Bacterial alginate biosynthesis – recent progress and future prospects

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Background

The extracellular polysaccharide alginate has been widely associated with chronic *Pseudomonas aeruginosa* infections in the cystic fibrosis lung. However, it is clear that alginate biosynthesis is a more widespread phenomenon. Alginate plays a key role as a virulence factor of plant-pathogenic pseudomonads, in the formation of biofilms and with the encystment process of *Azotobacter* spp.

Understanding the genetics, biosynthesis and physiological roles of alginate has been greatly aided by a multidisciplinary approach involving molecular biologists, microbial physiologists and biochemists. Furthermore, the study of alginate biosynthesis has provided a valuable, and more general, insight into the organization and regulation of genes in non-enteric Gram-negative bacteria.

Progress with research into alginate has been rapid over the past few years and there is now a coherent and rounded story to tell. Therefore, it is timely to review this research area and to identify the potential for future developments.

Alginate-producing bacteria

The polysaccharide alginate was first isolated from marine macroalgae last century, but it was approximately 80 years later that a bacterial source (*P. aeruginosa*) of the polysaccharide was identified (Linker & Jones, 1966). The association of mucoid forms, i.e. alginate-producing strains, of *P. aeruginosa* with chronic lung infections in patients with cystic fibrosis is now well-established, and is recognized as a major cause of morbidity and mortality in these individuals. Mucoid, alginate-producing strains of *P. aeruginosa* have also been isolated, albeit much less frequently, from other cohorts of patients, e.g. bronchiectatics and those with urinary tract or middle ear infections (McAvoy et al., 1989), although not normally from individuals with infected burn sites. Mucoid *P. aeruginosa* has also been isolated from the equine guttural pouch of a mare with a chronic mucopurulent nasal discharge (Govan et al., 1992). Although the overwhelming number of mucoid isolates of *P. aeruginosa* have been obtained from clinical samples, it is clear that alginate production is important in a much wider context. It is likely that many environmental isolates of *P. aeruginosa* are also mucoid but rarely identified as such because of the use of inappropriate isolation media (Grosse et al., 1995). The difficulties of isolation bedevilled early attempts to isolate mucoid strains from clinical isolates and as a result the true rate of occurrence was greatly underestimated, and it is likely that the same is currently true of environmental isolates. Alginate biosynthesis is probably a key factor in the establishment of stable biofilms of *P. aeruginosa* in a wide range of environmental situations.

Alginate production is fairly widespread amongst rRNA homology group I pseudomonads. Probes for three alginate structural genes (*algA*, *algC* and *algD*) and one regulatory gene (*algR1*) of *P. aeruginosa* hybridize to DNA fragments from *Pseudomonas syringae* pv. *glycinea*, *Pseudomonas viridiflava* and *Pseudomonas corrugata* (Fett et al., 1992) and other members of rRNA homology group I (Fialho et al., 1990). Genomic DNA from representatives of groups II–IV gave very weak or no hybridization with the probes, except for *algC*, indicating that the ability to produce alginate is restricted to members of rRNA homology group I. This substantiates earlier physiological studies in which alginate was isolated from *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas mendocina* (Govan et al., 1981) and *P. syringae* (Fett et al., 1986; Gross & Rudolph, 1987). Alginate is also synthesized by *Azotobacter vinelandii* as part of the encystment process. The mature cysts are surrounded by two discrete alginate-rich layers which enable the dormant cells to survive long periods of desiccation. Strains of *Azotobacter chroococcum* also produce alginate (Cote & Krull, 1988). In the *Azotobacter*, abnormalities in alginate
production result in impaired encystment, which demonstrates the close interdependence of these two processes.

**Alginate structure and properties**

Alginate is composed of the uronic acid β-D-mannuronate and its C-5 epimer α-L-guluronate. These monomers may be arranged in homopolymeric (polymannuronate or polyguluronate) or heteropolymeric block structures (Fig. 1). In addition, bacterial alginates are normally O-acetylated on the 2 and/or 3 position(s) of the D-mannuronate residues. Consequently, bacteria produce a range of alginates with different block structures and degrees of O-acetylation. The high molecular mass of bacterial alginate and the negative charge ensure that the polysaccharide is highly hydrated and viscous.

It is well-established that alginates from *P. aeruginosa* do not contain polyguluronate blocks (Sherbrock-Cox *et al.*, 1984) whereas those from *A. vinelandii* may do so. The block structure and degree of O-acetylation determine the physicochemical properties of alginate. Alginates which contain polyguluronate form rigid gels in the presence of Ca$^{2+}$ and are therefore important in structural roles, e.g. the outer cyst wall of *A. vinelandii*. Conversely, an absence of polyguluronate, as in *P. aeruginosa*, produces relatively flexible gels in the presence of Ca$^{2+}$. Furthermore, extensive O-acetylation of alginate increases the water-binding capacity of the polysaccharide, which may be significant in enhancing survival under desiccating conditions.

**Genetics of alginate biosynthesis**

The genes for alginate biosynthesis are all located on the bacterial chromosome, with no evidence to date of plasmid involvement. At least 24 genes have been directly implicated in alginate biosynthesis in *P. aeruginosa* (Table 1) and there is good evidence that others may also be involved, e.g. *glpM* (Schweizer *et al.*, 1995). It is difficult to be precise about the number of genes that are solely associated with alginate biosynthesis as it is now clear, for example, that some of the control genes act globally and encode proteins such as alternative σ factors (Yu *et al.*, 1995). Other ‘alg’ genes, e.g. *algA,C*, are also essential for LPS biosynthesis (Goldberg *et al.*, 1993). The majority of the genes are organized into three major clusters located at approximately 9, 34 and 68 min on the *P. aeruginosa* chromosome (Table 1).

Virtually all of the genes directly catalysing the synthesis of alginate are located at 34 min on the *P. aeruginosa* chromosome (Fig. 2) and the order and direction of transcription have been unequivocally established. Expression is under the control of the *algD* promoter and in essence this region (*algD*-*algA*) acts as an operon (Chitnis & Ohman, 1993), although there is sequence-based evidence for weak promoters within the gene cluster. Although most of the genes are essential for alginate biosynthesis, those encoding the epimerase and O-acetyltransferase(s) can be inactivated providing that essential genes downstream of the mutation are expressed in *trans*. One of the alginate biosynthetic genes, *algC*, is located at 10 min on the PAO map and is transcribed independently of the 34 min region.

The role of a gene (*algL*) encoding an alginate lyase in the biosynthetic operon (Boyd *et al.*, 1993; Monday & Schiller, 1996; Schiller *et al.*, 1993) is unknown. It is not clear why a degradative enzyme should be expressed concurrently with the biosynthetic enzymes. One suggestion has been that the lyase may be involved in excising polysaccharide fragments from the biosynthetic complex, although there is no real evidence to support this contention. It is clear though that overexpression of the *algL* gene product (May & Chakrabarty, 1994) or other alginate lyases (Gacesa & Goldberg, 1992) results in release of planktonic bacteria from biofilms. A similar arrangement of genes has been described in *Pseudomonas syringae* (Penaloza Vazquez *et al.*, 1997) and *Azotobacter vinelandii*, although in the latter there is good evidence for a specific *algD*-independent promoter which controls expression of *algL* (Lloret *et al.*, 1996).

The regulation of alginate biosynthesis is complex and involves specific gene products and those that act more globally. The five genes *algU* (*algT*), *mucA* (*algS*), *mucB* (*algN*), *mucC* (*algM*) and *mucD* (*algW*) located at 68 min on the chromosome comprise the main switch controlling the conversion between non-mucoid and mucoid forms of *P. aeruginosa*. On the basis of sequence analysis (Martin *et al.*, 1994), *algU* is a member of the $\sigma^5$ class of sigma factors, i.e. analogous to $\sigma^E$ of *Escherichia coli*, and is essential for alginate production.
Subsequent studies have established that algU and rpoE are functionally equivalent (Yu et al., 1995) and that AlgU forms complexes with RNA polymerase (Schurr et al., 1995). AlgU causes an increase in alginate biosynthesis by a direct action on the algD promoter (see below) and indirectly by up-regulating transcription of another regulatory gene, algR (Martin et al., 1994). The mucA, mucB (Schurr et al., 1996) and mucD (Boucher et al., 1996) gene products are negative regulators of AlgU and muc mutations result in overproduction of alginate (Fig. 2). An analogous set of genes associated with the control of alginate production has been described for A. vinelandii (Table 2) (Martinez-Salazar et al., 1996).

Table 1. Alginate genes of P. aeruginosa

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location on PAO map (min)</th>
<th>Gene product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alg8</td>
<td>34</td>
<td>Polymerase/export function?</td>
<td>Maharaj et al. (1993)</td>
</tr>
<tr>
<td>alg44</td>
<td>34</td>
<td>Polymerase/export function?</td>
<td>Maharaj et al. (1993)</td>
</tr>
<tr>
<td>algA</td>
<td>34</td>
<td>Phosphomannose isomerase/GDPmannose pyrophosphorylase</td>
<td>Darzins et al. (1985)</td>
</tr>
<tr>
<td>algB</td>
<td>13</td>
<td>Member of ntrC subclass of two-component regulators</td>
<td>Goldberg &amp; Dahnke (1992)</td>
</tr>
<tr>
<td>algC</td>
<td>10</td>
<td>Phosphomannomutase</td>
<td>Coyne et al. (1994)</td>
</tr>
<tr>
<td>algD</td>
<td>34</td>
<td>GDPmannose dehydrogenase</td>
<td>Deretic et al. (1987)</td>
</tr>
<tr>
<td>algE</td>
<td>34</td>
<td>Outer-membrane porin?</td>
<td>Rehm et al. (1994)</td>
</tr>
<tr>
<td>algF</td>
<td>34</td>
<td>O-Acetylation</td>
<td>Franklin &amp; Ohman (1993)</td>
</tr>
<tr>
<td>algG</td>
<td>34</td>
<td>Mannuronan C-5-epimerase</td>
<td>Franklin et al. (1994)</td>
</tr>
<tr>
<td>algH</td>
<td>?</td>
<td>Unknown function</td>
<td>Schlichtman et al. (1995)</td>
</tr>
<tr>
<td>algI</td>
<td>34</td>
<td>O-Acetylation</td>
<td>Franklin &amp; Ohman (1996)</td>
</tr>
<tr>
<td>algJ</td>
<td>34</td>
<td>O-Acetylation</td>
<td>Franklin &amp; Ohman (1996)</td>
</tr>
<tr>
<td>algK</td>
<td>34</td>
<td>Alginate translocator?</td>
<td>Aarons et al. (1997)</td>
</tr>
<tr>
<td>algL</td>
<td>34</td>
<td>Alginate lyase</td>
<td>Schiller et al. (1993)</td>
</tr>
<tr>
<td>algM</td>
<td>68</td>
<td>Regulator?</td>
<td>Boucher et al. (1996)</td>
</tr>
<tr>
<td>algN</td>
<td>68</td>
<td>Anti σ factor?</td>
<td>Martin et al. (1993a)</td>
</tr>
<tr>
<td>algP</td>
<td>9</td>
<td>See algR3</td>
<td>Deretic &amp; Konyecsni (1990)</td>
</tr>
<tr>
<td>algQ</td>
<td>9</td>
<td>See algR2</td>
<td>Roychoudhury et al. (1992)</td>
</tr>
<tr>
<td>algR2</td>
<td>9</td>
<td>Protein kinase or kinase regulator</td>
<td>Roychoudhury et al. (1992)</td>
</tr>
<tr>
<td>algR3</td>
<td>9</td>
<td>Histone-like transcription regulator</td>
<td>Kato et al. (1990)</td>
</tr>
<tr>
<td>algS</td>
<td>68</td>
<td>Anti σ factor</td>
<td>Martin et al. (1993b)</td>
</tr>
<tr>
<td>algT</td>
<td>68</td>
<td>See algU</td>
<td>Martin et al. (1994)</td>
</tr>
<tr>
<td>algU</td>
<td>68</td>
<td>Homologue of E. coli σ8 global stress response factor</td>
<td>Martin et al. (1994)</td>
</tr>
<tr>
<td>algW</td>
<td>68</td>
<td>Homologue of serine protease (HtrA)</td>
<td>Boucher et al. (1996)</td>
</tr>
<tr>
<td>algX</td>
<td>34</td>
<td>Unidentified function but high sequence similarity to algI</td>
<td>Monday &amp; Schiller (1996)</td>
</tr>
<tr>
<td>algZ</td>
<td>9</td>
<td>AlgR cognate sensor</td>
<td>Baynham &amp; Wozniak (1996)</td>
</tr>
<tr>
<td>mucA</td>
<td>68</td>
<td>See algS</td>
<td>Martin et al. (1993b)</td>
</tr>
<tr>
<td>mucB</td>
<td>68</td>
<td>See algN</td>
<td>Martin et al. (1993a)</td>
</tr>
<tr>
<td>mucC</td>
<td>68</td>
<td>See algM</td>
<td>Boucher et al. (1996)</td>
</tr>
<tr>
<td>mucD</td>
<td>68</td>
<td>See algW</td>
<td>Boucher et al. (1996)</td>
</tr>
</tbody>
</table>

*The histidine protein kinase of AlgB at 13 min on the chromosome had originally been designated algK but has subsequently been renamed kinB to avoid confusion (D. Ohman, personal communication).*

Genes located at a region spanning 9–13 min on the P. aeruginosa chromosome, algR(algR1), algQ(algR2) and algP(algR3), and algB modulate the production of alginate and have been described as auxiliary regulators of mucoidy (Govan & Deretic, 1996). The best characterized of these regulators is algR, which is transcribed in response to the protein AlgU. AlgR binds to three sites upstream of the algD promoter and, in conjunction with AlgU, up-regulates transcription of algD and the down-
Fig. 2. Organization and control of alginate genes in *P. aeruginosa*. Transcription of the biosynthetic genes located at 34 min on the chromosome is under the control of the *algD* promoter which is up-regulated by AlgU and AlgR. AlgU, which is the equivalent of the extreme heat shock sigma factor $\sigma^E$ (RpoE) of enteric bacteria, also up-regulates expression of *algR*, *algU* and *rpoH*. AlgS (MucA), AlgN (MucB) and AlgW (MucD) attenuate the expression of, or the activity of, AlgU.

**Table 2. Alginate genes of *A. vinelandii***

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>alg8</em></td>
<td>Polymerase?</td>
<td>Mejia Ruiz et al. (1997)</td>
</tr>
<tr>
<td><em>alg44</em></td>
<td>Polymerase/export function?</td>
<td>Mejia Ruiz et al. (1997)</td>
</tr>
<tr>
<td><em>algA</em></td>
<td>Phosphomannose isomerase/GDPmannose pyrophosphorylase</td>
<td></td>
</tr>
<tr>
<td><em>algD</em></td>
<td>GDPmannose dehydrogenase</td>
<td>Campos et al. (1996)</td>
</tr>
<tr>
<td><em>algE1-5</em></td>
<td>Mannuronan C-5-epimerases</td>
<td>Ertesvag et al. (1995)</td>
</tr>
<tr>
<td><em>algG</em></td>
<td>Mannuronan C-5-epimerase</td>
<td>Rehm et al. (1996)</td>
</tr>
<tr>
<td><em>algL</em></td>
<td>Alginate lyase</td>
<td>Lloret et al. (1996)</td>
</tr>
<tr>
<td><em>algU</em></td>
<td>Homologue of <em>E. coli</em> $\sigma^E$ global stress response factor</td>
<td>Martinez-Salazar et al. (1996)</td>
</tr>
<tr>
<td><em>mucA</em></td>
<td>Anti $\sigma$ factor</td>
<td>Martinez-Salazar et al. (1996)</td>
</tr>
<tr>
<td><em>mucB</em></td>
<td>Anti $\sigma$ factor</td>
<td>Martinez-Salazar et al. (1996)</td>
</tr>
<tr>
<td><em>mucC</em></td>
<td>Regulator?</td>
<td>Martinez-Salazar et al. (1996)</td>
</tr>
<tr>
<td><em>mucD</em></td>
<td>Homologue of serine protease (HtrA)</td>
<td>Martinez-Salazar et al. (1996)</td>
</tr>
</tbody>
</table>

AlgR also promotes expression of *algC*. The efficiency of AlgR is increased by phosphorylation by the cognate kinase AlgQ. AlgB also modulates *algD* expression and based on sequence analysis is a member of the NtrC subclass of two-component regulators (Wozniak & Ohman, 1991) but the *algB* and *algR* regulatory systems appear to operate independently of each other (Wozniak & Ohman, 1994). A number of additional genes, e.g. *algP* and *algZ*, are also involved in alginate regulation. Analysis of sequence data indicates that *algZ* encodes a sensory component of a signal transducer system but that it lacks several expected motifs typical of histidine protein kinases (Yu et al., 1997). A comprehensive account of the interrelationships of the regulators is beyond the scope of this article, but has recently been reviewed (Govan & Deretic, 1996).

**Biosynthesis of alginate**

A convincing pathway for alginate biosynthesis in *A. vinelandii* was first proposed by Pindar & Bucke (1975) based on the assay of individual enzyme activities. However, the corresponding enzymes in *P. aeruginosa* have proved more difficult to assay directly and the pathway has been elucidated using a combination of complementation analyses and gene cloning/overexpression studies. Although the initial steps in the
pathway are indisputable (Fig. 3), and the same for *A. vinelandii* and pseudomonads, there is still considerable debate about the final stages of biosynthesis and export of alginate.

The starting point for alginate biosynthesis is fructose 6-phosphate (Fig. 3). Radiolabelling studies have established that six-carbon growth substrates are oxidized via the Entner-Doudoroff pathway and that the resultant pyruvate ([1 mol (mol hexose)]⁻¹) is ultimately channelled into alginate biosynthesis (Lynn & Sokatch, 1984). More detailed analyses of the labelling patterns in alginate indicate that the pyruvate derived from the oxidation of sugars is fed into the TCA cycle prior to synthesis of fructose 6-phosphate and alginate (Narbad et al., 1988). This key role for the TCA cycle in alginate biosynthesis is supported by more recent genetic studies using non-mucoid mutants of *P. aeruginosa*. Levels of the phosphorylated (active) form of the key TCA cycle enzyme succinyl-CoA synthetase are reduced in algR2 mutants (Schliktman et al., 1994). This effect is specific as the activities of the other TCA enzymes are unaffected. The normally rare occurrence of mucoid forms of *P. aeruginosa* in culture can be significantly increased by growth on energy-poor media (Terry et al., 1992). However, there is a recent report that glucose can stimulate *algD* transcription and alginate production (Ma et al., 1997) but this contradicts earlier work which proposed that glucose repression of *algD* occurs (Devault et al., 1991).

Alginate biosynthesis occurs in response to energy deprivation yet the synthesis of the polysaccharide is an energy-demanding process. It has been suggested that the *algR2*-controlled expression of succinyl-CoA synthetase and nucleoside-diphosphate kinase may result in a decreased pool size of GTP and hence less available GDPmannose for alginate biosynthesis (Schliktman et al., 1994). However, quantification of the nucleotide sugars indicates that GDPmannose is present in great excess (Tatnell et al., 1993) even in constructs overexpressing GDPmannose dehydrogenase (Tatnell et al., 1994). More recent experiments using ¹³C-NMR have clearly established the key role of the Entner-Doudoroff pathway in the oxidation of hexoses and the obligatory requirement of triose intermediates in alginate biosynthesis (Beale & Foster, 1996). These authors conclude that the pyruvate derived from the oxidation of hexoses feeds into alginate biosynthesis via the formation of oxaloacetate and subsequent gluconeogenesis. This adds weight to earlier data using ¹⁴C-labelled precursors which implied an obligatory role for the TCA cycle in the conversion of glucose to alginate (Narbad et al., 1988).

The initial steps in the alginate biosynthesis pathway are essentially those of general carbohydrate metabolism and the intermediates are widely utilized. In particular, the steps up to and including GDPmannose are common to alginate and LPS biosynthesis (Goldberg et al., 1993). This and other evidence clearly indicates a crucial role...
for GDPmannose dehydrogenase (algD) in the biosynthesis of alginate. The algD gene is proximal to the promoter on the alginate ‘operon’ and expression is tightly controlled (Schurr et al., 1993). Analyses of nucleotide sugar pools and exopolysaccharide production clearly indicate that GDPmannose dehydrogenase is the kinetic control point in the alginate pathway (Tatnell et al., 1994).

The initial polymeric product of the alginate biosynthetic pathway is polymannuronan. Although the alginate polymerase has not been isolated, it has been assumed that polymerization and secretion across the inner membrane are accomplished simultaneously (May & Chakrabarty, 1994). A porin-like protein, AlgE, has been implicated in the transport of polymannuronan across the bacterial membranes (Rehm et al., 1994). A precursor form of AlgE is located in the cytoplasmic membrane but the mature protein appears to be primarily an outer-membrane protein detectable only in mucoid forms of *P. aeruginosa*. Another gene, algK, which has been cloned and sequenced, is considered to be a putative alginate translocator (Aarons et al., 1997). Although the polymerase has not been isolated, it is clear from 13C-NMR data that polymerization occurs from the non-reducing end of the polymer (Beale & Foster, 1996). The gene alg8 has been implicated as encoding the polymerase based on sequence similarity to a glycosyltransferase, NodC, of Azorhizobium caulinodans (Mejia Ruiz et al., 1997).

Once in the periplasmic space, alginate is O-acetylated by the combined action of the algF, algI and algJ gene products. Mutants deficient in any one of these three genes are unable to produce O-acetylated alginate, but the epimerization process and overall yields of alginate appear to be unaffected (Shinabarger et al., 1993; Franklin & Ohman, 1996). Acetyl-CoA is almost certainly the primary donor of O-acetyl groups for alginate modification; however, this metabolite is confined to the cytoplasm whereas the O-acetylation process occurs in the periplasm. Therefore, at least one of the algF, algI or algJ gene products is likely to be involved in transport of O-acetyl groups across the cell membrane into the periplasmic space. Sequence data indicate that AlgI is probably a membrane-bound protein and may fulfill this role (Franklin & Ohman, 1996). AlgF has a signal peptide, which is processed by *E. coli*, indicating that it is a periplasmic protein (Shinabarger et al., 1993) and therefore could be the O-acetyltransferase. AlgJ shows remarkable similarity (30% identity, 69% similarity) to another gene product, AlgX (Monday & Schiller, 1996), of unknown function but essential for alginate biosynthesis. However, neither AlgJ nor AlgX shows any significant similarity to other proteins in the databases and therefore their function in the O-acetylation process remains unresolved at this stage. The observation that a cell suspension of *P. syringae* is able to O-acetylate seaweed alginate suggests that this event is periplasmic or extracellular (Lee & Day, 1995). It is likely that the O-acetylation of mannuronate residues is catalysed by at least two enzymes each specific for either the 2- or 3-hydroxyl on the sugar ring. However, at this stage it is not known which gene products might be involved in determining these specific modifications.

Epimerization of D-mannuronate to L-guluronate residues in alginate is catalysed by a single gene product (AlgG) in *P. aeruginosa* (Franklin et al., 1994). The alginites produced by *P. aeruginosa* (Sherbrook-Cox et al., 1984) and other pseudomonads (Conti et al., 1994) are devoid of regions ('blocks') of polyguluronate. Also, the proportion and the nearest neighbour frequency of the two uronic acids in alginate appear to be constant for a particular strain of *P. aeruginosa* regardless of environmental conditions (Sherbrook-Cox et al., 1984). Deletion or disruption of the algG gene does not prevent alginate biosynthesis providing that other genes downstream of algG are expressed in trans. The epimerase has a signal peptide of 35 amino acids and is located in the periplasm. No cofactors or other proteins appear to be required for polymer-level epimerization to occur, but O-acetylation of mannuronate residues prevents epimerization (Franklin et al., 1994).

In *A. vinelandii*, where alginate forms an integral part of the cyst wall, the alginate may contain blocks of polyguluronate and the composition of the polysaccharide varies depending on environmental conditions (Larsen & Haug, 1971a). Specifically, the epimerization process is influenced by the concentration of Ca2+ in the growth medium (Larsen & Haug, 1971b) with the proportion of polyguluronate increasing with increasing Ca2+ concentration. Genetic analysis has revealed a family of five related epimerase genes in *A. vinelandii* which have rather confusingly been designated algE (Ertesvag et al., 1994, 1995) (the algE gene of *P. aeruginosa* encodes a putative outer-membrane porin). The multiple forms of the epimerase gene appear to be derived from a common ancestor with good evidence for events such as gene duplication, gene fusions and possibly gene conversions and unequal crossing over (Ertesvag et al., 1995).

Whereas the *P. aeruginosa* algG gene product has a molecular mass of 55 kDa, the *A. vinelandii* algE genes encode products ranging from 57.7 kDa (AlgE4) to 191 kDa (AlgE3). Notwithstanding the large differences in molecular mass, there is significant sequence similarity between the *P. aeruginosa* epimerase and defined regions of the *A. vinelandii* enzymes. The five *A. vinelandii* gene products each contain three distinct types of structural motif. The ‘A modules’, of which there are one (AlgE2, 4 and 5) or two (AlgE1, 3) per protein, show significant sequence similarity to the *P. aeruginosa* epimerase with approximately 55, 50 and 45% similarity over regions of 34, 46 and 96 amino acids (Ertesvag et al., 1995). The ‘R modules’, of which there are one to seven copies per gene, are typical of Ca2+-binding proteins. Each R module contains four to six repeats of a nine-amino-acid sequence. The proteins have a short consensus sequence, designated the ‘S module’, at the C-terminus.

More recently, a new mannuronan C-5-epimerase
(AlgG), which has 66% sequence identity to the P. aeruginosa algG gene, has been cloned from A. vinelandii (Rehm et al., 1996). Ca²⁺ activates the enzyme whereas the presence of Zn²⁺ causes complete inhibition. There is no conclusive evidence of signal peptide cleavage in the A. vinelandii AlgG protein and over-expression of algG in E. coli results in the formation of a partly insoluble product (Rehm et al., 1996).

The O-acetylation and epimerization of alginate are key determinants of the final structure and properties of this polysaccharide. However, the temporal order of these two reactions has not been unequivocally established, although experiments in vitro have established that the epimerase cannot utilize O-acetylated mannuronate residues as substrate (Franklin et al., 1994). There is no evidence as to how these two reactions may be controlled in P. aeruginosa and it is quite possible that the final structure of the alginate is simply the result of the relative rates of two simultaneous reactions. In contrast, it is clear from experiments in vitro that the different epimerases of A. vinelandii have the potential to produce different products. AlgE4 produces alginate with a relatively high proportion of guluronate residues (45%) but no polyguluronate blocks whereas AlgE2 introduces polyguluronate block structures (Ertesvag et al., 1995). The cyst structure in dormant A. vinelandii cells with the relative abundance of polyguluronate in the outer (exine) and polymannuronate in the inner (intine) layers (Page & Sadoff, 1975) indicates that there is a complex series of events leading to the formation of this structure. On the basis of ¹³C-NMR data, epimerization occurs while polymerization (Beale & Foster, 1996).

Role of alginate in the surface attachment of P. aeruginosa

Alginate is a key component of biofilms of P. aeruginosa and certain other pseudomonads. Gene fusion experiments have clearly established that expression of the algC (Davies et al., 1993; Davies & Geesey, 1995) and algD (Hoyle et al., 1993; Rice et al., 1995) genes is up-regulated following adherence of pseudomonads to solid substrata, with a concomitant increase in alginate production. However, alginate biosynthesis is not the only metabolic change which occurs during the transition from the planktonic to the sessile state, but is just one of a number of co-ordinately regulated events leading to the formation of a biofilm; for example, there are significant changes in the profile of outer-membrane proteins (Costerton et al., 1990) and in the expression of other virulence factors such as elastase (lasB) (Mohr et al., 1990). The expression of lasB and algD are inversely related (Mohr et al., 1990). Alginate biosynthesis and biofilm formation are triggered by a number of environmental factors including high osmolarity (Berry et al., 1989; Zielinski et al., 1992; Singh et al., 1992), ethanol (Devault et al., 1990) and nitrogen or phosphate limitation (Devault et al., 1989). The presence of copper can stimulate alginate biosynthesis in P. syringae (Kidambi et al., 1995) but not in P. aeruginosa (Leitão & Sá-Correia, 1997a) although expression of algA, algC, algD and algR genes is up-regulated. Similarly, high oxygen tension enhances expression of these alg genes in P. aeruginosa but does not result in increased alginate production (Leitão & Sá-Correia, 1997b).

Alginate clearly has an important role in the establishment of biofilms once bacteria have attached to an inert surface; for example, mucoid strains of P. aeruginosa are significantly more difficult to remove from agar surfaces than are non-mucoid strains (Boyd & Chakrabarty, 1994). Analysis of P. aeruginosa strains containing algC-lacZ constructs, in conjunction with fluorescence microscopy, demonstrates that expression of algC is up-regulated only after initial attachment of cells to Teflon or glass substrata (Davies & Geesey, 1995). Cells that failed to up-regulate alginate biosynthesis typically detached from the surface. The implication of these results is that alginate plays a role in the consolidation of a biofilm rather than in the initial adhesion event.

Furthermore, the mechanism may be different depending on whether P. aeruginosa is attaching to biological or non-biological surfaces. The trigger for alginate biosynthesis on non-biological surfaces may be the attachment process itself or the result of a secondary physiological effect such as alterations in cellular energy status (Boyd & Chakrabarty, 1995). Attachment of P. aeruginosa to inert surfaces such as Teflon is likely to be by simple hydrophobic interactions rather than pilus mediated.

Alginate (Baker, 1990) and pili with receptors to cell surface glycoproteins (Ramphal et al., 1991) both play a part in the attachment of P. aeruginosa to biological surfaces. Mucoid P. aeruginosa attaches to tracheal epithelium some 10–100-times better than do non-mucoid organisms (Marcus & Baker, 1985) but binding is significantly inhibited by anti-alginate antibodies or by polylysine (Baker, 1990). Also, mucoid P. aeruginosa grown in the presence of sublethal doses of amino-glycoside antibiotics produces less alginate and attaches less well to surfaces (Geers & Baker, 1987). Together, these data clearly indicate some role for alginate in the initial attachment of P. aeruginosa to cell surfaces. However, interactions between the bacterial pili and cell surface glycoprotein or glycolipid receptors probably play the primary role in attachment to biological surfaces (Baker, 1990).

The importance of alginate as a virulence factor and for the formation of stable biofilms by P. aeruginosa and related organisms has prompted studies into the use of alginates as potential anti-pseudomonal agents. Treatment of mucoid P. aeruginosa with alginate or anti-alginate antibodies results in the release of planktonic bacteria from artificial surfaces (Mai et al., 1993a) and infection foci (Bayer et al., 1992). Alginate lyase treatment of mucoid P. aeruginosa improves the efficacy of
administered antibiotics (Bayer et al., 1992) and enhances phagocytosis by host cells (Eftekhar & Speert, 1988; Mai et al., 1993b). The latter effect appears to be a direct result of the reduction in viscosity of alginate (Mai et al., 1993b) rather than any specific influence on chemoattractants such as interleukin 8 (Konig et al., 1995).

Although alginate is an important virulence factor in the infection of plants by some phytopathogenic pseudomonads, the precise purpose of the polysaccharide remains unresolved. The picture is further complicated as many of the phytopathogenic pseudomonads produce more than one exopolysaccharide in vitro. For example, P. syringae pv. glycinea produces levan when grown on sucrose, but an O-acetylated alginate with glucose as the carbon source, yet alginate is the only polysaccharide isolated from soy bean leaves which have been infected with this pathovar (Osman et al., 1986). Screening of 214 plant-associated fluorescent pseudomonads revealed that a variety of different polysaccharides are produced by these organisms, with only a minority producing alginate (Fett et al., 1989). However, a more detailed study of phytopathogenic P. syringae pathovars demonstrated that alginate production was always associated with bacterial-induced water-soaked lesions on the host plant leaves (Fett & Dunn, 1989). Circumstantially, alginate appears to be an important contributing factor to the successful infection of plant hosts by phytopathogenic bacteria, but whether the polysaccharide is involved in adhesion, the formation of a biofilm or some other process remains undetermined. The potential for commercial production of alginates by fluorescent pseudomonads has been explored with 6–10% conversion of glucose or fructose into polysaccharide achieved (Fett & Wijeyer, 1995).

Future prospects

Most of the research on bacterial alginates has been driven by the desire to understand the pathology of mucoid P. aeruginosa infections in the lungs of patients with cystic fibrosis. The use of stable laboratory strains of mucoid P. aeruginosa together with molecular biological techniques has greatly facilitated research in this area over the last 15 years or so. Not only has this resulted in a better understanding of the pathology of mucoid P. aeruginosa infections but it has also resulted in significant improvements in the treatment and welfare of cystic fibrosis patients. Furthermore, the research has provided significant insights into the global and specific regulatory mechanisms which control the biosynthesis, modification and degradation of bacterial exopolysaccharides. The results have had an impact far beyond that of cystic fibrosis itself into areas such as biofilm formation, lipopolysaccharide biosynthesis and differentiation processes such as encystment in A. vinelandii.

Despite the very significant progress of recent years there are still many questions that remain unanswered, and there are clearly several areas which are ripe for investigation. Although the pathway for alginate biosynthesis has been elucidated, there is still no real evidence to establish how the various components of the biosynthetic/export machinery are arranged in the membranes and periplasmic space. Models have been proposed (Boyd & Chakrabarty, 1995) but experimental evidence is still awaited. Also, the mechanisms of epimerization and O-acetylation are not yet understood. Clearly, the process is complex, particularly in A. vinelandii, where, for example, there are many forms of the epimerase (Ertesvag et al., 1995; Rehm et al., 1996). These and many other questions will undoubtedly be answered in the not too distant future.

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References


Bacterial alginate


