Characterization of the *Pseudomonas aeruginosa* Alginate (*alg*) Gene Region II

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Pseudomonas aeruginosa region II alginate genes are involved in the biosynthesis of the uronic acid containing exopolysaccharide, alginic acid. We have subcloned and overexpressed various DNA fragments contained within region II in an attempt to further characterize and more precisely localize the genes involved in alginate production. Overexpression of the genes controlling alginate biosynthesis within region II was accomplished by placing various cloned restriction fragments under the transcriptional control of the hybrid *trp-lac (tac)* promoter, and plasmid encoded proteins were examined in a maxicell expression system. We correlated various region II plasmid constructions with the ability to complement specific alginate negative (*alg*) mutants and code for polypeptides. Several proteins suspected of being involved in alginate production were encoded by sequences within region II. The results of this study further reveal that the transcriptional orientation of the *alg* loci within region II appears to be in the direction from *argF* to *pmi*. The specific activities of phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMP), two enzymes involved in the formation of the alginate precursor GDP-mannuronic acid, were measured in region II *alg* mutants and in cells overexpressing cloned segments from region II. Based on these enzyme measurements, we conclude that the remaining region II *alg* genes do not encode either PMM or GMP. These results support the suggestion that the remaining *alg* genes in region II are likely to be involved in post GDP-mannuronic acid processing events such as mannuronic acid transport, polymerization, secretion, epimerization and acetylation.

INTRODUCTION

Mucoid strains of *Pseudomonas aeruginosa* isolated from the sputa of cystic fibrosis (CF) patients secrete copious amounts of an exopolysaccharide known as alginic acid (Evans & Linker, 1973; Mian *et al.*, 1978). These strains are recognized by the gross morphologic aspects of the colonies, which have a raised, highly refractile and confluent appearance on agar solidified media. This mucoid phenotype is thought to represent the transformation of the typical *P. aeruginosa* into an encapsulated variant (Doggett *et al.*, 1971). These mucoid variants are rarely, however, found in non-CF infections despite prolonged colonization in several sites (Doggett, 1979; Iacocca *et al.*, 1963). The exopolysaccharide conferring the mucoid phenotype

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Abbreviations: PMI, phosphomannose isomerase; PMM, phosphomannose mutase; GMP, GDP-mannose pyrophosphorylase; GMD, GDP-mannose dehydrogenase; CF, cystic fibrosis; PIA, *Pseudomonas* Isolation Agar; IPTG, isopropyl β -D-thiogalactopyranoside.

Fig. 1. (a) Alginic acid biosynthetic pathway. F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDP-M, GDP-mannose; GDP-MA, GDP-mannuronic acid. (b) Genetic map of region II. The line at the top represents chromosomal DNA sequences from *alg* region II. The lines below the genetic map represent region II subclones in either pCP13 (solid rectangles), pMMB22 or pMMB24 (solid rectangles with *Ptac*) or pKK 223-3 (open rectangles). *Ptac*, hybrid *trp-lac* promoter; B, *Bgl*II; Bam, *Bam*HI; C, *Clal*; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; X, *Xho*I; Xb, *Xba*I; Xm, *Xma*I.

on these morphological variants is composed of selectively acetylated β -1,4-linked D-mannuronic acid and its C5 epimer L-guluronic acid (Linker & Jones, 1966). This polysaccharide is similar to the extracellular polymer which is produced by *Azotobacter vinelandii* (Gorin & Spencer, 1966) and certain species of brown algae (Drummond *et al.*, 1962).

Based on the pathway proposed by Lin & Hassid (1966), a pathway for alginate biosynthesis has been suggested to operate in *P. aeruginosa* (Piggott *et al.*, 1981; Sutherland, 1982). The

glycolytic intermediate fructose 6-phosphate is first converted to mannose 6-phosphate and then to mannose 1-phosphate by the action of the enzymes phosphomannose isomerase (PMI; EC 5.3.1.8) and phosphomannose mutase (PMM; EC 5.4.2.8), respectively (Fig. 1*a*). The next step involves the formation of guanosine-5'-diphosphomannose (GDP-mannose) from mannose 1-phosphate by GDP-mannose pyrophosphorylase (GMP; EC 2.7.7.13). GDP-mannose is then oxidized by GDP-mannose dehydrogenase (GMD; EC 1.1.1.132) to GDP-mannuronic acid, and alginic acid is then formed after polymerization of the mannuronic acid residues, epimerization of selected mannuronic acid residues to guluronic acid, and acetylation.

There are several genes, the function of which are essential for producing alginic acid in *P. aeruginosa*. These genes are positioned on the *P. aeruginosa* chromosome in at least two main regions. Region I is located at 19 min and has been postulated to contain an alginate regulatory element (Darzins & Chakrabarty, 1984). In addition, DNA within this region has recently been shown to be capable of undergoing amplification resulting in the establishment of the mucoid phenotype, and also to be capable of activating a promoter that controls the expression of the GDP-mannose dehydrogenase gene (Deretic *et al.*, 1986, 1987*b*). Region II has been mapped at 45 min of the *P. aeruginosa* PAO chromosome and contains a tight clustering of several genetic loci involved in alginate biosynthesis (Darzins *et al.*, 1985*b*). This region (Fig. 1*b*) has been shown to contain the phosphomannose isomerase (*pmi*) and GDP-mannose dehydrogenase (*gmd*) genes, whose products catalyse the first and last steps, respectively, in the production of GDP-mannuronic acid from fructose 6-phosphate (Darzins *et al.*, 1985*a*; Gill *et al.*, 1986; Deretic *et al.*, 1987*a*). In addition, the nucleotide sequence of the *P. aeruginosa* *pmi* gene as well as overexpression and identification of the gene product have recently been reported (Darzins *et al.*, 1986; Gill *et al.*, 1986).

The function of the remaining region II alginate genes, however, is largely unknown, except that they are important in the biosynthesis of alginic acid, since mutations in such genes render the cells phenotypically Alg⁻. In order to begin to understand the functional organization of the genes involved in the synthesis of this polysaccharide, we have characterized the gene products of region II by overexpressing the alginate genes using strong promoters.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *alg* mutants used in this study for genetic complementation were obtained by chemical mutagenesis of the stable mucoid strain 8830 and were described by Darzins & Chakrabarty (1984). *P. aeruginosa* strains were routinely grown on Pseudomonas Isolation Agar (PIA) plates (Difco) or in Luria broth (LB) liquid medium (Miller, 1972). Antibiotic concentrations used for maintaining plasmids in *P. aeruginosa* were 50 µg tetracycline ml⁻¹ and 300 µg carbenicillin ml⁻¹ (for growth on LB liquid medium) and 400 µg tetracycline ml⁻¹ and 1 mg carbenicillin ml⁻¹ (for growth on PIA plates). M9 minimal medium used for cultivating *Escherichia coli* was described by Miller (1972).

Manipulation of recombinant DNA. Plasmid DNA was isolated using a modification (Chatterjee *et al.*, 1981) of the method described by Casse *et al.* (1979). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and were used according to the manufacturers specifications. Calf intestine alkaline phosphatase, linker DNA and T4 infected *E. coli* polynucleotide kinase were obtained from Boehringer Mannheim. For end-labelling [γ -³²P]ATP was purchased from Amersham. Routine DNA manipulation, agarose gel electrophoresis and DNA end-labelling were done as described by Maniatis *et al.* (1982).

Genetic analysis of alginate biosynthetic genes. Triparental matings were done as described by Figurski & Helinski (1979) by using pRK2013 as a helper plasmid to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa*. Equal volumes of each parent were filtered onto a 0.45 µm filter (Millipore). The filters were placed onto a LB agar plate and incubated for 5–8 h before the filters were washed with 0.9% saline. Cells were plated on PIA selective plates supplemented with the appropriate antibiotic to determine the ability of the various region II constructs to complement various Alg⁻ mutants. IPTG (isopropyl β -D-thiogalactopyranoside; final concentration 5 mM) was incorporated into agar plates to derepress the *Ptac-lacI*^r system when required.

Maxicell analysis of *alg* gene products. Polypeptides synthesized from plasmid encoded genes were labelled by a modification of the *E. coli* maxicell technique described by Sancar *et al.* (1979). The *E. coli* maxicell strain CSR603 harbouring the relevant plasmid was grown to an OD₆₀₀ of 0.5 in M9 minimal medium containing 0.6% glucose, 1% Casamino acids and 5 µg thiamin ml⁻¹. After UV irradiation cycloserine was added to a final concentration of 200 µg ml⁻¹ to kill those cells surviving the UV treatment. The cultures were then incubated for an additional 16 h at 37 °C with aeration. Cells were collected by centrifugation and washed with Hershey's buffer (Worcel & Burgi,

Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Description	Reference
<i>E. coli</i>		
AC80	<i>thr leu met hsdR hsdM</i>	Chakrabarty <i>et al.</i> (1978)
DH1	F ⁻ <i>recA1 endA1 gyrA96 thi-1</i>	Low (1968)
CSR603	<i>recA uvrA6 phr-1</i>	Sancar <i>et al.</i> (1979)
<i>P. aeruginosa</i>		
8821	<i>his-1 Alg</i> ⁺	Darzins & Chakrabarty (1984)
8822	<i>his-1 alg-1</i> (spontaneous)	
8830	<i>his-1 Alg</i> ⁺ (stable)	
8835	<i>his-1 alg-5</i>	
8838	<i>his-1 alg-8</i>	
8846	<i>his-1 alg-16</i>	
8857	<i>his-1 alg-27</i>	
8874	<i>his-1 alg-44</i>	
8887	<i>his-1 alg-60</i>	Darzins <i>et al.</i> (1985 <i>b</i>)
8891	<i>his-1 alg-67</i>	
8893	<i>his-1 alg-71</i>	
8897	<i>his-1 alg-76</i>	
Plasmids		
pCP13	IncP Tc ^r Km ^r Cos ⁺	Friedman <i>et al.</i> (1982); Darzins & Chakrabarty (1984)
pKT240	IncQ Km ^r Ap ^r	Bagdasarian <i>et al.</i> (1983)
pMMB22	IncQ Ap ^r <i>Ptac lacI</i> ^q	
pMMB24	IncQ Ap ^r <i>Ptac lacI</i> ^q	
pKK223-3	ColE1 rep Ap ^r <i>Ptac</i>	Amman <i>et al.</i> (1983)

1974) lacking sulphate. After 1 h of sulphate starvation the cells were suspended in Hershey's buffer containing 0.6% glucose and 1 mM each of proline, leucine, threonine and arginine. IPTG was added to a final concentration of 1 mM when needed. [³⁵S]Methionine [Amersham; 1250 Ci mmol⁻¹ (46.25 TBq mmol⁻¹)] was added to a final concentration of 5 µCi ml⁻¹ (185 kBq ml⁻¹) and incubation was continued for another hour at 37 °C. The cells were washed twice with 100 mM-potassium phosphate buffer, pH 7.0, resuspended in loading dye and the proteins were denatured by boiling at 100 °C for 2 min. Samples were subjected to SDS-PAGE as described by Dreyfuss *et al.* (1984). [³⁵S]Methionine-labelled proteins were visualized by fluorography by using the water-soluble fluor sodium salicylate (Sigma) (Chamberlain, 1979).

Enzyme assays. The preparation of cell free extracts and the determination of PMM, GMP and GMD activities were done according to the methods described by Sa'-Correia *et al.* (1987). The protein concentration of the extracts was estimated according to the method of Bradford (1976). The protein assay reagent was obtained from Bio-Rad. Bovine serum albumin (BSA) was used as the protein standard. All phosphorylated sugars, nucleotides, sugar nucleotides and enzymes were obtained from Sigma.

RESULTS

Overexpression and size determination of alg gene products in the alg-8 and alg-44 region

Our laboratory has recently demonstrated that the activities of the four alginate biosynthetic enzymes – PMI, PMM, GMP and GMD – are normally present at low levels in mucoid *P. aeruginosa* cell free extracts (Sa'-Correia *et al.*, 1987). The two genes for two alginate biosynthetic enzymes, namely *pmi* and *gmd*, have been characterized and sequenced only after the appropriate genes were cloned downstream of the *trp-lac* hybrid promoter (*tac*), so that hyperproduction after IPTG induction of the gene products allowed clear detection of the relevant enzymic activities (Darzins *et al.*, 1986; Gill *et al.*, 1986; Deretic *et al.*, 1987*a*). These results indicated that the identification of each remaining *alg* gene function could ultimately depend on the ability to overproduce each gene product. The possible problem of low gene expression was, therefore, overcome by using plasmids which overexpressed *alg* functions from a strong promoter.

The recombinant plasmid pAD2 has previously been shown to restore alginate producing capabilities to several *alg* mutants (Darzins & Chakrabarty, 1984). The 9.5 kb *Hind*III fragment of pAD2, which resides within region II, was subcloned into the *tac* promoter-containing broad host range expression vector pMMB24. The resulting plasmids, pSK402 (Fig. 1*b*) and pSK401, contained the *tac* promoter at opposite ends of the pAD2 *Hind*III fragment. In the absence of IPTG, plasmids pSK402 and pSK401 were able to complement only those mutants previously shown to be complemented by pAD2 and no additional *alg* mutants were complemented upon IPTG induction. The DNA fragment of a subclone of pAD2 (designated pSK2005, Fig. 1*b*), which complemented only one of these *alg* mutants (*alg-5*), is located most distal from the phosphomannose isomerase gene (*algA*) nearest the *argF* gene on the *P. aeruginosa* chromosome (Darzins *et al.*, 1985*a*). Further subcloning of an internal 2.7 kb *Cla*I–*Xma*I fragment of the DNA insert of pSK2005 resulted in the construction of plasmid pSK123 which placed the *tac* promoter next to the *Cla*I site (Fig. 1*b*). Plasmid pSK123 was able to restore *alg-5* to Alg⁺ but only in the presence of IPTG. An analogous plasmid construct which placed the *tac* promoter adjacent to the *Xma*I site was unable to complement *alg-5* even in the presence of IPTG. These results suggest that the promoter of the *alg-5* complementing gene may reside upstream of the *Cla*I site and that the transcriptional orientation of *alg-5* is from the *Cla*I site toward the *Xma*I site. These observations are in agreement with the direction of transcription and sequence analysis of this fragment which has recently been shown to contain the gene (*gmd*) encoding GDP-mannose dehydrogenase (Deretic *et al.*, 1987*a*).

Located immediately downstream of *gmd* are two distinct alginate loci, *alg-8* and *alg-44*. Plasmid pSK2001, which contains a 4.2 kb *Xho*I–*Pst*I fragment of pSK402, complements *alg-8* (Darzins *et al.*, 1985*b*). The 4.2 kb fragment of pSK2001 was subcloned into pMMB24 and the resulting plasmids, designated pSK2021 (Fig. 1*b*) and pSK2022 (not shown), placed the *tac* promoter adjacent to the *Xho*I and *Pst*I sites, respectively. Plasmid pSK2021 was capable of complementing *alg-8* only upon IPTG induction of the *tac* promoter while pSK2022 failed to restore alginate production in the same mutant even upon IPTG induction. Furthermore, pSK2021 was also capable of complementing *alg-44* upon IPTG induction. Plasmid pAD5059 (Fig. 1*b*), which shares common region II sequences with pSK2021, complements only *alg-44* upon IPTG induction (Darzins *et al.*, 1985*b*). An additional plasmid that further localized the *alg-44* complementation function was constructed. A 1.9 kb *Bgl*II–*Pst*I fragment of the 4.2 kb *Xho*I–*Pst*I fragment of pSK2021 was subcloned into the *tac* containing, broad host range expression vector, pMMB22. The resulting construct, designated pSK311 (Fig. 1*b*), contained the *tac* promoter adjacent to the *Bgl*II site and was capable of complementing *alg-44* to Alg⁺ upon IPTG induction. These results, therefore, demonstrate that both *alg-8* and *alg-44* reside within the 4.2 kb *Xho*I–*Pst*I fragment of pSK2021 and further suggest that the direction of transcription of these two *alg* loci is in the same direction as that found for *alg-5* (*gmd*).

The plasmid-encoded gene products of the *alg-8* and *alg-44* region were examined in the *E. coli* plasmid maxicell system and labelled with [³⁵S]methionine. We were unable, however, to detect the synthesis of any unique, non-vector polypeptides encoded by plasmids pSK2021 and pSK311. Similarly, plasmid pAD5059 is only able to direct the synthesis of a faint 51 kDa polypeptide (data not shown). In an attempt to better visualize the *alg* gene products the DNA fragments of pSK2021, pSK311 and pAD5059 were recloned into the ColE1 based, high copy number, constitutive expression (*tac*) vector pKK223-3. This resulted in the construction of plasmids pSK2023, pSK315 and pSK341, respectively (Fig. 1*b*). When compared to pKK223-3 encoded proteins (Fig. 2, lane A), pSK2023 directed the synthesis of a unique 41 kDa polypeptide (Fig. 2, lane B). pSK315, which contains sequences capable of complementing *alg-44*, directed the synthesis of two consistently reproducible new polypeptides with apparent molecular masses of 41 and 15 kDa (Fig. 2, lane C). The other small molecular mass proteins (Fig. 2), however, did not migrate in a consistent manner from one experiment to the next and are believed to represent degraded fragments of the existing polypeptides. DNA sequence analysis of a 1.5 kb *Bgl*II–*Kpn*I DNA fragment contained within the 1.9 kb *Bgl*II–*Pst*I fragment of pSK315 revealed an open reading frame capable of coding for a 40.6 kDa protein (S.-K. Wang, unpublished data). An additional open reading frame coding for a 14 kDa protein was

Fig. 2. SDS-PAGE of proteins synthesized and labelled in maxicells. Autoradiogram of plasmid-encoded polypeptides. Lanes: A, pKK223-3; B, pSK2023; C, pSK315; D, pSK434; E, pSK341; F, pSK430; G, pSK530. Molecular mass marker proteins (kDa) are indicated at the right.

also found to overlap with that of the larger open reading frame. In order to examine the possible function of the 14 kDa polypeptide, plasmid pSK331 was constructed (Fig. 1*b*). pSK331 was obtained by cloning the 0.98 kb *SalI*-*KpnI* fragment of pSK315 into pMMB24 with the *SalI* site adjacent to the *tac* promoter. This plasmid retained the entire 14 kDa open reading frame but remained unable to complement *alg-44*. This result demonstrates that it is the 41 kDa protein and not the 14 kDa polypeptide synthesized by pSK315 that most likely is the *alg-44* gene product. Protein hydrophobicity analysis of the *alg-44* gene product demonstrated the presence of three hydrophobic domains (S.-K. Wang, unpublished data). In addition, the N-terminal sequence of the polypeptide does not show any homology with *E. coli* signal peptides suggesting that the *alg-44* gene product may indeed be a membrane bound protein. However, further subcloning and overexpression studies of specifically that portion of pSK2023 responsible for *alg-8* complementation will be needed for a more definitive gene product assignment.

pSK341, which contains both pSK315 and downstream sequences, was found to direct the synthesis of two additional polypeptides with apparent molecular masses of 51 and 54 kDa (Fig. 2, lane E). This result suggested the possible existence of additional coding sequences downstream of the *alg-44* locus. The analysis of plasmid pSK434 (Fig. 1*b*), which contained the 1.8 kb *XmaI*-*EcoRI* fragment of pSK341 and was therefore devoid of *alg-44* coding sequences, supported this conclusion. This plasmid also encoded two polypeptides with apparent molecular masses of 51 and 54 kDa (Fig. 2, lane D).

Fig. 3. SDS-PAGE of proteins synthesized and labelled in maxicells. Autoradiogram of plasmid-encoded polypeptides. Lanes: A, no plasmid; B, pMMB22; C, pMMB24; D, pISC10; E, pSK700; F, pAD4047; G, labelled molecular mass marker proteins (kDa).

Overexpression and size determination of alg gene products in the alg-76 region

Previous cloning and complementation studies have tentatively localized the *alg-76* complementing activity downstream of the *alg-44* locus (Darzins *et al.*, 1985*b*). Plasmid pSK431 (Fig. 1*b*) contains a 3.8 kb *Xma*I–*Hind*III fragment from near the centre of the region II *alg* cluster and was capable of restoring alginate producing capabilities to *alg-76*. The 3.8 kb fragment of pSK431 was cloned into the expression vector pKK223-3 so as to place the *tac* promoter next to the *Xma*I site and the resulting plasmid was designated pSK430. A maxicell analysis of this plasmid revealed the synthesis of two unique polypeptides with apparent molecular masses of 51 and 54 kDa (Fig. 2, lane F). This result pointed out the possibility that two coding regions may be contained within the 3.8 kb *Xma*I–*Hind*III fragment of pSK430. In order to identify which of these polypeptides could correspond to the *alg-76* gene product, plasmid pSK530 was constructed. The 3.8 kb fragment of pSK431 was isolated, digested with *Bgl*II and the 2.4 kb *Bgl*II–*Hind*III fragment was cloned into both pCP13 and pKK223-3 resulting in the construction of pAD318 and pSK530, respectively (Fig. 1*b*). pAD318 was capable of complementing *alg-76* to Alg⁺ and pSK530 directed the synthesis of a protein with an apparent molecular mass of 54 kDa (Fig. 2, lane G). These results first suggest that the gene product for *alg-76* is a 54 kDa protein and second, that an additional coding sequence lies between the *alg-44* and *alg-76* loci.

Overexpression and size determination of alg gene products in the alg-60 region

Work from this laboratory has previously demonstrated that the *alg-60* structural gene resides within a 3.2 kb *Xho*I–*Hind*III fragment located downstream of the *alg-76* locus (Darzins *et al.*, 1985*b*). A 3.4 kb *Xba*I–*Hind*III fragment containing the entire 3.2 kb *alg-60* complementing

sequence was cloned into the broad host range expression vector pMMB24 and the resulting plasmid was designated pISC10 (Fig. 1*b*). This construct, which contained the *tac* promoter adjacent to the *Hind*III site, was capable of complementing *alg-60* to Alg⁺. Compared with pMMB24, pISC10 encoded two additional faint protein bands which migrated at 55 and 43 kDa (Fig. 3, lanes C and D). In order to distinguish which of these polypeptides could correspond to the *alg-60* gene product plasmid pSK700 was constructed. A 1.8 kb *Xba*I-*Eco*RI fragment from the 3.4 kb fragment of pISC10 was subcloned into pMMB22 placing the *tac* promoter adjacent to the *Eco*RI site (Fig. 1*b*). The resulting plasmid, designated pSK700, encoded a unique 43 kDa protein (Fig. 3, lane E). Plasmid pAD4047, which contains the DNA fragment of pSK700 and those contiguous sequences up to and including the *pmi* gene (Darzins *et al.*, 1986; Fig. 1*b*), also directed the synthesis of the 43 kDa polypeptide (Fig. 3, lane F). Since both pSK700 and pAD4047 failed to complement *alg-60* the 55 kDa protein synthesized by pISC10 is a possible candidate for the *alg-60* gene product. Inactivation of *alg-60* by some sort of insertion or deletion with a concomitant loss of complementing ability and the synthesis of the 55 kDa polypeptide, however, will be required for final confirmation. The 43 kDa polypeptide most likely represents another coding sequence located downstream of *alg-60*.

The remaining 3.6 kb of DNA between the 43 kDa coding region and the *pmi* gene represented by the recombinant plasmid pSK810 (Fig. 1*b*) failed to produce any detectable polypeptides in maxicell studies (data not shown). However, pSK810 and pSK700 had a profound effect on the alginate producing capability of strain 8821. Cells of strain 8821 harbouring these plasmids grew slowly and became phenotypically non-mucoid when grown on agar plates containing carbenicillin in the presence of IPTG. Restreaking of the non-mucoid colonies onto fresh agar plates containing antibiotic but lacking IPTG resulted in the restoration of the mucoid phenotype. The control transconjugants obtained using only the expression vector used to construct pSK700 and pSK810 were not prevented from producing exopolysaccharide. These results suggest that even though no polypeptides were detected in the maxicell analysis of pSK810, some gene(s) exist within the cloned fragment of pSK810 whose product(s), when present in excess, interfere with growth and polysaccharide production.

Quantification of alginate biosynthetic enzymes

Since assays for detecting low levels of PMI, PMM, GMP and GMD in *P. aeruginosa* have recently been described (Sa'-Correia *et al.*, 1987) we attempted to further characterize the four *alg* loci (*alg-60*, *alg-76*, *alg-44* and *alg-8*), their possible corresponding complementing gene products and the two additional coding sequences located within *alg* region II downstream of the *alg-44* and *alg-60* loci. Even though the *gmd* gene has just recently been identified, specific activities of PMM, GMP and GMD were determined in the four *alg* mutants and in a mucoid and non-mucoid strain harbouring appropriate region II overproducing constructs.

Table 2 shows that all four mutants contained PMM, GMP and GMD levels that were similar to those values obtained for either the stable mucoid parent strain 8830 or the unstable mucoid strain 8821. The only exceptions were *alg-8* and *alg-44* which had levels of GMD that were consistently lower than the value for 8830. The non-mucoid phenotype of these two mutants, however, could not be attributed to the lower activities of GMD for the following reason. Plasmid pSK311 was able to restore alginate producing capabilities to *alg-44*. The GMD level of *alg-44* harbouring pSK311, however, was not significantly different from the value of GMD obtained with *alg-44* alone (Table 2). In addition, overexpression of pSK311 by IPTG induction did not induce significant changes in the specific activity of GMD. Similar results were obtained with plasmid pSK2021, the recombinant plasmid capable of complementing *alg-8* (data not shown).

In order to ascertain the effect of overproducing various region II protein products on the level of the alginate biosynthetic enzymes the following region II constructs were introduced into the mucoid 8821 strain and a spontaneous non-mucoid revertant strain (8822): pSK402 for any additional *alg* loci which may reside upstream of *alg-5*, pSK123 for *alg-5*, pSK2021 for *alg-8* and *alg-44*, pSK311 for *alg-44*, pAD5059 for *alg-44* and the 51 kDa coding sequence, pSK431, for the 51 kDa coding sequence and *alg-76*, pISC10 for *alg-60* and the 43 kDa coding sequence and

Table 2. Specific activities of PMM, GMP and GMD in mucoid and non-mucoid *P. aeruginosa* strains1 mU is defined as the formation of 1 nmol NADPH or NADH min⁻¹.

Strain/plasmid	Phenotype	PMM	GMP	GMD
		[mU (mg protein) ⁻¹]		
8821	Mucoid	2.0	1.2	8.0
8830	Mucoid	1.6	2.3	10.6
8838 (<i>alg-8</i>)	Non-mucoid	1.5	2.5	2.5
8874 (<i>alg-44</i>)	Non-mucoid	1.5	2.4	3.7
8897 (<i>alg-76</i>)	Non-mucoid	1.0	1.2	8.1
8887 (<i>alg-60</i>)	Non-mucoid	0.8	0.9	9.6
8874/pSK311	Mucoid	1.4	2.1	3.2
8874/pSK311*	Mucoid	1.2	2.1	2.4

* IPTG added to 5 mM (final concentration).

pSK 700 for the 43 kDa coding sequence. Plasmids pSK2021, pSK311, pAD5059, pSK431 and pISC10 failed to have any effect on either PMM, GMP, or GMD activities in either 8821 or 8822 upon IPTG induction (data not shown). The non-mucoid strain 8822 harbouring plasmid pSK 123 showed a dramatic increase in GMD activity [68 mU (mg protein)⁻¹ compared to 9 mU (mg protein)⁻¹ without IPTG induction]. This is consistent with the finding that pSK123 harbours the structural gene for GMD. The only other region II construct which had any effect on the alginate biosynthetic enzyme activities was pSK 700. Strain 8822 harbouring pSK 700 was found to have a reproducible 3.5-fold increase in the GMD activity from 2 mU (mg protein)⁻¹ to 7 mU (mg protein)⁻¹ upon IPTG induction.

DISCUSSION

In this study, the *P. aeruginosa* alginate biosynthetic gene cluster, designated region II, was further characterized. Gene expression studies with several region II constructs revealed that the transcriptional orientation of the known *alg* loci was unidirectional, with transcription proceeding from *gmd* (located proximal to *argF*) to *pmi* (Fig. 1b). Two additional, possibly *alg* related, polypeptides were encoded from within the *alg* gene cluster and the genes encoding these additional proteins appeared to be also transcribed in the same orientation as the other *alg* genes within region II.

One of these additional polypeptides was coded for by a 1.8 kb *Xma*I–*Eco*RI fragment located between *alg-44* and *alg-76*. Plasmids pSK434 and pAD5059 consistently produced two polypeptides, 54 and 51 kDa in size. Since 1 kb of DNA can (roughly) code for a 37 kDa polypeptide it seems unlikely that the 1.8 kb DNA fragment of pSK434 could harbour two non-overlapping genes coding for a 54 and a 51 kDa protein. A possible explanation for this result is that these two polypeptides share the same open reading frame but initiate translation at different points along the open reading frame. Another possibility is that the 54 kDa polypeptide may be the precursor form of the 51 kDa polypeptide which was formed by a post-translational modification (i.e., cleavage of a small peptide). As of yet, no *alg* mutants have been isolated which map to this locus. However, we believe that since the DNA fragment encoding this polypeptide is flanked by two *alg* loci involved in alginate biosynthesis (*alg-44* and *alg-76*) it is likely that this polypeptide is also involved in the production of alginate acid.

The second additional polypeptide (43 kDa) was found to be encoded by DNA sequences immediately downstream of *alg-60*. Overproduction of the 43 kDa protein by plasmid pSK 700 in 8822 resulted in the 3-fold increase of GMD activity to near wild-type mucoid levels suggesting that this polypeptide may act as an activator for GMD expression. Overexpression of pSK 700 did not have an effect in the mucoid 8821 strain as no further increases in GMD activity were detected. Even though chemical induced mutants defective in alginate biosynthesis have

not been isolated in this locus, we believe that the following justification is reason enough to consider that this region may also be involved in exopolysaccharide production.

Several smaller segments of the 3.6 kb fragment between the 43 kDa coding region and the *pmi* gene have been cloned into the ColE1 based, narrow host range vector, pBR325. These plasmids cannot replicate autonomously in *P. aeruginosa* and their maintenance within *P. aeruginosa* is solely dependent on their ability to integrate into the chromosome by recombination at the site of homology (Darzins *et al.*, 1985b). The integration of these constructs into the chromosome of the mucoid strain 8830 resulted in tetracycline resistant transconjugants which had lost the mucoid phenotype (data not shown). This result indicates that the plasmid insertions either disrupted crucial genes involved in alginate biosynthesis or interrupted the normal process of transcription through this region. This is consistent with the recent report that the *pmi* gene may be part of a single regulatory unit which has at its 5' end the 43 kDa coding region (Darzins *et al.*, 1986). Our laboratory has recently shown that the overproduction of PMI causes the derepression of PMM and GMP activity but not of GMD (Sa'-Correia *et al.*, 1987). Since the presence of GMD activity is indispensable for alginate production it is important to derepress GMD activity also. It is thus likely that the 43 kDa polypeptide plays a role in the derepression of the GMD activity.

The ability of a cloned DNA fragment to inhibit exopolysaccharide synthesis in other bacterial systems has previously been reported. In *E. coli* the biosynthesis of capsular polysaccharide (colanic acid) is negatively regulated by the *lon* gene product which is in turn positively regulated by the *OmpT* gene. The product of the *OmpT* gene is a 40 kDa major outer membrane protein known as *a* (also known as 3b or M2) (Gayda *et al.*, 1979). Overproduction of the *OmpT* gene product has led to the repression of colanic acid synthesis (Berg *et al.*, 1976; Gayda *et al.*, 1979). Similarly, wild-type *Rhizobium* strains produce an exopolysaccharide thought to be involved in the symbiotic relationship between plant and bacterium. A region of the symbiotic plasmid pRP2JI when cloned at an increased copy number inhibited exopolysaccharide synthesis (Borthakur *et al.*, 1985). It has been proposed that the *psi* (polysaccharide inhibition) gene is a regulatory gene that acts to repress the expression of gene(s) involved in exopolysaccharide synthesis in the plant associated bacteroid. It is possible that the products from pSK700 and pSK810 responsible for inhibiting alginate production could be somehow negatively regulating exopolysaccharide synthesis or could be sterically interfering with the polymerization and/or secretion processes. *P. aeruginosa*, in addition to producing a very viscous exopolysaccharide, also produces an enzyme (alginate) capable of degrading this polymer. Alginate activity is believed to be responsible for the loss in viscosity of alginate during later stages of cell growth (Linker & Evans, 1984). However, the genetic aspects of alginate production remain unclear.

Except for the *pmi* and *gmd* genes the functions of the remaining genes in region II are still not fully understood. One thing that becomes clear, however, is that the remaining genes do not appear to encode either PMM or GMP activities. This conclusion is supported by the finding that all of the *alg* mutants mapped to region II contained wild-type levels of PMM and GMP and that overproduction of region II gene products (excluding PMI) failed to demonstrate increased levels of these two enzymes. The results of this study strongly point to the fact that the remaining genes present within region II may be involved in the conversion of GDP-mannuronic acid to alginate, catalysing such steps as polymerization of mannuronic acid residues and secretion, epimerization and acetylation of the resulting polymer.

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