that led to higher viscosity solutions probably had the higher degree of polymerization (Sutherland, 1990). Viscosity was not proved to be dependent on the acetyl content of the polymer and it is likely that the mannuronic acid content of all the alginates was close to 100% (Sutherland, 1990).

The optimal temperatures for alginate production, high levels of enzyme activity and high viscosity were coincident for the low-alginate-producing strain 8821. Strain 8821M, however, produced higher alginate yields within the range 20–25 °C, below the optimal temperature for growth (35 °C), while the alginates that led to the highest viscosity were synthesized at 30 °C. The temperature for maximal activity of GDP-mannuronic acid biosynthetic enzymes was also 30 °C. Competition between growth and alginate synthesis could possibly explain these results, since alginate production was growth-associated. Therefore, the yield of biopolymer might be higher at temperatures suboptimal for cell growth, although exhibiting lower molecular mass.

The acetyl content of the alginates appeared to be related to the maximal specific growth rate. Since the slower growing cells exhibited the higher acetyl content,

and considering the activated precursor acetyl-CoA as the probable source of acetyl in the final polymer (Sutherland, 1990), the degree of acetylation might reflect acetyl-CoA availability.

The synthesis of alginate enzymes was nearly independent of temperature in strain 8821, in which *algD* transcription was repressed. In contrast, temperature significantly affected the synthesis of all four enzymes in strain 8821M, which were stimulated at temperatures below the optimum for growth. A similar pattern of temperature regulation for extracellular proteases and periplasmic phosphatases was recently observed in the psychrotrophic *Pseudomonas fluorescens*, where growth temperature rather than growth rate regulated synthesis (Gugi *et al.*, 1991). The control exerted by growth temperature on GMD synthesis could not be explained by regulation of the *algD* promoter. In fact, considering the decrease of recombinant plasmid pVD2X copy number with increasing temperature, the transcription of *algD* appeared to be more active at higher temperatures (35 °C). The activation at high temperatures of the transcription of the glutamine transport system (*glnQH*) operon of *Bacillus stearothermophilus* was also recently reported (Wu & Welker, 1991). The dependence of alginate enzyme synthesis on growth temperature should be considered as the result of temperature effects on the transcription rate of the alginate genes on the chromosome (Deretic *et al.*, 1991).

Although the number of plasmids per chromosome is primarily a function of the plasmid genetic make up, it can be strongly influenced by host strain physiology and growth conditions (Zabriskie & Arcuri, 1986). Several authors have reported that the plasmid copy number decreased with increasing growth rate (Zabriskie & Arcuri, 1986). The number of recombinant plasmids derived from pMMB24 was also found to increase in *P. aeruginosa* when growth temperature decreased (J. H. Leitão, A. M. Fialho and I. Sá-Correia, unpublished results), consistent with the pattern of dependence reported here for pVD2X copy number. Temperature effects on plasmid copy number are possibly related to growth kinetics; decreasing copy number accompanying growth rate increases with increasing temperature. These results show that caution should be taken in the interpretation of results obtained under different environmental conditions when using transcription fusions in plasmids.

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