0273-2289/94/4901—0051$05.60

Studies on Some Enzymes of Alginic Acid Biosynthesis in Mucoid and Nonmucoid *Azotobacter chroococcum* Strains

A. PECIÑA AND A. PANEQUE*

Instituto de Bioquimica Vegetal y Fotosintesis, Universidad de Sevilla-Consejo Superior de Investigaciones Cientificas, Apdo. 1113, 41080 Sevilla, Spain

Received February 24, 1993; Accepted January 25, 1994

ABSTRACT

Measurements of enzymes involved in alginate biosynthesis were straightforward in mucoid (alginate-positive) *Azotobacter chroococcum* ATCC 4412 crude extracts. At the stationary growth phase, where the production of the exopolysaccharide was greatest, the enzymes phosphomannose isomerase and GDP-mannose pyrophosphorylase increased markedly, whereas phosphomannomutase and GDP-mannose dehydrogenase kept the high activity levels measured in the acceleration growth phase. In nonmucoid (alginate-negative) *A. chroococcum* and *A. vinelandii* strains, the activities of phosphomannose isomerase and GDP-mannose pyrophosphorylase were rather low or, in some cases, undetectables. Except in *A. chroococcum* MCD1, which exhibited a low activity, phosphomanomutase was high in the nonmucoid *Azotobacter* strains, and GDP-mannose dehydrogenase reached a significant activity level in two out of four nonmucoid strains tested. The results suggest that derepression of phophomannose isomerase and GDP-mannose pyrophosphorylase is a *sine qua non* condition for alginate formation by *A. chroococcum*.

Index Entries: Alginate; biosynthetic enzymes; mucoid strains; nonmucoid strains; *Azotobacter chroococcum*.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

The production of microbial exopolysaccharides has been identified as an attractive and potential growth area for the fermentation industry (*1,2*). These compounds are commercially useful for producing gels and modifying the rheological properties of aqueous systems. Since these biopolymers can be readily available independent of sunlight and climates, they have the potential to replace the polysaccharides from marine algae traditionally used in the food, textiles, pharmaceutical, and oil industries (*1*).

Among the exopolysaccharides, microbial alginate is the product of two groups of bacteria: *Azotobacteriaceae* and *Pseudomonadaceae* (*3–6*). Although production of alginate by *Azotobacter* is important under a biotechnological point of view, a purpose of the research in *P. aeruginosa* is to look for inhibitors of alginate synthesis with potential use as therapeutic agents, because the biopolymer is a major virulence factor in cystic fibrosis patients infected with this bacterium (*7*). Bacterial alginates are acetylated polymers of β-1,4-linked residues of D-mannuronic acid and its C-5 epimer, L-guluronic acid. Their arrangement as well as their relative amounts vary greatly in alginates from different sources. Evidence has been presented for the location of *O*-acetyl groups on some of the D-mannuronosyl residues (*1*).

In certain species of marine algae (*8*) and in the bacteria *P. aeruginosa* (*9*) and *Azotobacter vinelandii* (*10*) the biosynthetic pathway of alginic acid has been studied. The results of these studies demonstrated considerable similarity in the pathway leading to the synthesis of GDP-mannuronic acid in each organism. In the pathway for alginate byosynthesis, the glycolytic intermediate fructose 6-phosphate is first converted to mannose 6-phosphate by the action of phosphomannose isomerase (PMI). Mannose 1-phosphate is then formed by the action of phosphomannomutase (PMM) and further esterified with GTP by GDP-mannose pyrophosphorylase (GMP). Polymannuronic acid is then synthesized by the action of GDP-mannose dehydrogenase (GMD) and alginate polymerase. A bifunctional protein is responsible for the activities of PMI and GMP in *P. aeruginosa*, the microorgaism from which the gene cluster involved in the synthesis of alginate has been characterized (*11*).

In a previous work from our group, the effect of environmental conditions on the biopolymer production by *A. chroococcum* was investigated (*12*). Under the experimental conditions used in that work, which are the same as we use in the present article, only a type of alginate-like exopolysaccharide was produced by *A. chroococcum* (*12*, and refs. therein). Since no research on the biochemical and genetic characterization of the alginate biosynthesis in this bacterium has been done, we have examined now some of the enzymes involved in the process in *A. chroococcum* ATCC 4412, a particularly mucoid, alginate-producing *Azotobacteriaceae*.

The results suggest that the sequence of reactions leading to the biosynthesis of alginate, as stated above, takes place also in *A. chroococcum*. In a comparative study using different strains of *A. chroococcum* and *A. vinelandii*, the *A. chroococcum* ATCC 4412 strain was shown to exhibit the highest activity levels of PMI and GMP and the largest amount of alginate produced.

MATERIALS AND METHODS

Microorganisms

Azotobacter chroococcum strain ATCC 4412 (from the Valencia University Collection, Valencia, Spain), *A. chroococcum* strain MCD1 (a kind gift of R. Robson, Brighton, UK), *A. vinelandii* strain UW (a gift of W. J. Brill, University of Wisconsin, Madison, via the Department of Genetic, University of Sevilla, Spain), *A vinelandii* ATCC 12837 (a gift of B. R. Glick, Waterloo, Ontario), and *A. vinelandii* pGSS15 (a gift of B. R. Glick also, and that is a transformant of *A. vinelandii* ATCC 12837 constructed by himself with the broad-host-range plasmid pGSS15) were used throughout this study.

Growth Media

Stock cultures of bacteria were maintained on slants containing a combined nitrogen-free medium (*13*) supplied with 6% (w/v) sucrose as sole carbon and energy source, and solidified with 2% (w/v) Difco agar. A preculture of *A. chroococcum* was made by inoculating 100 mL of the nitrogen-free medium with an appropiate amount of stock culture in a 250-mL conical flask and shaking it at 30°C under air in a orbital shaker at 200 rpm. After 10–12 h, an appropriate volume of this cell suspension was transferred into 100 mL liquid medium in a 200 mm height × 40 mm diameter glass tube with pointed bottom and narrow (20-mm) mouth. Air at a rate of 147 L/L culture/h was then bubbled through it.

Polysaccharide Determination and Preparation of Cell Extracts

Cell suspensions were harvested by centrifugation at 10,000*g* for 10 min at 4°C. Sedimentation was improved by adding NaCl and EDTA to final concentrations of 100 and 10 m*M*, respectively. The supernatant was precipitated with 3 vol of propan-2-ol and washed with a mixture of propan-2-ol:water (3:1). The exopolysaccharide was determined after drying in an oven at 80°C to constant weight. The cell sediment was washed with 50 m*M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $MgCl_2$, and resuspended at a cell density of 1 g/mL of sonication buffer (50 m*M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $MgCl_2$, 1 m*M* D,L-dithiothreitol,

and 1 m*M* phenyl-methylsulfonyl fluoride). Cells were disrupted by sonication (20 kHz; 75 W) for 5 min (in 30-s periods) with a Branson sonifier model B 12. The homogenate was centrifuged at 33,000*g* for 45 min at 4°C, and the resulting supernatant freed from sugars and small molecules by fast filtration through a Sephadex G-25 gel using the method described by Penefsky (*14*). Enzyme assays were performed on fresh extracts prepared in this way.

Analytical Procedures

Activities of PMM, PMI, GMP, and GMD were assayed in crude extracts by recording increases in optical density at 340 nm (owing to the reduction of NAD or NADP) at 30°C in a Hewlett Packard Diode Array Spectrophotometer model 8452 A. Enzyme activities were calculated from the initial linear rates of cofactor reduction after subtraction of endogenous activities (which were measured in assays without substrate). Control assays lacking only the extracts were also carried out.

One unit of enzymatic activity was defined as that which reduced 1 μmol of NADP or NAD/min under the specified assay conditions. Specific activities are given as mU/mg of protein. Enzymes used in coupled reactions were added in excess relative to the enzymatic activity under measurement.

PMI activity was assayed by the method of Slein (*15*). The reaction mixture, in a total volume of 1 mL, contained 50 m*M* Tris/HCl buffer, pH 7.5, 10 m*M* $MgCl_2$, 0.5 U each of phosphoglucoisomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH), 1.0 μmol of NADP, and 0.12–1.2 mg of protein. The reaction was initiated by the addition of 3.0 μmol of D-mannose-6-phospate.

PMM acctivity was assayed by the method described by Pindar and Bucke (*10*). The reaction mixture, in a total volume of 1 mL, contained 50 m*M* Tris/HCl buffer, pH 7.5, 10 m*M* $MgCl_2$, 0.5 U each of PGI, PMI, and G6PDH, 1.0 μmol of NADP, 0.25 μmol of α-D-glucose-1,6-diphosphate, and 1.2-2.5 mg of protein. The reaction was initiated by the addition of 3.0 μmol of α-D(+)-mannose-1-phosphate.

GMP activity was assayed by the method of Munch-Petersen (*16*). The reaction mixture, in a total volume of 1 mL, contained 50 m*M* Tris/HCl buffer, pH 7.5, 10 m*M* $MgCl_2$, 0.1 μmol of ADP, 2 μmol of pyrophosphate, 5 μmol of sodium fluoride, 0.5 U each of hexokinase, nucleoside-5′-diphosphate kinase, and G6PDH, 0.8 μmol of glucose, 1.0 μmol of NADP, and 0.12–1.2 mg of protein. The reaction was initiated by the addition of 0.4 μmol of GDP-D-mannose.

GMD activity was assayed by the method of Preiss (*17*). The reaction mixture, in a total volume of 1 mL, contained 50 m*M* Tris/HCl buffer, pH 7.5, 10 m*M* $MgCl_2$, 1.0 μmol of NAD, and 0.12–1.2 mg of protein. The reaction was initiated by the addition of 1.0 μmol of GDP-D-mannose.

Table 1
Effect of Period of Growth on Specific Activities of Alginate Biosynthetic Enzymes and Alginate Production in *A. chroococcum* ATCC 4412

	Specific activities, mU/mg[a]				
Growth, h	PMI	PMM	GMP	GMD	Alginate, g/L
16	0.5–3	10–20	1–3	0.8–4	0.6
24	0.5–3	10–20	1–2	0.2–0.7	0.6
48	7–17	7–20	2–5	0.2–2	5–8

[a]Five extracts from differents batches of bacteria at the indicated period of growth were prepared; duplicate assays were performed to within 3% for each extract. Protein concentrations in the crude enzyme preparations used were within the limits of 8.3–8.8, 17–20, and 0.5–2 mg/mL, at 16, 24, and 48 h, respectively. The results show the range of activities encountered.

The protein concentration of extracts was estimated by the method of Lowry et al. (*18*), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

We had previously reported that, in *A. chroococcum*, alginate production increased markedly after the cessation of growth (*12*). To check whether changes in polysaccharide concentration could be correlated with changes in the presumable biosynthetic activities, the effect of period of growth in which cells were harvested on enzyme activities and alginate production was examined. When the mucoid *A. chroococcum* ATCC 4412 strain was harvested either in the acceleration (16 h of growth) or midexponential (24 h) growth phase, the alginate released to the medium was 0.6 g/L, and PMI, PMM, GMP, and GMD activities were easily detectable (Table 1). Though the growth conditions were different, these activities were undetectable in *A. vinelandii* before the stationary growth phase (*19*). Table 1 indicates also that in the stationary growth phase (48 h of growth), exopolysaccharide production (5–8 g/L) was ten times higher than in the previous growth phases, PMI activity increased strongly, and GMP nearly doubled. No clear trend of changes in GMD activity was obtained. In this context, it is worth mentioning that in *P. aeruginosa*, GMD activity follows the growth curve (*20*), whereas *A. vinelandii*, as stated above, is undetectable in exponentially growing cells and reaches maximum levels in stationary-phase cells (*19*).

These data, therefore, support the existence of a close relationship between alginate production by *A. chroococcum* and the activity levels of PMI and GMP. To investigate this relationship further, nonmucoid strains of *A. chroococcum* and *A. vinelandii* were analyzed for PMI, PMM,

Table 2
Specific Activities of Alginate Biosynthetic Enzymes in Strains ATCC 4412 and MCD1 of *A. chroococcum* and UW, ATCC 12837 and ATCC 12837 pGSS15 of *A. vinelandii*, and Exopolysaccharide Production by These Strains

	Specific activities, mU/mg[a]				
Strain	PMI	PMM	GMP	GMD	Alginate, g/L
A. chroococcum					
ATCC 4412 (5)	7–17	7–20	2–5	0.2–2	5–8
MCD1 (3)	0.7–1.5	0.7–0.8	0.6–2	nd	0.1–0.3
A. vinelandii					
UW (4)	nd	7–9	nd	nd	0
ATCC 12837 (4)	0–1	30–40	nd	0.2–1	0.3–0.5
ATCC 12837 pGSS15 (4)	nd	20–25	nd	2–3	0.3–0.6

[a]The number of extracts prepared are shown in parentheses after each strain; duplicate assays were performed to within 3% for each extract. Protein concentrations of the crude enzyme preparations used were within the following limits: *A. chroococcum* ATCC 4412, 0.5–2 mg/mL; *A. chroococcum* MCD1, 2.5–3 mg/mL; *A. vinelandii* UW 15–20 mg/mL; *A. vinelandii* ATCC 12837, 8–10 mg/mL; *A. vinelandii* ATCC 12837 pGSS15, 7–9 mg/mL. The results are the range of activities encountered. nd: not detected.

GMP, and GMD activities and alginate production. In addition, *A. vinelandii* cells that had been transformed with the broad-host-range plasmid pGSS15 and appeared as large and mucoid colonies (*21*) was used. Table 2 shows that *A. chroococcum* MCD1 (*22*), a nonmucoid strain prepared by curing a derivative of a nongummy variant of *A. chroococcum* ATCC 4412, exhibited only 10% of the PMI and PMM activities found in its parent strain, approx 50% of GMP activity, and undetectable GMD activity. Alginate produced by MCDl was rather low, 0.1–0.3 g/L. In the nonmucoid *A. vinelandii* UW strain, which displayed appreciable PMM activity and no alginate production, PMI, GMP, and GMD activities were lacking or at least, under the detection limits of the assay methods.

Table 2 illustrates also that in *A. vinelandii* strain ATCC 12837, PMI activity was rather low as compared with that present in *A. chroococcum* ATCC 4412 and that GMP activity was absent. In this strain, measurable GMD and quite high PMM activities did not promote a significant alginate production (0.3–0.5 g/L). *A. vinelandii* pGSS15, constructed from *A. vinelandii* ATCC 12837 as referred to above, did not exhibit PMI and GMP activities at all, whereas PMM and GMD reached rather high activity levels. Genetic transformation of *A. vinelandii* had been described to perturb the organism's metabolic capabilities severely with the production of extracellular slime being the only cellular process that seemed to be augmented (*21*). Under the experimental conditions used in our laboratory, however, only a low amount (0.3–0.6 g/L) of alginate was produced by the transformed cells, as seen in Table 2. This is not surprising

because, for example, in *Pseudomonas* NCIB 11264 (*23*) and in *A. chroococcum* (*12*), the amount of polysaccharide produced is dependent, among other things, on environmental factors.

Taken together, these results suggest that the alginate biosynthetic pathway in *A. chroococcum* is similar to the proposed pathway for *A. vinelandii* (*10*). Since the nonmucoid strains we used exhibited low or, in some cases, undetectable levels of PMI and GMP, which were high in the alginate-producing *A. chroococcum* wild-type cells, our data suggest that repression or loss of activation of the synthesis of both enzymes is responsible for the absence of mucoidy as is the case for *P. aeruginosa* (*11*) and *A. vinelandii* (*19,24*). Obviously, for the alginate biosynthesis to take place, an adequate activity level of all of the enzymes involved, including those responsible for polysaccharide polymerization and excretion, has to be derepressed. Finally, the parallelism observed in PMI and GMP through the growth phases is in agreement with the hypothesis that, in *P. aeruginosa,* the phosphomannoisomerase gene codes for a single protein with two separate enzymatic activities, namely PMI and GMP (*7,11*).

ACKNOWLEDGMENTS

This work was supported by grant BIO91/0914 from Comisión Interministerial de Ciencia y Tecnologia, and by a financial help from Plan Andaluz de Investigación (Spain). We thank M. G. de la Vega for her collaboration during the first stages of this work, A. Garzón for his help in maintaining some strains used in this work, and J. de la Cruz for his assistance with the computer.

REFERENCES

1. Sutherland, I. W. (1985), *Ann. Rev. Microbiol.* **39,** 243–270.
2. Gacesa, P. (1988), *Carbohydr. Polymers* **8,** 161–182.
3. Sanford, P. A., Cottrell, I. W., and Pettitt, D. J. (1984), *Pure Appl. Chem.* **56,** 879–892.
4. Sutherland, I. W. (1986), *Microbial Sci.* **3,** 5–8.
5. Evans, L. R. and Linker, A. (1973), *J. Bacteriol.* **116,** 915–924.
6. Deretic, V., Gill, J. F., and Chakrabarty, A. M. (1987), *Biotechnol.* **5,** 469–477.
7. Zielinski, N. A., Mahara, R., Roychoudhury, Danganan, C. E., Hendrickson, W., and Chakrabarty, A. M. (1992), *J. Bacteriol.* **174,** 7680–7688.
8. Lin, T. Y. and Hassid, W. Z. (1966), *J. Biol. Chem.* **241,** 5284–5297.
9. Piggott, N. H., Sutherland, I. W., and Jarman, T. R. (1981), *Eur. J. Appl. Microbiol. Biotechnol.* **13,** 179–183.
10. Pindar, D. F. and Bucke, C. (1975), *Biochem. J.* **152,** 617–622.
11. Sa-Correia, I., Darzins, A., Wang, S-K., Berry, A., and Chakrabarty, A. M. (1987), *J. Bacteriol.* **169,** 3224–3231.

12. De la Vega, M. G., Cejudo, J., and Paneque, A. (1991), *Appl. Biochem. Biotechnol.* **30,** 273–284.
13. Cejudo, J., De la Torre, A., and Paneque, A. (1984), *Biochem. Biophys. Res. Commun.* **123,** 431–437.
14. Penefsky, H. S. (1977), *J. Biol. Chem.* **252,** 2891–2899.
15. Slein, M. W. (1955), *Methods Enzymol.* **1,** 299–306.
16. Munch-Petersen, A. (1962), *Methods Enzymol.* **5,** 171–174.
17. Preiss, J. (1964), *J. Biol. Chem.* **239,** 3127–3132.
18. Lowry. O. H., Rosenbrough, N. J., Farr, A. L., and Randall R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
19. Horan, N. J., Jarman, R. T., and Dawes, E. A. (1981), *J. Gen. Microbiol.* **127,** 185–191.
20. Pugashetti, B. K., Vadas, L., Prihar, H. S., and Feingold, D. S. (1983), *J. Bacteriol.* **153,** 1107–1110.
21. Glick, B. R., Brooks, H. E., and Pasternak, J. J. (1985), *J. Bacteriol.* **162,** 276–279.
22. Robson, R. L. Chesshyre, J. A., Wheeler, C., Jones, R., Woodley, P. R., and Postgate, J. R. (1984), *J. Gen. Microbiol.* **130,** 1603–1612.
23. Williams, A. G. and Winnpenny, J. W. T. (1980), *J. Gen. Microbiol.* **116,** 133–141.
24. Horan, N. J., Jarman, T. R., and Dawes, E. A. (1983), *J. Gen. Microbiol.* **129,** 2985–2990.