Characterization of the \textit{Pseudomonas aeruginosa} Alginate (\textit{alg}) Gene Region II

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\textit{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 \textit{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 (\textit{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 \textit{alg} loci within region II appears to be in the direction from \textit{argF} to \textit{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 \textit{alg} mutants and in cells overexpressing cloned segments from region II. Based on these enzyme measurements, we conclude that the remaining region II \textit{alg} genes do not encode either PMM or GMP. These results support the suggestion that the remaining \textit{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.

\section*{INTRODUCTION}

Mucoid strains of \textit{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 \textit{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 \textit{P. aeruginosa} into an encapsulated variant (Doggett \textit{et al.}, 1971). These mucoid variants are rarely, however, found in non-CF infections despite prolonged colonization in several sites (Doggett, 1979; Iacocca \textit{et al.}, 1963). The exopolysaccharide conferring the mucoid phenotype

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\begin{small}
\textit{Abbreviations}: PMI, phosphomannose isomerase; PMM, phosphomannose mutase; GMP, GDP-mannose pyrophosphorylase; GMD, GDP-mannose dehydrogenase; CF, cystic fibrosis; PIA, \textit{Pseudomonas Isolation Agar}; IPTG, isopropyl \textit{\beta}-\textit{D}-thiogalactopyranoside.
\end{small}
\end{flushright}
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 Prac) or pKK223-3 (open rectangles). Prac, hybrid irp-lac promoter; B, BglII; Bam, BamHI; C, CiaI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SstI; Sa, SalI; X, XhoI; Xb, XbaI; Xm, Xmal.

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. 1a). 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, 1987b). 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., 1985b). This region (Fig. 1b) 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., 1985a; Gill et al., 1986; Deretic et al., 1987a). 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 culturing *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 *Plac–lacI* 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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>E. coli AC80</td>
<td>thr leu met hsdR hsdM</td>
<td>Chakrabarty et al. (1978)</td>
</tr>
<tr>
<td>DH1</td>
<td>F' recA1 endA1 gyrA96 thi-1</td>
<td>Low (1968)</td>
</tr>
<tr>
<td>CSR603</td>
<td>recA uvrA6 phr-1</td>
<td>Sancar et al. (1979)</td>
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<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8821</td>
<td>his-1 Alg+</td>
<td>Darzins &amp; Chakrabarty (1984)</td>
</tr>
<tr>
<td>8822</td>
<td>his-1 alg-l (spontaneous)</td>
<td></td>
</tr>
<tr>
<td>8830</td>
<td>his-1 Alg+ (stable)</td>
<td></td>
</tr>
<tr>
<td>8835</td>
<td>his-1 alg-5</td>
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</tr>
<tr>
<td>8838</td>
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<td>8897</td>
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<tr>
<td>Plasmids</td>
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<td>pCP13</td>
<td>IncP Tc' Km' Cos+</td>
<td>Friedman et al. (1982); Darzins &amp; Chakrabarty (1984)</td>
</tr>
<tr>
<td>pKT240</td>
<td>IncQ Km' Ap'</td>
<td>Bagdasarian et al. (1983)</td>
</tr>
<tr>
<td>pMMB22</td>
<td>IncQ Ap' Ptac lacI'</td>
<td></td>
</tr>
<tr>
<td>pMMB24</td>
<td>IncQ Ap' Ptac lacI'</td>
<td></td>
</tr>
<tr>
<td>pKK223-3</td>
<td>ColE1 rep Ap' Ptac</td>
<td>Amman et al. (1983)</td>
</tr>
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</table>

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 (lac), 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). 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 HindIII 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 HindIII 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 ClaI–XmaI fragment of the DNA insert of pSK2005 resulted in the construction of plasmid pSK123 which placed the tac promoter next to the ClaI 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 XmaI 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 ClaI site and that the transcriptional orientation of alg-5 is from the ClaI site toward the XmaI 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 XhoI–PstI 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 XhoI and PstI 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 BglII–PstI fragment of the 4.2 kb XhoI–PstI 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 BglII 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 XhoI–PstI 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 BglII–KpnI DNA fragment contained within the 1.9 kb BglII–PstI 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
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. 1b). 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. 1b), which contained the 1.8 kb Xmal–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. 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.
**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., 1985b). Plasmid pSK431 (Fig. 1b) contains a 3·8 kb XmaI–HindIII 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 XmaI 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 XmaI–HindIII 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 BglII and the 2·4 kb BglII–HindIII fragment was cloned into both pCP13 and pKK223-3 resulting in the construction of pAD318 and pSK530, respectively (Fig. 1b). 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 XhoI–HindIII fragment located downstream of the alg-76 locus (Darzins et al., 1985b). A 3·4 kb XbaI–HindIII 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. 1b). This construct, which contained the tac promoter adjacent to the HindIII 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 XbaI–EcoRI fragment from the 3.4 kb fragment of pISC10 was subcloned into pMMB22 placing the tac promoter adjacent to the EcoRI site (Fig. 1b). 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. 1b), 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. 1b) 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 strains

1 mU is defined as the formation of 1 nmol NADPH or NADH min⁻¹.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Phenotype</th>
<th>PMM [mU (mg protein)⁻¹]</th>
<th>GMP [mU (mg protein)⁻¹]</th>
<th>GMD [mU (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8821</td>
<td>Mucoid</td>
<td>2.0</td>
<td>1.2</td>
<td>8.0</td>
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<td>8830</td>
<td>Mucoid</td>
<td>1.6</td>
<td>2.3</td>
<td>10.6</td>
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<td>8838 (alg-8)</td>
<td>Non-mucoid</td>
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<td>2.5</td>
<td>2.5</td>
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<td>8874 (alg-44)</td>
<td>Non-mucoid</td>
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<td>1.2</td>
<td>8.1</td>
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<td>8887 (alg-60)</td>
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<td>0.9</td>
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<td>2.1</td>
<td>3.2</td>
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<tr>
<td>8874/pSK311*</td>
<td>Mucoid</td>
<td>1.2</td>
<td>2.1</td>
<td>2.4</td>
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</table>

* IPTG added to 5 mM (final concentration).

P. aeruginosa alginate (alg) region II

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 XmaI–EcoRI 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 alginic 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 pSK700 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 pSK700 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

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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 pmr gene have been cloned into the CoIE1 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., 1985 b). 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 pmr 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 RP2JII 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 (alginase) capable of degrading this polymer. Alginase 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 alginase production remain unclear.

Except for the pmr 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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