**REVIEW
ARTICLE****Bacterial alginate biosynthesis – recent progress and future prospects**

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Keywords: alginate, exopolysaccharide, *Pseudomonas*, *Azotobacter***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.

Fig. 1. Structure of alginate. The uronic acids β -D-mannuronate (top left) and α -L-guluronate (top right) are assembled into (1-4)-linked blocks of polymannuronate (centre), polyguluronate (bottom) or mixed sequences (not shown). Normally, bacterial alginates are O-acetylated at the 2 and/or 3 positions. Alginate from *P. aeruginosa* is devoid of polyguluronate, whereas that from *A. vinelandii* may comprise any or all of these structures.

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 of 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 σ^E class of sigma factors, i.e. analogous to *rpoE* of *Escherichia coli*, and is essential for alginate production.

Table 1. Alginate genes of *P. aeruginosa*

Gene	Location on PAO map (min)	Gene product	Reference
<i>alg8</i>	34	Polymerase/export function?	Maharaj <i>et al.</i> (1993)
<i>alg44</i>	34	Polymerase/export function?	
<i>algA</i>	34	Phosphomannose isomerase/ GDPmannose pyrophosphorylase	Darzins <i>et al.</i> (1985)
<i>algB</i>	13	Member of <i>ntnC</i> subclass of two-component regulators	Goldberg & Dahnke (1992)
<i>algC</i>	10	Phosphomannomutase	Coyne <i>et al.</i> (1994)
<i>algD</i>	34	GDPmannose dehydrogenase	Deretic <i>et al.</i> (1987)
<i>algE</i>	34	Outer-membrane porin?	Rehm <i>et al.</i> (1994)
<i>algF</i>	34	O-Acetylation	Franklin & Ohman (1993)
<i>algG</i>	34	Mannuronan C-5-epimerase	Franklin <i>et al.</i> (1994)
<i>algH</i>	?	Unknown function	Schlichtman <i>et al.</i> (1995)
<i>algI</i>	34	O-Acetylation	Franklin & Ohman (1996)
<i>algJ</i>	34	O-Acetylation	Franklin & Ohman (1996)
<i>algK*</i>	34	Alginate translocator?	Aarons <i>et al.</i> (1997)
<i>algL</i>	34	Alginate lyase	Schiller <i>et al.</i> (1993)
<i>algM</i>	68	Regulator?	Boucher <i>et al.</i> (1996)
<i>algN</i>	68	Anti σ factor?	Martin <i>et al.</i> (1993a)
<i>algP</i>	9	See <i>algR3</i>	Deretic & Konyecsni (1990)
<i>algQ</i>	9	See <i>algR2</i>	Roychoudhury <i>et al.</i> (1992)
<i>algR1</i>	9	Regulatory component of two-component sensory transduction system	Kato & Chakrabarty (1991)
<i>algR2</i>	9	Protein kinase or kinase regulator	Roychoudhury <i>et al.</i> (1992)
<i>algR3</i>	9	Histone-like transcription regulator	Kato <i>et al.</i> (1990)
<i>algS</i>	68	Anti σ factor	Martin <i>et al.</i> (1993b)
<i>algT</i>	68	See <i>algU</i>	Martin <i>et al.</i> (1994)
<i>algU</i>	68	Homologue of <i>E. coli</i> σ^B global stress response factor	Martin <i>et al.</i> (1994)
<i>algW</i>	68	Homologue of serine protease (HtrA)	Boucher <i>et al.</i> (1996)
<i>algX</i>	34	Unidentified function but high sequence similarity to <i>algJ</i>	Monday & Schiller (1996)
<i>algZ</i>	9	AlgR cognate sensor	Baynham & Wozniak (1996)
<i>mucA</i>	68	See <i>algS</i>	Martin <i>et al.</i> (1993b)
<i>mucB</i>	68	See <i>algN</i>	Martin <i>et al.</i> (1993a)
<i>mucC</i>	68	See <i>algM</i>	Boucher <i>et al.</i> (1996)
<i>mucD</i>	68	See <i>algW</i>	Boucher <i>et al.</i> (1996)

*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).

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).

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 σ^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*

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

stream genes. 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 inter-

relationships 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

Fig. 3. Alginate biosynthetic pathway. The genes indicated are those of *P. aeruginosa*. **algA* encodes the bifunctional enzyme phosphomannose isomerase/GDPmannose pyrophosphorylase.

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 (Schlichtman *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 (Schlichtman *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 polymannuronate. 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 polymannuronate 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 ^{13}C -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 fulfil 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 alginates produced by *P. aeruginosa* (Sherbrock-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 (Sherbrock-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 Ca^{2+} in the growth medium (Larsen & Haug, 1971b) with the proportion of polyguluronate increasing with increasing Ca^{2+} 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 Ca^{2+} -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). Ca^{2+} activates the enzyme whereas the presence of Zn^{2+} 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 alginates 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 ^{13}C -NMR data, epimerization occurs while polymannuronate is being synthesized, and in the same direction as 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 aminoglycoside 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 alginases as potential anti-pseudomonal agents. Treatment of mucoid *P. aeruginosa* with alginase 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 soybean 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 & Wijey, 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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