The *Azotobacter vinelandii* mannuronan C5-epimerases: their biological functions and new tools useful for their future *in vivo* biotechnological application.

by

Magnus Steigedal

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Department of Biotechnology, Faculty of Natural Science and Technology, Norwegian University of Science and Technology.

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Abstract

Alginate, an industrially widely used polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G), is produced by brown algae and certain bacteria (e.g. pseudomonads and *Azotobacter vinelandii*). The alginate monomers are grouped in blocks of M, G or MG. G-blocks enable the polymer to form gels by crosslinking (using cations like Ca^{2+}). Commercially, alginates are utilized for their viscosifying and gel-forming properties. At present all commercially available alginates are harvested from brown algae, but with an ever increasing range of possible applications for the polymer alginate production from bacteria are now also being investigated.

Alginate in *A. vinelandii* is produced as an exopolysaccharide released into the growth medium in vegetatively growing cells, but under certain adverse environmental conditions the organism is able to undergo a differentiation process by which it develops into a desiccation resistant and morphologically distinct form designated cyst, which is surrounded by a rigid coat in which alginate is a major component. The alginate from both vegetatively growing cells and cysts contain G-blocks and therefore probably has a potential for commercial production. For all alginate producers the G residues are introduced at the polymer level by mannuronan C5-epimerases. *A. vinelandii* encode one Ca^{2+}-independent periplasmic epimerase (AlgG) and seven secreted Ca^{2+}-dependent epimerases (AlgE1-7). All the AlgE epimerases have been expressed recombinantly in *Escherichia coli* and their properties have been extensively studied *in vitro.* In contrast, much less is known about their *in vivo* functions and this Ph.D project was undertaken to develop a better understanding of their biological function and to generate a knowledge basis that later may be used to modify *A. vinelandii* genetically so that it can become an *in vivo* producer of bacterial alginates with predetermined properties.

To elucidate the *in vivo* roles of the AlgE epimerases in *A. vinelandii* the genes encoding each of them were individually disrupted. These interruptions had no clear effect on the structure of the alginates produced, or on the morphology of cells grown in shake-flasks (RA1 medium), but when the same cells were grown in fermentors (PM1 medium), the *algE3* mutant (strain MS159) alginates contained only 8% G, in contrast to the 25 % found in alginates from wild-type cells grown under the same conditions. This result indicated that the phenotypes of the mutants may be significantly dependent on the growth conditions (Paper 2).

Based on the results from the single *algE* disruptions it appeared likely that more clear effects on the alginate structure and *A. vinelandii* biology would become more apparent if more than one epimerase gene was inactivated in one single strain. All *algE* genes with the exception of *algE5* are clustered in the *A. vinelandii* chromosome, and this made it possible to delete the entire cluster,

generating strain MS163. In this strain the *algE5* gene was then interrupted, generating strain MS163171. Fermentor-grown cells of strain MS163 were found to produce alginates containing only 6% G, and the polymer products from strain MS163171 contained nearly undetectable levels of G (below 2%). In addition strain MS163171 was incapable of forming cystlike structures in RA1 medium and was unable to withstand desiccation in standard cyst-testing experiments. This observation is almost certainly linked to the inability of this strain to make G-blocks (Paper 2).

In conclusion, the experiments with the *algE* mutants for the first time directly showed that the AlgE epimerases are responsible for nearly all alginate epimerization in *A. vinelandii*. In addition this work shows that the AlgE epimerases are essential for the ability of the cells to differentiate into desiccation resistant cysts (Paper 2). The conclusions reached in these experiments are in complete agreement with the results obtained in a parallel study of a transport system needed for export of the AlgE epimerases. The studies of this system constitute a minor part of this PhD work, but it is important for the independent evidence further substantiating the conclusions on the role of the AlgE epimerases (Paper 1).

A very important spin-off of these results is that strain MS163171 seems to represent a very good starting point for production of tailor-made alginates, by constructing a new series of strains in which separate selected *algE* genes are expressed at carefully controlled levels.

Even from the start of this Ph.D project it was realized that to utilize *A. vinelandii* for production of alginates with predetermined structures, a new gene expression system was needed. No such specialized tools existed then, and it was therefore decided to try to identify and develop a new broad-host-range gene expression system that could be used in *A. vinelandii.* The new system was found in *Acinetobacter sp*. and is the promoter of *chnB* together with its positive regulator ChnR. The system was called *Pb*. *Pb* was tested for ability to induce luciferace in response to an externally added inducer and was shown to be inducible in several species (Paper 3). The *Pb*-promoter was planned to be used in combination with another system previously developed in our group, and which is based on the *Pm* promoter and its cognate positive regulator XylS. An example of such a combined use is illustrated in paper 3, where the technically more convenient alginate-producer *Pseudomonas fluorescens* is used as a test organism. This model experiment showed that the two systems in combination could be used to control alginate monomer structure. The prospects for future use of these systems to construct sophisticated production systems for alginate production in *A. vinelandii* therefore now seem promising.

Table of Contents

1. Introduction ... 1
1.1 Alginate- structure, applications and sources ... 1
1.1.1 Structure properties ... 1
1.1.2 Alginate applications ... 4
1.1.3 Sources of alginate ... 4
1.2 Azotobacter vinelandii – Metabolism and life-cycle ... 6
1.2.1 Characteristics, nitrogen fixation and secondary metabolite production ... 6
1.2.2 Cell-cycle - Focus on cyst formation ... 8
1.3 Alginate production by bacteria: biosynthesis, genetics and post-polymerization processing ... 11
1.3.1 Alginate biosynthesis in bacteria ... 11
1.3.2. Genetics and regulation of alginate synthesis ... 14
1.3.3. Alginate post-polymerization processing ... 16
- Acetylation ... 16
- Degradation ... 16
- Epimerisation ... 19
1.3.4 Factors influencing alginate production in *A. vinelandii* ... 23
1.4 Tailoring microorganisms using genetic- and metabolic-engineering tools ... 25
1.4.1 Analysis and design: Identifying the right targets by "ome" analyses ... 25
1.4.2 Perturbation: Molecular biology tools for experimantal metabolic- and genetic-engineering in bacteria. ... 27
- Broad-host-range vector systems ... 28
- Regulating protein expression in metabolic engineering: Desirable promoter features ... 29
2. Aims of the study ... 32
3. Summary of Results, and Discussion ... 33
3.1 AlgE epimerases are needed for cell diferentiation and to control alginate structurein vivo in A. vinelandii ... 33
3.1.1 *A. vinelandii* forms Ca^{2+}-dependent cyst-like structures during growth in RA1-medium ... 33
3.1.2 Identification and characterization of the secretion apparatus for the AlgE-type mannuronan C5-epimerases in *A. vinelandii*. ... 36

3.1.3 Creating single disruption mutants of *algE1-7 in A. vinelandii* 37
- Effect on cell morphology and alginate production 37
- The effects on alginate structure are different in fermentor-grown (PM1-medium) cells compared to cells grown in shake-flasks (RA1-medium)................................. 39
-The possible effects on cyst formation of individual *algE* gene disruptions were too small to be quantified. 40
3.1.4 Generating deletion mutants of multiple *algE1-7* genes in *A. vinelandii* 40
- Dramatic effects on cell morphology and alginate structure in RA1-medium grown cells.. 40
- Effect on the cells ability to make cysts.. 42
3.1.5 Strain MS163171 has a potential to be used for *in vivo* production of alginates with predetermined structures. 42
3.2 Promoter systems for use in gram-negative bacteria 44
3.2.1 Construction of a new promoter system suitable for regulated gene expression in gram-negative bacteria. 45
3.2.2 Development of a screening method for identification of *Pm* promoter mutants with a low background expression, based on a *Pm* promoter mutant library. 48
4. Concluding remarks 51
5. References 53

List of Manuscripts

Paper 1

Martin Gimmestad, Magnus Steigedal, Helga Ertesvåg, Soledad Moreno, Bjørn Erik Christensen, Guadalupe Espìn and Svein Valla. **"Identification and characterization of an *Azotobacter vinelandii* Type I secretion system responsible for export of the AlgE-type mannuronan C5-epimerases"**. Manuscript

Paper 2

Magnus Steigedal, Håvard Sletta, Soledad Moreno, Bjørn Erik Christensen, Trond Erling Ellingsen, Guadalupe Espìn, Helga Ertesvåg and Svein Valla. "**The *Azotobacter vinelandii* AlgE mannuronan C5-epimerase family is essential for the *in vivo* control of alginate monomer composition and for functional cyst formation**." Manuscript

Paper 3

Magnus Steigedal and Svein Valla. **"The *Acinetobacter sp. chnB* promoter together with its cognate positive regulator ChnR is an attractive new candidate for metabolic engineering applications in bacteria"**. Manuscript

1. Introduction

1.1 Alginate- structure, applications and sources

1.1.1 Structure properties

E. E. C. Stanford, a British chemist, described the polymer alginate in a patent dated in 1881 (254). It was first named algic acid and algin and just a few of its properties were known. Over the next hundred years after its discovery more of the interesting properties of the polymer were discovered. In the current literature alginate is said to be a family of linear (1→4)-linked copolymers of β-D-mannuronic acid (M-unit) and α-L-guluronic acid (G-unit) (Figure 1) (70, 111, 112). The monomers can be arranged in widely varying sequential structures, depending on the tissue or organism from which it was isolated (Table 1 and 2). As a result of this the polymer structure is often described as consisting of only M-residues (M-blocks), stretches of alternating monomers (MG-blocks) or stretches of only G-residues (G-blocks) (Figure 1) (108, 112). Alginates may contain a varying content of these substructures even when isolated from the same source. The M-residues of alginates from bacteria can be acetylated at the O-2-and/or O-3 positions (44, 239) and the degree of acetylation is reported to be between 4% and 57% (238). As seen in Figure 1, the chair conformation is different for each of the two monomers: $^4C_1$ for M-residues and $^1C_4$ for G-residues. This property can be used to analyze alginate molecules and X-ray diffraction analysis showed that G-blocks form buckled and rod-like structures, while M-blocks form more flat ribbon-like structures (7, 8). The composition of alginates can be further determined by partial acid hydrolysis and subsequent fractionation, enabling quantification of the three different block structures (111, 112). Grasdalen *et al.* (95, 97, 98) developed the currently most used method of assigning block compositions of alginates using ^{1}H-NMR spectroscopy. This technique gives information about the monad (M and G alone), diad (MM, MG, GM and GG) and G-centered triad (GGG, MGG, GGM, and MGM) frequencies. M-centered triads can be identified using ^{13}C-NMR (96, 97). All these techniques give a view on the relative amount of M and G in the alginate and to some degree also provide information about the monomer sequence. To elucidate more of the sequence of the polymer work has been initiated to use enzymatic polymer degradation by alginate lyases (lyases are described in Chapter 1.3.3) (120).

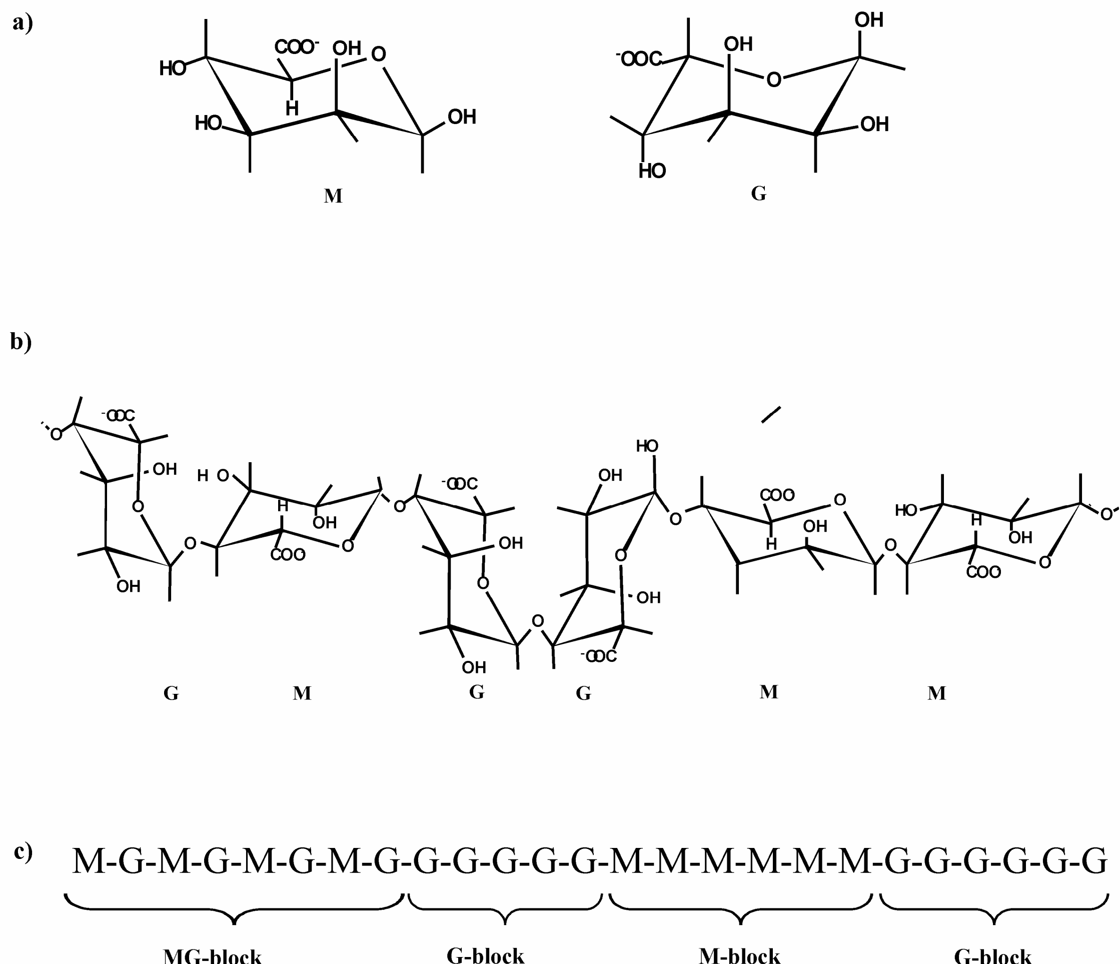


Figure 1: Alginate building blocks and polymer structure. a) Stereochemical drawings of alginate monomers: β-D-mannuronic acid (M) and α-L-guluronic acid (G). **b)** Structure of GG-, GM-, MM-, and MG-bonds in the alginate polymer chain. **c)** Alginates may contain M-blocks, G-blocks and MG-blocks of variable lengths.

Structure and molecular weight are properties dictating the functional properties of the alginate. Even at low concentrations high molecular weight alginates generate highly viscous solutions. This viscosity is mainly a function of the molecular weight (245), but the composition also contributes because of the intrinsic flexibility increase that is observed in the order GG < MM < MG (247, 248). Another interesting property, which is determined by the composition of the alginates, is their ability to make gels and bind cations. (244). The gels can be formed under two different physiological conditions. One is in an acidic environment where the M-residues are responsible for the gelation (246), and the other is a consequence of

the selective binding of cations. The diaxially linked G-residues form cavities that are the binding sites for divalent ions. These binding sites and binding sites from other parts of the polymer form junctions in the gel network, which is described as “The egg-box model”, shown in Figure 2 (94).

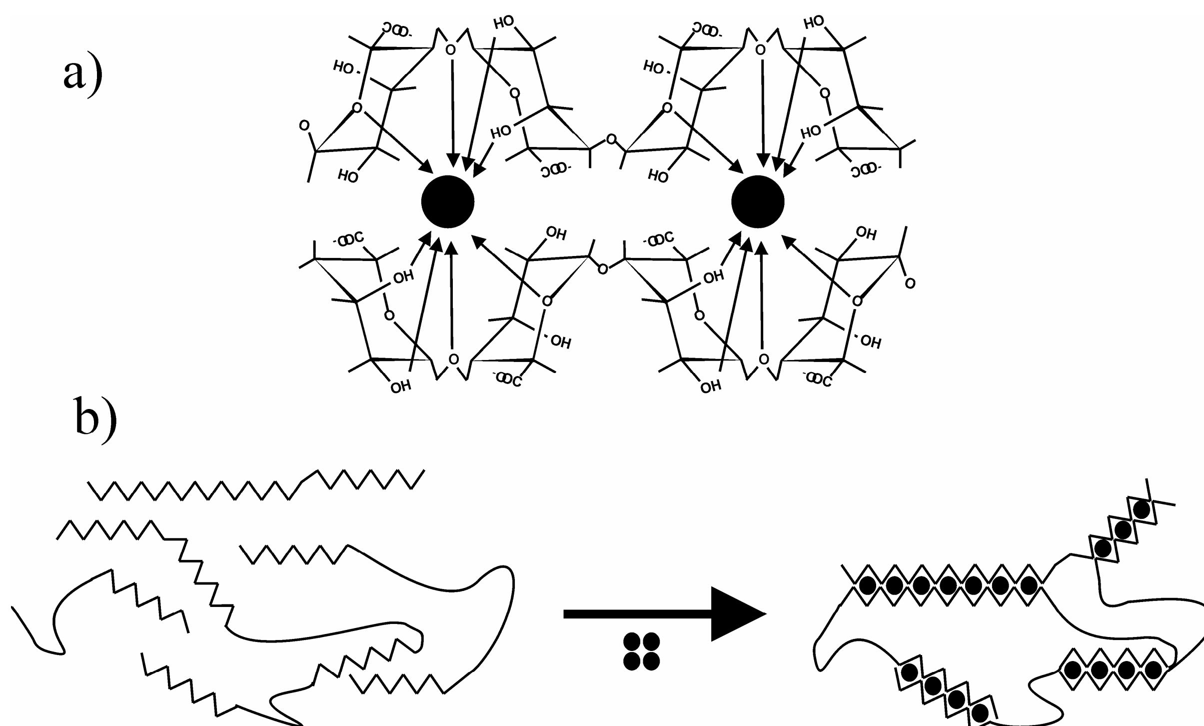


Figure 2: The “egg box model”: A model describing the interactions between G-block alginate and divalent cations: a) The G-blocks of the alginate can form junctions by interactions between the positive charged divalent ions and the negative charged and polarized regions of the alginates. **b)** Addition of divalent cations (e.g. Ca^{2+}) (●) to alginate solution lead to cooperative intra- and inter-molecular associations of G-blocks eventually leading to gel-formation.

This model makes it easy to understand the observation that the strength of this gel network depends on the distribution and length of the G-blocks, and Stokke *et al.* (258) reported the minimum continuous stretch of G-residues to form gel to be 8 (for more than 500 units residues). M-block and MG-block alginate was previously believed to be unable to gel at low concentrations due to an unsuitable chain geometry (94), but more recent studies show that structures containing alternating structures contribute to the gel-forming ability even at low concentrations (57). M-block alginates can form soft gels if the alginate concentration is high enough (58).

1.1.2 Alginate applications

Historically alginate has been used in many applications. In the 1881 patent of Stanford (254) the polymer was suggested for use as thickening agent, both in food applications, as dressings, and in the textile industry. These applications are still valid and the ability of alginates to retain water and their gel-forming, viscosifying and stabilizing properties are important for these applications (246). In medical applications alginate has been used since the 1950s and in recent years new and promising medical applications have been found. The ability of alginates to make stable hydrogels makes the material useful as 3D growth scaffolds in cell culturing (214) (e.g. tissue engineering of bone marrow (279) and regeneration of peripheral nerves (106)). The immune-stimulating effects by M-rich alginates (192) are utilized in other applications, e.g. in wound dressings (277) or because of their anti-tumor activity (75). G-rich alginate, on the other hand, does not stimulate the immune response (240) and this can be exploited in applications of alginates for encapsulation of cells for transplantation (191). Systems where this latter approach has been utilized include diabetes mellitus (251, 252), liver failure (162, 234), hemophilia B (123), colon cancer (291), brain cancer (205, 267, 278), and cells for testosterone supplement (160). Successful implantating of alginate-covered cells in large animal models is still not developed to a satisfactory level (107). This is mainly due to the instability of the capsule and consequent bio-incompatibility (47). The use of alginates modified in their chemical composition has shown promising results for the production of more stable capsules (213). These observations have increased the demand for developing alginates with compositions previously unknown in nature. For such purposes the enzymes described in Chapter 1.3.3 have an interesting potential.

1.1.3 Sources of alginate

Industrially alginate is mainly produced by extraction from brown algae. In these organisms the polymer comprise up to 40% of the dry matter and gives the plant its mechanical strength and flexibility (246). The alginate harvested from algae varies in composition according to species and from which part of the plants the polymer is extracted (F_G (fraction of G) varies from 0.78 to 0.10) (Table 1). The reason for the variation is the biological role of the polymer as structural components in the algae, e.g. alginate with high G-content is needed to provide rigidity in the stipe and outer cortex whereas a high M-content is needed in the more flexible blades (246).

Table 1: Composition and sequence parameters of some algal alginates (241).

Source		F_G[1]	F_{GG}[1]	$F_{MG, GM}$[1]	F_{MM}[1]
Elachista fucicola		0.78	0.68	0.1	0.12
Dictyosiphon foeniculaceus		0.67	0.61	0.06	0.27
Laminaria hyperborea					
	Blade	0.55	0.38	0.17	0.28
	Stipe	0.68	0.56	0.12	0.20
	Outer cortex	0.75	0.66	0.09	0.16
Scytosipkon lomentaria (204)		0.50	0.41	0.09	0.41
Macrocystis pyrifera		0.39	0.10	0.23	0.38
Durvillea antartica		0.29	0.15	0.14	0.57
Ascophyllum nodosum	Fruiting body	0.10	0.04	0.06	0.84

[1] F_G, F_{GG}, $F_{MG, GM}$ and F_{MM} is fraction of G, GG, MG, GM and MM respectively.

Bacteria are also known to produce alginate, and the first evidence of this was reported in 1964 in a paper describing a mucoid variant of the opportunistic pathogen *Pseudomonas aeruginosa* (154). These bacteria colonize the pulmonary tract of cystic fibrosis patients (171). The alginate biofilm produced protects the bacterium from dehydration and also protects it both from the hosts immune system and from antibiotic treatment. Other *Pseudomonas* strains like *P. fluorescens, P. putida, P. mendocina* (92) and *P. syringae* (140) also produce alginate. The alginates produced by pseudomonades are different from those produced by brown algae, by lacking G-blocks (238) and having acetyl groups (209, 238). *Azotobacter vinelandii* and *Azotobacter chroococcum* also produce alginate in their vegetative growth stages, probably as protection against O_2, or heavy metals or to facilitate adhesion to surfaces (42, 89). These bacteria have a metabolic resting stage in their life cycle which is triggered by adverse environmental conditions. They then differentiate into metabolically dormant cells (cysts) which are surrounded by an alginate rich capsule (194, 221). This process will be described in more detail in chapter 1.2.2. Alginates produced by *A. vinelandii* are compositionally more similar to those extracted from brown algae in that they can contain both M-, G-, and MG-blocks, and the G-content can vary over a wide range (from 5-80%) as shown in Table 2.

Table 2: Composition of bacterial alginates (273).

Source	F_G	F_{GG}	d.a.[a]
A. vinelandii	0.05-0.80	0-0.75	0.02-0.6
A. chroococcum[42]	0.08-0.12	ND[b]	0.04-0.12
P. aeruginosa	0-0.5	0	0.2-1.2
P. syringae	0-0.5	0	0.2-1.2
P. mendocina	0-0.5	0	0.2-1.2
P. putida	0-0.5	0	0.2-1.2
P. fluorescens	0-0.5	0	0.2-1.2

[a] d.a = Degree of acetylation, maximum value for O-2 and O-3 acetylated alginates is 2.

[b]ND = Not determined

Azotobacter alginates also have acetyl groups at O-2 and/or O-3 on the mannuronic acid residues. This acetylation is always present in bacterial alginates, but the degree of acetylation varies over a wide range among different species, and also in different strains of the same species (Table 2). The role of acetylation has been proposed to be to protect the polymer against degradation and epimerization (238). Acetylation has been most thoroughly investigated for *P. aeruginosa* due to its role in cystic fibrosis. Here it is believed to have a role in biofilm formation by strengthening the exopolymer interactions and determining the structure of the mature biofilm (268). The biosynthesis of alginates in bacteria will be covered in more detail in Chapter 1.3.1.

1.2 Azotobacter vinelandii – Metabolism and life-cycle

1.2.1 Characteristics, nitrogen fixation and secondary metabolite production

Bergeys manual describes *A. vinelandii* as a gram negative, mobile, soil living, obligate aerobic, chemoheterotrophic organism that is able to fix molecular nitrogen (265). To utilize atmospheric nitrogen the organism has three nitrogenases (reviewed in (59)). Nitrogen fixation is a quite energy consuming process and the nitrogenase complexes are easily destroyed by oxygen (188). Being an obligate aerobe, *A. vinelandii* should potentially have problems fulfilling the requirement of an oxygen free environment for the nitrogen fixation and several hypotheses have been proposed to explain how the cells still can manage this problem. It is known that *A. vinelandii* has a very high respiration rate, leading to two important effects. First, it generates large amounts of energy, which would supply the

nitrogenase and second, it consumes oxygen. This oxygen consumption was believed to be sufficient to keep the internal parts of the cell anoxic even at high ambient O_2 concentrations. Furthermore, it has been believed that a sudden increase in ambient oxygen levels, that would saturate the initial protection mechanism, would lead to the formation of an inactive but O_2 tolerant form of the nitrogenase complex that could be re-activated when the internal oxygen levels again normalize (43). More recently it has been argued that this protection can only be valid at low ambient oxygen levels and that other mechanisms must be active at higher levels (188). One possible protection measure is that the nitrogenase complex has the possibility to reduce O_2 without being inactivated by creating H_2O_2 which in turn is removed by superoxide dismutase (SOD) or catalases (266). This hypothesis is strengthened by the finding of an increase in SOD and catalase levels in cultures where oxygen levels are increased but this increase may also be a direct response to the increase in oxygen concentration (56). Another way of protecting the nitrogenase might be through the production of alginate. Polymer production and the amount of G introduced in the alginate increases when the levels of oxygen are increased. By this way the bacterium can make a capsule around the cells that functions as a diffusion barrier (219). The alginate production and other uses of this polymer in *A. vinelandii* will be covered in more detail in Chapter 1.3.

In addition to alginate *A. vinelandii* also produces two other industrially important secondary metabolites: 5-alkylresorciols (phenolic lipids) (210), which has a potential in biotechnology (132, 144), and poly-β-hydroxybutyric acid (PHB), which can be used for preparation of biodegradable plastics (270). PHB accumulates in many bacteria as energy and carbon source (reviewed by Anderson and Dawes (4)) and in *A. vinelandii* internally stored PHB granules function as a carbon and energy source during encystment of the cells (221, 256, 288), a process that will be described in the next chapter. This poly-ester is synthesized in three steps from acetyl-CoA, shown in Figure 3, where β-Ketothiolase catalyzes the first reaction, giving acetoacetyl-CoA. This compound is reduced by acetoacetyl-CoA reductase to β-hydroxybutyryl-CoA, a NADPH consuming process. The final step in the synthesis is catalyzed by PHB synthase (164). This process is extracting carbon from the TCA cycle, thereby leading to storage of both energy and carbon. What also can be seen in Figure 3 is that PHB synthesis may compete with alginate synthesis (Chapter 1.3.4).

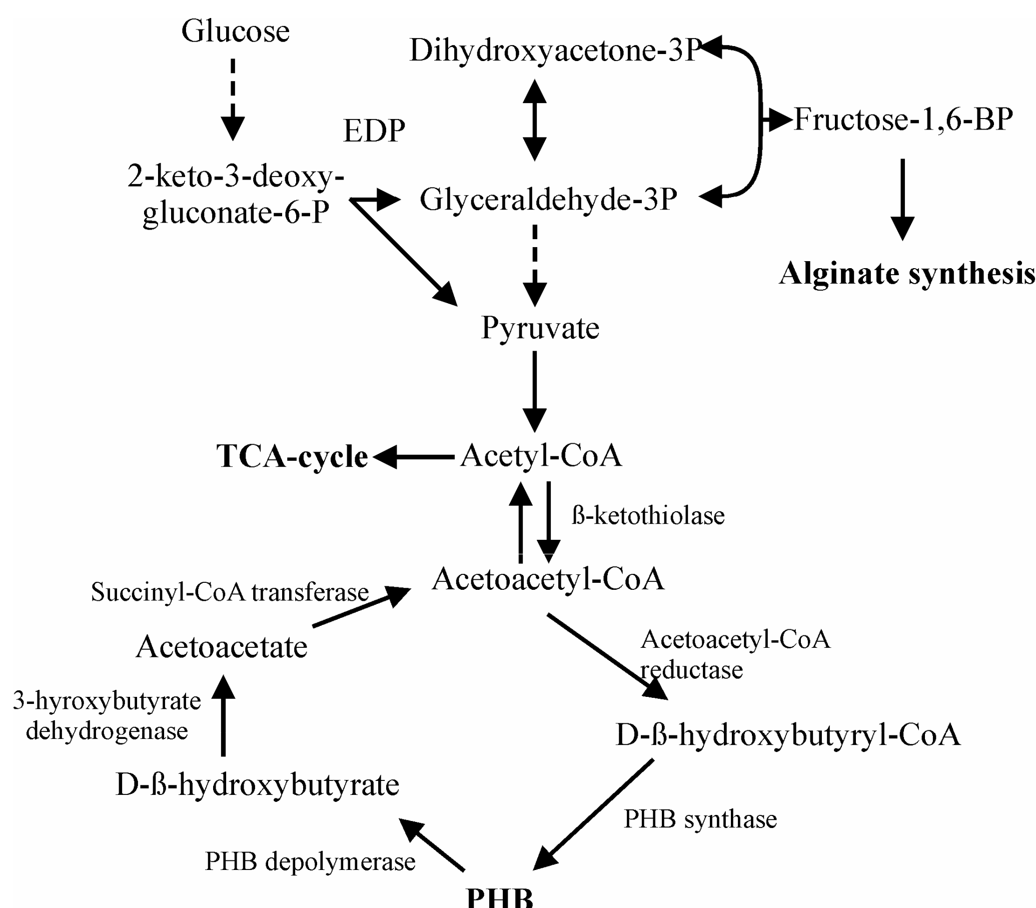


Figure 3: Glucose catabolism, alginate and poly-β-hydroxybutyrate (PHB) biosynthetic pathways in *A. vinelandii.* EDP: Entner-Duodorof pathway (Adapted from (222, 233))

1.2.2 Cell-cycle - Focus on cyst formation

A. vinelandii is in the laboratory generally seen as large, rod-shaped cells at their vegetative stage (287). The organism however, has a complex life cycle and therefore can be seen in a large variety of morphological stages (88, 221). The most studied stages include two forms of resting cells, the cyst stage (reviewed by Sadoff (221)) and the filterable cellulae stage (88). When *A. vinelandii* experience adverse environmental conditions an encystment process begins (see Figure 4). This process morphologically starts by the rounding of the cells and a rapid PHB accumulation (Figure 4b) (116). Prior to this morphological change the biochemical processes have changed by shutting down DNA synthesis (117), and reducing N_2 fixation to very low levels (117). Morphological changes continue to appear after cyst induction and the next stage is the start of the development of an outer coating around the cells. This coat is built from membrane like bleeds that come from the cell surface and contains alginate. The membrane bleeds break away from the cell surface, combine and form sheet like structures (Figure 4d), resulting in the formation of a dense compact coat, called

the exine (Figure 4e) (287). Over time a viscous material develops in the area between the cell wall and the exine, called the intine. At first this intine layer is a thin empty area, but over time and development of the cyst, it increases in size and becomes filled with granules. The increase in the size of the intine correlates with a decrease in the size of the central body (Figure 4e) (287). Three to five days after encystment has been induced the cyst has reached its mature stage, where it has an improved tolerance against UV-irradiation, desiccation and some chemicals (250). The chemical composition of the cysts are different from the vegetative cells in that the latter has a higher protein, but lower carbohydrates and lipid content (149).

The capsule components can be separated and the alginate composition can be analyzed both for vegetative cells and cysts. Such analyses have shown that the vegetative cell capsule has an M/G ratio of 1.84 and most of the alginate is either MG or MM. In contrast to this the exine has an M/G ratio of 0.45 and the alginate is dominated by G-blocks. The intine alginate is more similar to the vegetative capsule in its structure (221). When the cells are destined to encystment the process can still be aborted (Figure 4c), for instance by lack of calcium (193) (257) or addition of glucose (150). The alginate of cells undergoing abortive encystment also has been analyzed and was shown to have an unusual high M/G ratio of 5.94 and almost exclusively MM diads in the alginate (221), which is not surprising for the cells grown in low Ca^{2+} concentrations for reasons that will be covered in Chapter 1.3.3 on epimerisation. For the encystment process to occur there are at least two factors that are of central importance. The first being that alginate production must be present, shown by strains defective in synthesis of the polymer is unable to make cysts (173, 221). Second, a shift in metabolism from carbohydrate- to lipid metabolism is also essential (81). PHB accumulation is not essential for cyst formation, but is important for control of the induction of cyst formation, demonstrated in strains with blocked PHB synthesis inducing cysts even under non-inducing conditions (232). Mature cysts that experience suitable conditions for growth will undergo germination. In these conditions the central body of the cyst increases in size and the intine is reduced (Figure 4f) (287). The stored PHB decrease in amount and the respiration rate increases after 4-5 hours (148). This correlates with the onset of DNA synthesis and nitrogen fixation (156). After this change in metabolism the exine of the cyst breaks and the central body is released and enters the vegetative stage of the life cycle (Figure 4 g-h) (287).

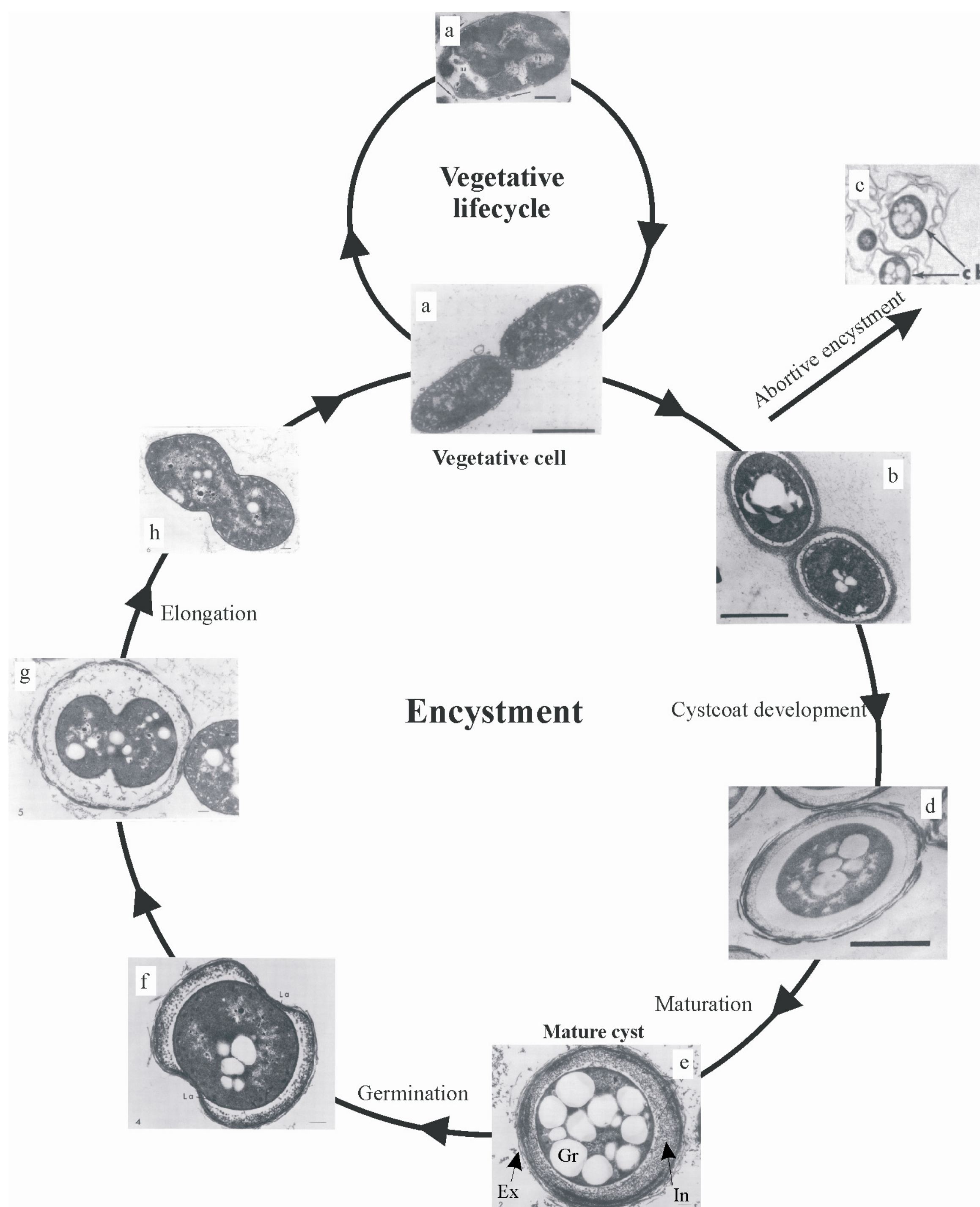


Figure 4: Encystment of *A. vinelandii* illustrated by electron microscopic pictures. a) vegetative cell (221, 276) , b) Encystment starts with rounding of the cells (221), c) abortive encystment (150), d) Exine development in encystment (151), e) Mature cyst (148), f) Germination of cyst (148), g) Further germination of cyst, initiation of cell division (148), h) development into vegetative cell (148). Gr, PHB-granules; Ex, exine; In, intine; For a more detailed description, see the text.

The encystment process can also be induced chemically by addition of n-butanol or the metabolite β-hydroxybutyrate, and this give a higher percentage of encystment than for cells undergoing encystment caused by other methods or environmental factors (150, 256, 287).

Although the cyst stage is the most studied part of the *A. vinelandii* life cycle four forms of the bacterium are observed when grown in dialyzed soil medium; vegetative cells, precysts, mature cysts and small filterable cells (12). This last morphological form is termed germinal cellulae and can grow and divide into large cells (vegetative cell morphology), and these cells are then able to differentiate into the germinal cellulae again. It has been proposed that in nature the germinal cellulae are the most prominent morphological stage and that the large cells are reproductive stages which are promoted in the laboratory setting (157).

1.3 Alginate production by bacteria: biosynthesis, genetics and post-polymerization processing

1.3.1 Alginate biosynthesis in bacteria

Alginate biosynthesis in bacteria has been investigated for several species. The studies of *P. aeruginosa* was pioneering due to its importance during chronic infections in patients suffering from cystic fibrosis (153). The biosynthesis has also been studied in other *Pseudomonas* species by genetic methods (68, 69), and at a physiological level (67, 90, 99, 100). The genes for alginate biosynthesis and regulation have also been identified in *A. vinelandii* (155). The biopolymer production in these bacterial strains share several similarities but also some differences. There are at least 13 genes directly involved in the biosynthesis of alginate in *P. aeruginosa* and in addition at least 17 genes are involved in regulation (Reviewed in (203)). An overview of the elements directly involved in alginate biosynthesis is given in Table 3, and elements involved in the regulation of synthesis are shown in Table 4. The biosynthesis of alginate can start with fructose-6-phospate (F-6-P) as substrate (158) (working model presented in Figure 5). This substrate can have its origin from several carbon sources; like from glucose via the Entner-Doudoroff pathway (in *A. vinelandii*) (16, 158, 180), using the metabolic intermediate glyceraldehyde 3-phosphate to generate fructose 1,6-bisfosphate which is dephosphorylated to F-6-P. *Pseudomonas sp.* can also use fructose to make F-6-P for alginate synthesis via a fructose phosphotransferase system (179).

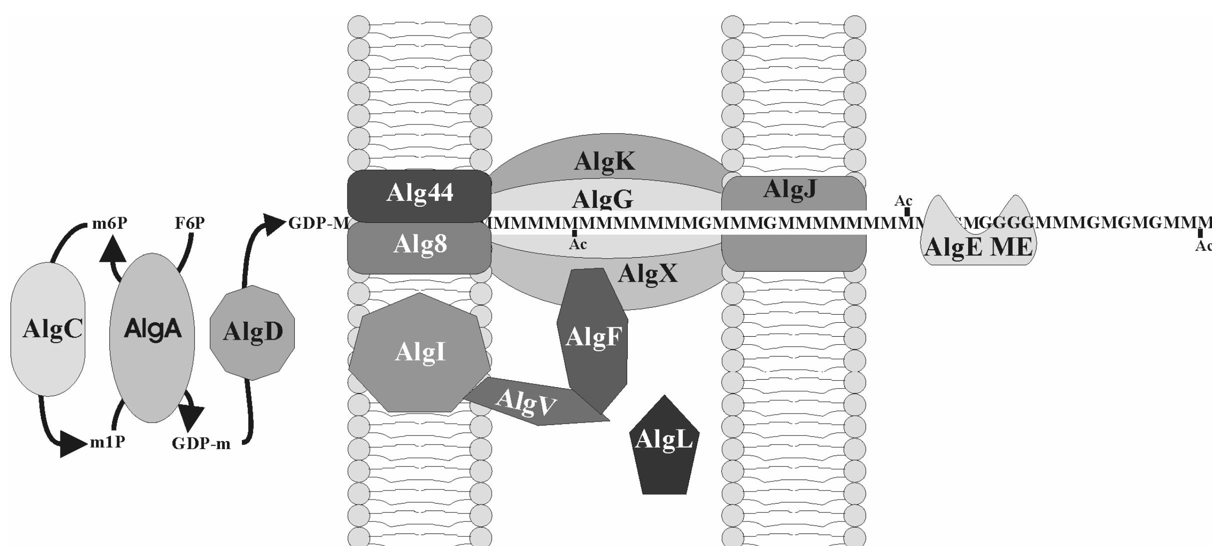


Figure 5: Alginate biosynthesis in bacteria (Examplified by *A. vinelandii*). Functions of proteins and references are listed in Table 3, for details on the process, see text. F-6-P, Fructose-6-phophate; m-6-P, Mannose-6-phosphate; m-1-P, Mannose-1-phophate; GDP-m, GDP-manose; GDP-M, GDP-Manuronic acid; AlgE ME, secreted AlgE1-7. Adapted from (83).

AlgA is the first enzyme in the biosynthesis and it has a dual role. It first converts the substrate to mannose-6-phospate (m-6-P), which is then converted to mannose-1-phopate (M-1-P) by AlgC, a phospomannomutase. The AlgA protein then converts m-1-P to GDP-mannose and AlgD finally converts GDP-mannose into GDP-mannuronic acid, the precursor for polymerization. The process was first proposed in brown algae (152) later similar pathways were identified in *A. vinelandii* (202) and in *P. aeruginosa* (201) (The genes were discovered later, see Table 3 for references). At the cytoplasmic membrane a complex including several proteins do the subsequent steps of the biosynthesis. Alg8 is together with Alg44 proposed to make the initial polymer of mannuronic acid from the GDP-mannuronic acid (161, 172). The further pathway for the mannuronan polymer is not known in full detail, but it has been proposed that three proteins, AlgG, AlgK and AlgX, form a complex by which the polymer can be transported through the periplasm (11, 126-128, 177, 211, 294). AlgG is also a C5-mannuronan epimerase, as described in more detail in Chapter 1.3.3. In the periplasm some of the residues in the polymer chain become O-acetylated. This reaction is catalyzed by a protein complex consisting of AlgI in the inner membrane, AlgJ (called AlgV in *A. vinelandii*) in the periplasm, and AlgF in the periplasm (74). Another protein in the periplasm, AlgL, has a more uncertain role and will be discussed in later chapters. The periplasmic protein complex that guides the polymer through the periplasm probably recruits the last protein involved in the polymer export, AlgJ (called AlgE in *Pseudomonas*). This is

an outer membrane protein which is an alginate specific ion channel, and is believed to be responsible for the transport of the polymer out of the periplasm and into the extracellular environment (93, 207).

Table 3: Elements involved in alginate biosynthesis in *Pseudomonas* sp. (P) and *Azotobacter* sp.(A). Some genes have alternative names in *A. vinelandii* (indicated in brackets).

Protein	Proposed function	*P*	*A*
AlgA	Phospomannose isomerase and GDP-mannose pyrophosphorylase	(171, 235)	(155)
AlgC	Phosphomannomutase and phosphoglucomutase activities.	(206, 292)	(82)
AlgD	GDP-mannose dehydrogenase	(249, 264)	(31)
AlgE (AlgJ)	Outer membrane, alginate specific ion channel responsible for transport.	(39, 93)	(207)
AlgF	Periplasmic protein responsible for O-acetylation.	(72, 74)	(275)
AlgG	Periplasmic-located C5-mannuronan epimerase that also protects the alginate polymer from lyase activity.	(36, 71, 84, 126)	(208)
AlgI	Inner membrane protein involved in alginate O-acetylation.	(74)	(275)
AlgJ (AlgV)	Membrane-associated, periplasmic protein involved in O-acetylation.	(74)	(275)
AlgK	Periplasmic protein required for proper polymer production.	(127, 294)	(173)
AlgL	Periplasmic alginate lyase, responsible for clearing the periplasmic space.	(1, 11, 128, 177, 228)	(61, 197)
AlgX	Periplasmic protein required for proper polymer production.	(177, 211)	(275)
Alg8	Membrane protein and hypothesized polymerase.	(161)	(172)
Alg44	Membrane protein required for alginate production.	(161)	(172)
PsmE/ AlgE1-7	Secreted mannuronan C5-epimerases.	(19)	(60, 62, 259)

1.3.2. Genetics and regulation of alginate synthesis

The genes for biosynthesis of alginate are organized similarly in all alginate-producing bacteria investigated so far (*P. aeruginosa (37), P. syringae* (200) and *A. vinelandii* (155)). They all have twelve genes in the pathway clustered together, as shown in Figure 6. In *P. aeruginosa* they are transcribed from a single promoter (37), but in *A. vinelandii* they are transcribed from several promoters (31, 155, 172, 173, 275).

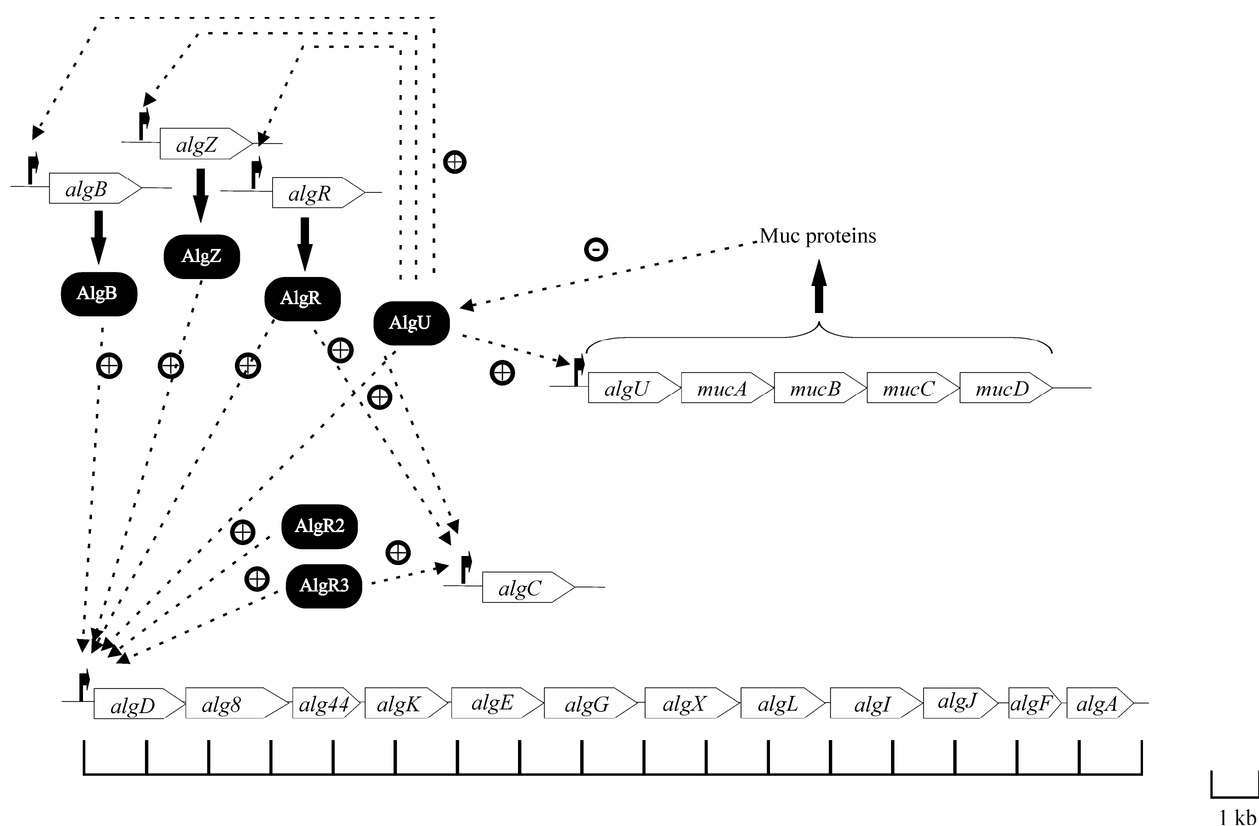


Figure 6: Organization and regulation of the alginate gene cluster in *P. aeruginosa*. Genes are denoted as white arrows and proteins are black ovals. Transcription and translation are indicated by thick vertical black arrows. Regulation is indicated with dashed arrows and promoters are indicated by curved arrows, +: positive regulation, -:negative regulation. See table 4 for further details of each regulatory protein.

Regulation of the polymer production involves several genes and regulatory units and is complex. Most of the knowledge on this originates from studies of *P. aeruginosa,* and the gene and gene products involved in the regulation are shown in Table 4.

Table 4: Elements involved in bacterial alginate production for *Pseudomonas sp* (P) and *Azotobacter sp* (A).

Protein	Proposed function*	P	A
AlgB	Positive activator of *algD* transcription.	(86, 285)	-
AlgH	Regulates alginate production, unknown mechanism.	(230)	
AlgP/AlgR3	Activator of *algD* transcription. Histone-like protein.	(136, 141)	
AlgR	Activates *algC* and *algD* transcription.	(53, 54, 183)	(185)
AlgT/AlgU	Alternative sigma factor involved in transcription of *algD, algR, algT, algZ* and *rpoH.*	(55, 115, 165, 284)	(168)
AlgQ/AlgR2	Activator of *algD* transcription.	(135, 230)	
FimS/AlgZ	Negative regulator of alginate production and hypothetical cognate sensor kinase for AlgR.	(280, 290)	
AlgZ	Activator of *algD* transcription.	(14, 15)	
CysB	Activator of *algD* transcription.	(51)	
IHF	Required for *algD* transcription, histone like protein.	(271, 286)	
KinB	Cognate sensor kinase for Alg, inner membrane protein.	(159)	
MucA	Anti-sigma factor, negative regulator of alginate production by sequestration of AlgT (AlgU), inner membrane protein.	(167)	(168, 184)
MucB/AlgN	Negative regulator of AlgT. Periplasmic protein.	(87, 166)	(168)
MucC/AlgM	Proposed negative regulator of algT, might act synergistically with MucA and MucB, Inner membrane protein.	(25, 170)	(168, 184)
MucD/AlgY	Negative regulator of AlgT (AlgU), works by removing activating factors.	(23)	(168)
RpoN/NtrA/σ^{54}	Involved in regulator of *algD* transcription and responsible for *algC* transcription.	(24)	

*Most of these data originates from studies in *P. aeruginosa*. For some of the mentioned factors the regulation have been shown to be different for other species.

In *P. aeruginosa* the expression from the biosynthetic operon is under the control of the *algD* promoter. An alternative sigma-factor, AlgT (AlgU), is an important inducer of this promoter and also induces the expression of other positive regulators of *algD* transcription, like AlgR, AlgB, AlgZ and AlgT (autoregulation) (91). AlgT is negatively regulated by the gene

products of *mucA, mucB, mucC* and *mucD.* For MucA and MucB this regulation is believed to act by sequestration of the AlgT protein, and an inactivation of the *muc* genes lead to deregulation of AlgT and thereby a deregulation of alginate production (87, 166, 167). The mechanism by which MucC and MucD work is stil uncertain (24, 25, 231). AlgR has a role in activation of the *algD* promoter under certain conditions. It binds within three regions of the *algD* promoter which lies up to 479 bp upstream of the transcription start point (176). AlgB is also a positive regulator of *algD* transcription but further details about this regulation are not known (86, 285). AlgZ is an activator with a binding site located far upstream of the transcription start point of *algD*. The protein has some similarity to other DNA binding proteins and is believed to be part of a complex with the RNA polymerase and AlgT (14, 15). AlgR3 (AlgP) and IHF have been shown to be required for *algD* transcription and they are believed to function by contributing to DNA looping (91). AlgR3 is also believed to control the expression of *algC*, which is located in another part of the chromosome and expressed from another promoter than the rest of the alg genes. This expression is in addition under the control of AlgR (76) and AlgT.

1.3.3. Alginate post-polymerization processing

- Acetylation

As previously mentioned the alginates become acetylated in the O-2 and/or the O-3 position on their way out of the cell. The proteins responsible for this are AlgI, AlgJ (AlgV) and AlgF. The genes encoding these proteins are located adjacent to each other in the alginate biosynthesis cluster (Figure 6), and the proteins are proposed to be individually essential for the acetylation process in *P. aerugionosa.* AlgI, AlgJ and AlgF are hypothesized to form a protein complex which performs the acetylation (72-74, 236, 275). In *P. fluorescens* a more recent study show that only AlgF is essential for this process and mutant strains without the genes for AlgI and AlgJ can stil acetylate the alginate, but at a reduced level (83).

- Degradation

Alginate degrading enzymes are found in many organisms ranging from marine bacteria, soil living bacteria and marine algae and vertebrates (reviewed by Wong *et al.* (282)). Even the alginate biosynthesis gene cluster includes the gene for an alginate degrading enzyme, *algL* (61, 155, 228). This might seem paradoxical, but as will be discussed later in this chapter it

has an important role in the biosynthesis machinery. AlgL is an enzyme that is part of a large family of enzymes called alginate lyases. They catalyze the degradation of the polymer by a β-elimination mechanism, targeting the glycosidic 1→4 O-linkage between monomers. This leads to double bond formation between carbon 4 and 5 in the ring from which the 4-O-glycosidic bond is eliminated (111). A proposed mechanism for this reaction is shown in Figure 7 (77, 78), and part of this mechanism is probably shared by the epimerization reaction described later in this chapter.

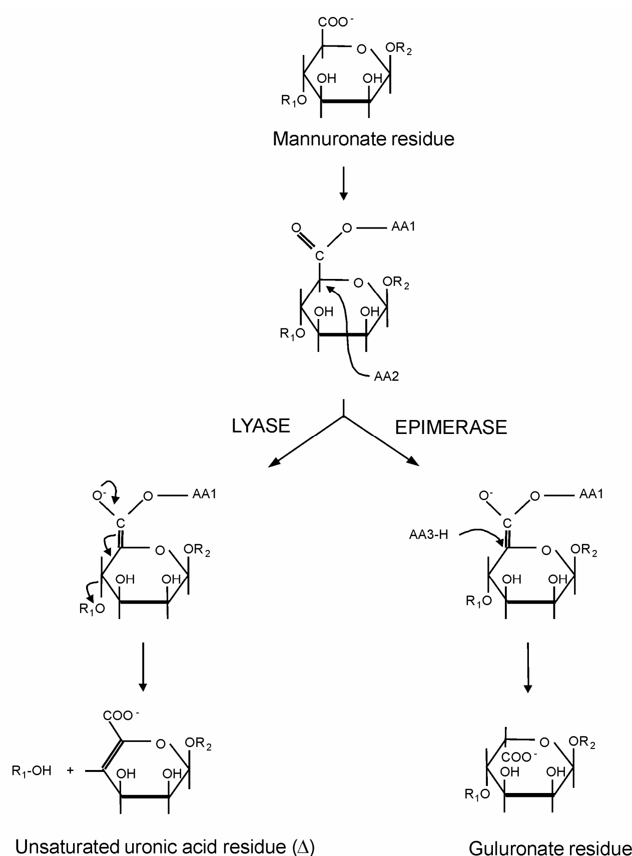


Figure 7: Alginate lyase and epimerase reactions. The two processes have been proposed to share the first step in the reaction and a reaction intermediate is made. The alginate lyase product is then made by an β-elimination of the 4-O-glycosidic bond, while epimerization results in a replacement of the proton at C5 (77).

For the species that do not produce the polymer themselves the role of the alginate lyase is to degrade the alginate to provide a carbon- and energy-source. These lyases can be intracellular (145, 146, 227) or extracellular (225, 226, 262, 263). For organisms producing the polymer the alginate lyases have different roles, and is found both intracellularly, extracellularly and in the periplasm. For *A. vinelandii* and *A. chroococcum* the first alginases were reported as

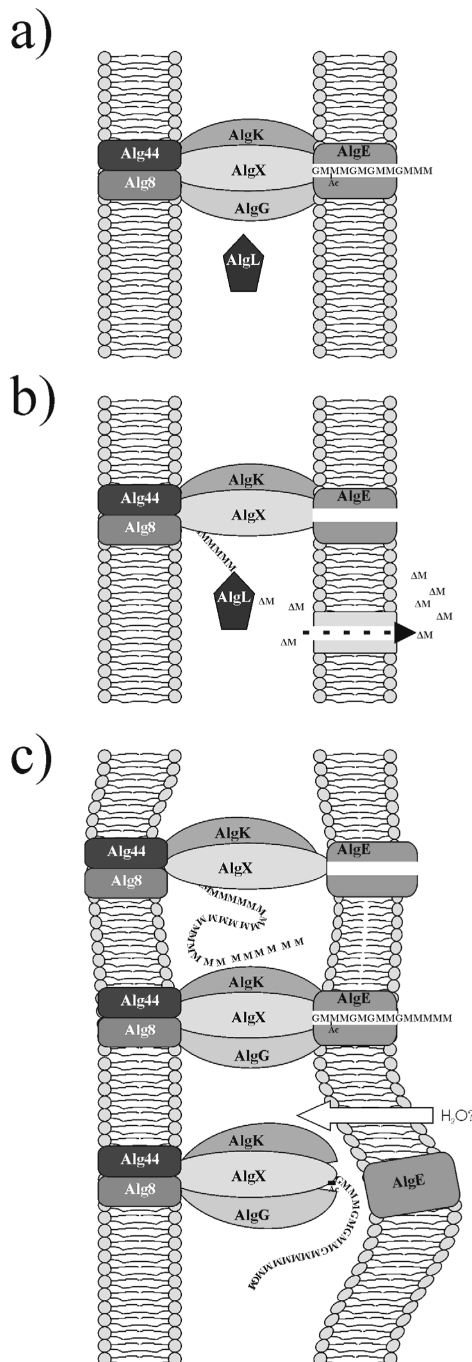


Figure 8: Modell for the function of AlgG and AlgL. (11)
a) Alginate is polymerized in the inner membrane and AlgG, AlgK and AlgX form an alginate transport scaffold that guide the polymer through the periplasmic space and protects the growing polymer chain from AlgL-mediated polymer degradation. b) When one of the alginate scaffold components is absent (e.g. AlgG), the alginate polymer leaks into the periplasmic space and becomes degraded by AlgL to dimers (ΔM). c) Absence of AlgL leads to accumulation of alginate in the periplasmic space which in turn leads to toxic swelling of this compartment.

periplasmic during vegetative growth, lost during the cyst stage and resynthesized upon germination (139). An example of these alginases was the Ca^{2+}-dependent alginate lyase AlgL isolated from *A. chroococcum* (197). A similar enzyme was later found in *A. vinelandii* (61) but this was reported to be independent of divalent cations. AlgL is also found in *P. fluorescens* and *P. aeruginosa* and its role in the alginate machinery has been investigated in these organisms. Alginate biosynthesis was first believed to be independent of AlgL and the role of AlgL was to determine the chain length of the alginate molecules (26). Another study contradicted this conclusion and showed that AlgL has an essential role in the biosynthesis and that the deletion of *algL* from the genome leads to toxic effects to the cells (177). Recent studies of this protein have revealed more information and it is now believed that AlgL is essential for alginate production. A proposed working model for AlgL is shown in Figure 8 (11, 128). The alginate polymer is, as earlier stated, believed to be transported through the periplasm by a protein complex, but in some cases this export fails and some alginate molecules become stranded in the periplasmic space. AlgL is proposed to be a scavenger of these stranded molecules (Figure 8b) and the polymer accumulation is probably the reason for the observed toxic effect of *algL* deletion-mutants of *Pseudomonads* (Figure 8c).

A. vinelandii has previously been reported to have two lyases, AlgL (61) and the bifunctional enzyme AlgE7 (260) which also has an epimerase function that will be described in the following chapter. Recent work in our group has lead to the discovery of three new lyases: AlyA1-3 where one (AlyA3) seems involved in alginate release from the cell and one (AlyA2) even seem to be essential for survival during certain growth conditions (83).

- Epimerisation

An interesting property of the alginate polymer is that it is synthesized as polymannuronic acid and then the conversion of mannuronic acid to guluronic acid is performed by mannuronan C-5 epimerases acting at the polymer level (110). This was first established for bacterial alginate production, but has also been found to be the case for algal alginate (186, 212). The first mannuronan C-5-epimerase to be partly characterized was AlgG (gene encoded in the *alg* gene cluster (Figure 6)) from *P. aeruginosa* (36). Its gene was identified by making mutant strains incapable of incorporating G in the alginate. A few years later the same group published more evidence for the AlgG protein to be a polymer level alginate C5-mannuronan epimerize by *in vitro* experiments using recombinant AlgG and poly-mannuronic acid as a substrate (71). They also proposed that the protein exists in two forms, and that a pre-AlgG has an N-terminal signal sequence directing the protein to the periplasmic space. For *A. vinelandii* it had been known that a secreted Ca^{2+}-dependent mannuronan C5-epimerizating enzyme existed (109) and the gene for such an protein, designated *algE*, was cloned and expressed (60). This protein catalyzed G-block formation in polymannuronic acid and was different from AlgG in that it was exported out of the cell, carrying out epimerization on the outside. The discovery of the gene for the first enzyme was followed by the discovery of a whole family of secreted enzymes of the AlgE type designated AlgE1-AlgE7 (62, 259). The different enzymes have been cloned and expressed in *E. coli* and extracts from such cultures can be used *in vitro* to epimerize mannuronan. In this way it has been discovered that each enzyme generate varying epimerization patterns (Table 5) (63). This variation in pattern can be explained by the modular structures found in these enzymes, see Figure 9. One of these modules, the A-module, is about 385 amino acids and is present as one or two copies in each enzyme. It has been shown, with AlgE1 as an example, that this module is responsible for the epimerization reaction, determination of the sequence distribution of G residues and binding of Ca^{2+} (64) This was later shown for A-modules of AlgE2, AlgE3 and AlgE4 (20, 63). The other module type, designated the R-module, is

present in one to seven copies in the enzymes and these units are recognized by their Ca^{2+}-binding motifs and they also have been shown to stimulate the epimerization reaction (64).

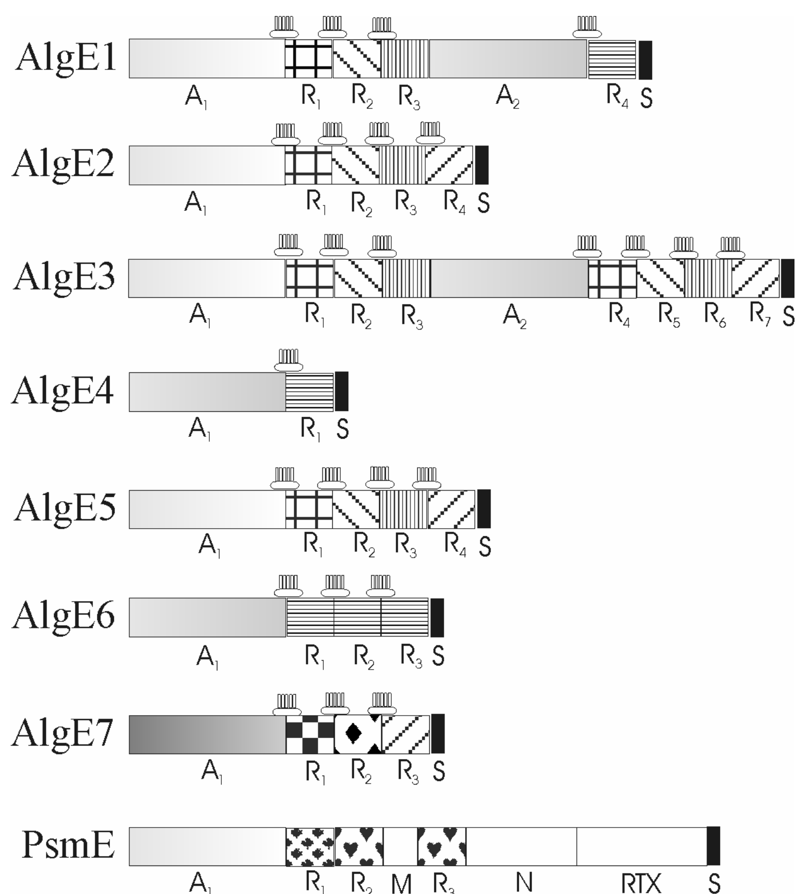


Figure 9: The modular structures of the secreted mannuronan C5-epimerases. Secreted mannuronan C5-epimerases are composed of different numbers of A- and R-modules. The A-modules can be grouped by degree of similarity and members of the same group are colored with identical shade of gray. Closely related R-modules are colored with identical patterns. S: conserved motif that is not part of the R-modules (19, 62, 259); M: a cadherin-like domain; N: Region possessing acetyl-hydrolase activity; RTX: domain related to hemolysin-ype calcium binding proteins.

Nonameric, glycine rich motifs are also found in the R-module. Such sequences are characteristic for proteins secreted via Type I secretion systems and suggest that the R-module may be responsible for targeting the enzyme for secretion (50, 60). Recent work, where the structure of the R-module was determined by NMR, has confirmed the findings of the R-modules Ca^{2+} binding capacity (293). This study also hypothesized that the R-module provides an elongation of the A-module and that the R-module is capable of binding alginate in contrast to previous suggestions based on atomic force microscopy (242).

Table 5: Epimerization Pattern of the different epimerases. Partially reproduced from (63).

Enzyme	F_G	F_{GG}	$F_{MG,GM}$	F_G/F_{MG}
AlgE1	0,47	0,23	0,25	1,9
AlgE1-1	0,49	0,36	0,13	3,8
AlgE1-2	0,41	0	0,41	1,0
AlgE2	0,40	0,27	0,13	3,1
AlgE2	0,63	0,45	0,18	3,5
AlgE3	0,28	0,1	0,18	1,6
AlgE3	0,65	0,46	0,19	3,4
AlgE3-1	0,42	0,29	0,13	3,2
AlgE3-1	0,65	0,47	0,18	3,6
AlgE3-2	0,43	0,03	0,40	1,1
AlgE4	0,36	0,04	0,33	1,1
AlgE5	0,43	0,28	0,15	2,9
AlgE6	0,42	0,26	0,17	2,5
AlgE6	0,78	0,57	0,21	3,7
AlgE7	0,34	0,09	0,25	1,4
PsmE (19)	0,86	0,78	0,07	12,3

Table 5 show that AlgE4 is the only epimerase that makes alternating structures in the alginate, but as seen in Figure 9 there are two enzymes with more than one A-module (AlgE1 and AlgE3) and the A-modules of these enzymes have been cloned individually (AlgE1-1, AlgE1-2, AlgE3-1 and AlgE3-2 in Table 5). Two of theses A-modules (AlgE1-2 and AlgE3-2) also generate such epimerization patterns. The other A-modules and enzymes all make G-blocks to varying extents. Interestingly at similar epimerization levels the length of the G-blocks varies for each enzyme (shown for AlgE1 and AlgE6) (120). The A-modules ability to create these patterns can partially be explained by the modes of action of the enzymes. All epimerases are hypothesized to work in a non-random fashion, either by a preferred attack mechanism, (AlgE2 (105)) or in a processive manner (AlgE4 (30, 105, 118, 242)). The processivity of AlgE4 on the substrate leads to the resulting alternating structure because of the physical structure of the alginate. To be processive on mannuronan and introduce G-blocks the enzyme would have to turn 180º which is energetically unfavorable. AlgE2 therefore seems to have lost its processivity and instead display a preferred attack mechanism, preferentially introducing a G next to a preexisting G. When the enzymes work on filling in MG-blocks they can display a processive mode of action and the varying G-block length introduced by the different enzymes can be explained by difference in prosessivity (120). These models require the enzyme to accommodate several residues of the

polymer and findings of the 3D structure of the A-module of AlgE4 supports this hypothesis (18, 19, 105). The differences in epimerization patterns have opened up the possibility to make alginate with defined structures *in vitro*, making these enzymes potentially interesting for production of high quality alginates for advanced biotechnology applications (e.g. more stable alginate capsules for transplantation mentioned in Chapter 1.1.2). The enzymes also display different optimum conditions for action and one (AlgE7) also possesses a lyase activity in addition to its epimerisation ability (260). Biologically the epimerases of *A. vinelandii* are believed to play a role in the encystment process. This is due to the fact that the different parts of the cyst structure contain alginates with different structure (221). A study trying to elucidate this connection showed that there is an increase in the amount of epimerases and that the presence of the individual epimerases vary during the events of encystment and germination (63). Recently preliminary information about the expression from the promoters of the epimerases have been found and these results also indicate a change in the pattern of expression from the *algE* promoters during encystment and germination processes (19).

A secreted mannuronan C-5 epimerase has also been found in a *P. syringae* strain. This enzyme, called PsmE, has a similar modular structure as the *A. vinelandii* enzymes (Figure 9), where the A-modules operate in the same manner as the A-modules of AlgE1-7. In addition PsmE is capable of deacetylating the alginate prior to epimerization and it also have some extra modules (Figure 9) (19).

The identification of the AlgE type epimerases led to the hypothesis that *A. vinelandii* has a different approach to epimerisation than *P. aeruginosa*. It was therefore somewhat surprising when an *algG* homolog was later found in what looked like an *alg* gene cluster physically organized in a similar manner to that in *P. aeruginosa* (208). This gene product had the same characteristics as *P. aeruginosa* AlgG and together with the identification of all the other gene homologs of the *P. aeruginosa alg* operon led to the belief that the alginate synthetic machinery was inherited from an common ancestor and that the *algE1-7* genes were incorporated into the *A. vinelandii* genome at a later stage to cope with cyst formation. As previously mentioned recent work in *P. aeruginosa* and *P. fluorescens* has shown that the AlgG protein has a dual role. In addition to its role as an epimerase it is also believed to be a structural component in the alginate synthesis machinery, where it is responsible for channeling the alginate through the periplasmic space and protecting it from the alginate

lyase (AlgL) (Figure 8) (84, 126). The epimerase activity of AlgG is not needed for alginate production in *P. fluorescens* and *P. aeruginosa,* as has been shown for AlgG point mutants that are defective in epimerization, but still make alginate efficiently (84).

1.3.4 Factors influencing alginate production in *A. vinelandii*

The production of microbial alginate would have the advantage of constant and unique composition and there would be no limitations on supply (218). Still such a process faces several challenges, such as production efficiency and product molecular weight and composition. As mentioned in Chapter 1.2.1. *A. vinelandii* also produces PHB and the production of alginate and PHB are competing processes. It has been reported that these two processes are difficult to separate in the wild-type organism (169) but genetic engineering might provide strains with more desirable production properties. Initial attempts at generating mutants impaired in the PHB synthesis pathway surprisingly only generated strains which produce less alginate than the wild-type (199). Other attempts have had more success in increasing the alginate production in yield, but the overall growth of these mutants is reduced, and the total amount of alginate produced is therefore also reduced or similar to the wild type (233).

The alginate production in *A. vinelandii* strongly reflects the biological function the polysaccharide has in this bacterium. As mentioned earlier (Chapter 1.2.2.), the alginates function as a structural material in the cysts that are made under unfavorable conditions. Interestingly the polymer is also produced during vegetative growth. This production is believed to function as a protective barrier against heavy metals, as an ion exchange system or as a coating for protection from other bacteria or viruses (102). It has also been postulated that the alginate in vegetative cells form a capsule that protects the cells from O_2 (219) (discussed in Chapter 1.2.1). It has been shown that the control of oxygen concentration is of great importance for optimal alginate production and also for biomass production (220). Limitation in oxygen levels leads to PHB synthesis that will lead to low alginate production (122, 196, 219, 220). More surprisingly, excess oxygen supply will also lead to a lowering in alginate production (122, 196). This is more difficult to explain, but it has been proposed that the alginate coating made by cells under conditions of high levels of oxygen supply prevents the diffusion of other medium components, thereby lowering the biomass and alginate production (219).

The critical impact oxygen levels have on the production of alginate has complicated the study of other factors important for production. This is evident for example from studies of the effect of different phosphate concentrations, where it was found that the results varied significantly between uncontrolled flask cultures and controlled microaerophilic conditions (220). Despite these difficulties several attempts to increase alginate productivity by altering media composition or cultivation conditions have been made (e.g. shaking speed (33, 40, 41, 121, 129, 190, 198, 224)). One way of influencing the alginate production is to vary the content of fixed nitrogenous compounds. This is interesting because it has been shown that addition of ammonium inhibits alginate production (28). The studies also concluded that by using different peptone sources in the medium the yield changed as much as 30%. Other very important nutrients for alginate production is phosphate, but as already mentioned, most of the studies have been done in shake flasks without oxygen control and therefore seem to be contradictory and difficult to interpret (196, 220). The effect of iron and/or molybdate has also been studied, and it was found that a limitation gave the highest production rates of alginate (5, 66). Both iron and molybdate are important for the nitrogen fixing machinery of *A. vinelandii* (3) and the effect on alginate production was shown to be most apparent in cells grown diazotrophically. This can probably be explained by the hypothesis that alginate protects the nitrogen fixing machinery from oxygen and when important co-factors of the machinery are limited the protection becomes even more critical, hence the increase in alginate production. Cations have a role in cross-linking alginate chains together, forming the protective capsule around the cells. A limitation of these cations might therefore be hypothesized to increase alginate production, but this seems not to be the case (for Ca^{2+}) (5, 187). It has been suggested that calcium ions are so important for the secreted epimerases of *A. vinelandii* that a limitation of this ion would lead to alginates with less efficient cell protection from oxygen, therefore lowering biomass and alginate production (218).

1.4 Tailoring microorganisms using genetic- and metabolic-engineering tools

Traditionally alteration in the production of secondary metabolites by microorganisms has been conducted by random mutagenesis of the entire genome, followed by screening strategies aimed at finding the desired mutants. These procedures are time-consuming and may also generate side effects by introducing unwanted secondary mutations that might not be visible until later when e.g. more realistic production parameters are being tested (217). A more rational approach has been developed and was termed metabolic engineering. Metabolic engineering has been described as "the improvement of cellular activities by manipulations of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (10). A description of the cycle of metabolic engineering is given in Figure 10.

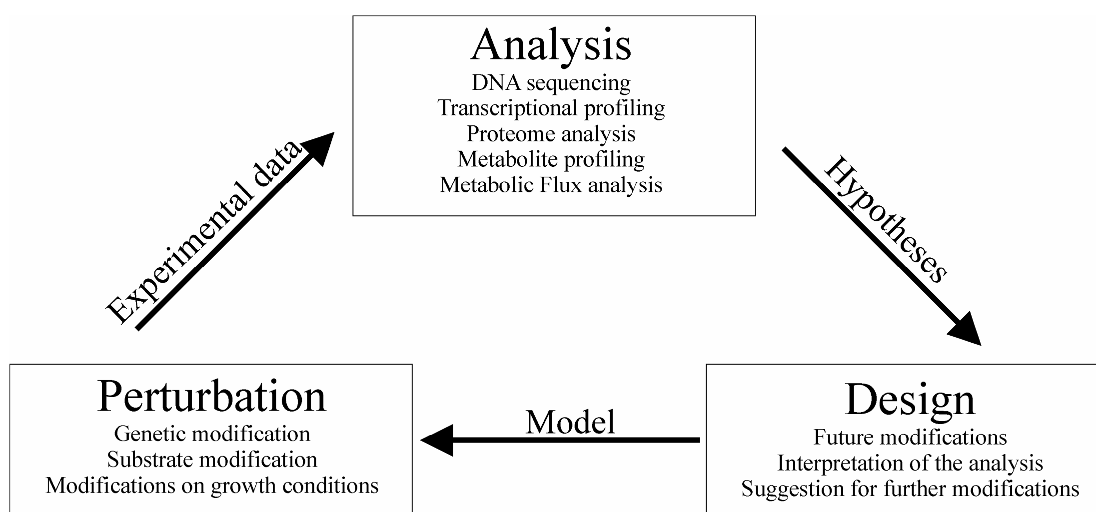


Figure 10: The cycle of metabolic engineering

1.4.1 Analysis and design: Identifying the right targets by "ome" analyses

In recent years more tools in functional genomics have been developed. These tools include DNA sequencing (genome sequencing and other large-scale sequencing projects), transcriptional profiling, proteome analysis, metabolite profiling and comparative flux analysis (Reviewed by Bro and Nielsen (29)). A summary of these techniques, their advantages and disadvantages and examples of their uses are given in Table 6.

Table 6: Functional genomics tools, their advantages, disadvantages and examples of use (Inspired by Bro and Nielsen (2003)).

Tool	Advantages	Disadvantages	Examples of finding targets for:
DNA sequencing - Genomic	Discovers all possible mutations	Expensive and requires extensive data analysis	Increased methionine production (216)
DNA sequencing - Selected genes/regions of the chromosome	Cheap and easy to analyze	Not all mutations responsible for observed phenotype may be discovered	Increased production of L-lysine (189)
Transcriptional profiling	All possible effects on mRNA level may be identified Simple to perform Relatively inexpensive	Difficult data-analysis No control over secondary effects	Increased IGF-I protein production. (38)
Proteome analysis	All possible effects on proteins that may be identified is found	Laborious analysis technique Risk of loosing info from proteins of low concentrations (e.g. regulatory proteins)	Increased production of serine-rich proteins (103) Increased recombinant production of antibody fragments (2)
Metabolite profiling	Relatively easy to perform Direct link to pathways	Interpretational difficulties Many secondary effects	Increased production of lovasatine (6)
Comparative flux analysis	Direct information obtained Easy to perform	Integrative information difficult to interpret and use on new systems	

The uses of these techniques are still in their infancy, but some research projects have utilized them with success. Transcriptional profiling has been used to enhance the production of insulin-like growth factor I fusion protein in *E. coli*. In this study the transcription profile was shown to be significantly altered for 529 genes and 200 of them was down-regulated. Two of these genes, *prsA* (responsible for the first step in biosynthesis of amino acids and nucleotides) and *glpF* (responsible for the first step in glycerol metabolism), were selected and coexpressed resulting in an increase in production from 1.8 to 4.3 g/L (38). Analyzing the proteome has also been successful. In one of these studies the goal was to increase production of serine-rich proteins, like leptin and interleukin-12. Protein profiling (2D-gel electrophoresis) detected that induction of production of the wanted protein resulted in a decrease in the amount of enzymes responsible for the synthesis of the serine family amino acids. Using this information a system, where *cysK* (gene for cystein synthase A) was co-expressed with the wanted protein was constructed, and this led to a two-fold increase in the production (103). To fully take advantage of this information good systems for integrating the different -omics are needed and also efficient tools for creating perturbations are needed (see next chapter).

1.4.2 Perturbation: Molecular biology tools for experimantal metabolic- and genetic-engineering in bacteria.

After the analysis and design phase a target for metabolic engineering is identified. This target might be deletion or introduction of a single gene, several genes, an operon or a combination of these targets. To effectively deal with the genetic manipulations of these targets a set of tools are needed. These tools include a vector system that allows reasonable transformation of genetic material to the strain of interest and a promoter system for expressing the proteins of interest at the desired level (182). Keasling has defined the ideal qualities of gene-expression tools for metabolic engineering: The promoters must show tight and consistent control, the expression system should be able to regulate several genes simultaneously at different levels, the vector should be stably maintained in the host for several generations and the system should represent a minimal metabolic burden to the cell (137). For most organisms there are not developed any specific tools that meet these criteria, and the remaining part of this chapter will cover broad-host range systems that can be used in several species.

- Broad-host-range vector systems

When targets for genetic manipulation have been identified, vectors for introducing the genetically modified elements are needed. These vectors can be pure cloning vectors, expression vectors or transposon vectors. Examples of these three types are given in Table 7.

Table 7: Examples of vectors for introducing genetic elements to gram-negative bacteria

Vector		Replicon	Characteristics[1]	Reference
Cloning				
	pJB3Cm6 series	RK2	Cm^R, Ap^R, LacZα, mob, mcs, *oriT*, IncP	(21)
	pRK415	RK2	Km^R, lacZα, mob, mcs, IncP	(138)
	pKT210 series	RSF1010	Cm^R, Sm^R, IncQ	(9)
	pUFR027 series	pSa	Km^R, LacZα, mcs, mob, IncW	(49)
Expression				
	pJB86n series	RK2	*xylS Pm,* Ap^R, mcs, mob, Tc^R, Km^R	(21, 22)
	pCF430 series	RK2	*araC,* P_{BAD}, Tc^R, mcs, mob	(181)
	pMMB22 series	RSF1010	*laclq,* P_{tac}. Sm^R, Ap^R	(178)
	pNM185	RSF1010	*xylS Pm,* Km^R, Sm^R, mcs,	(174)
	pJN105	pBBR1	*araC,* P_{BAD}, Gm^R, mcs, mob	(181)
Transposon				
	Tn5-TC1 series		Tc^R	(223)
	Mini-Tn5xylS/Pm::T7pol		Km^R, *tnp*$^-$, *xylS*, *Pm*, T7 RNA polymerase	(113)
	Mini-Tn5-Cm series		Cm^R, *tnp*$^-$	(52, 114)
	Tn5-B60		Nm^R, P_{tac} mobile promoter	(237)

[1] Cm^R, resistance to chlorampenicol; Ap^R, resistance to ampicillin; Km^R, resistance to kanamycin; Sm^R, resistance to spectinomycin; Tc^R, resistance to tetracycline; Gm^R, resistance to gentmycin; Nm^R, resistance to neomycin; LacZα, β-galactosidase complementation; mob, mobilization; mcs, multiple cloning site; *oriT*, origin of transfer; *xylS* and *Pm,*alkylbenzoate responsive regulator and promoter; *araC* and P_{BAD}, arabinose responsive regulator and promoter; *laclq and* P_{tac}., IPTG-responsive regulator and promoter; *tnp*$^-$, transposase negative transposon

One of the most important features of a vector is its ability to replicate and to be stably maintained in the host of interest. For this purpouse researchers have been using shuttle-vectors with two replicons, one for the strain they want to change and one for *E. coli* so they can do the manipulations there (269). A better solution often is broad-host-range plasmids. Historically, such plasmids with three main types of replicon have been used: RK2 (IncP), RSF1010 (IncQ) and pSa (IncW). These plasmids can replicate in a large variety of gram negative- and also some gram positive strains (45). More recent research in this field has led to the discovery of other alternative replicons which can be small in size and compatible with other plasmids. An example of this is pBBR1 and derivatives which are both small and stable and can be used in several species (13, 142, 143). The plasmids with all the above mentioned replicons are relatively stable in the cell, but without any selective pressure the cells will

gradually loose their plasmid. The challenge of instability has been proposed solved using transposons. Transposons are specific DNA sequences that catalyze their own movement to a location in the host chromosome. Besides stability another desirable feature of transposons is the fact that a low-copy system is established, and this might lead to better control over transcription (137). A disadvantage of transposons is that the sites of insertion are difficult to control leading to inactivation of important genes, or to site-specific variation in the expression from the transposon (253). An alternative approach to insert genes in the chromosomes at a specific site is to use homologous recombination. For this purpous there have been developed broad-host-range conditional suicide vector systems (134).

- Regulating protein expression in metabolic engineering: Desirable promoter features

Traditionally metabolic engineering has often been performed by increasing the expression level of one "rate limiting" enzyme or by reducing the flux to unwanted side reactions through gene deletions (195, 199). These attempts often have had minimal success and the reason have been proposed to originate from the complex nature of most biochemical pathways. Modeling of pathways trying to identify "rate limiting" steps have shown that success of metabolic engineering might require the regulation of several genes at the same time (65, 255). After the analysis and design phase several targets for genetic manipulation should have been identified and to effectively regulate the targeted genes a suitable promoter tool is needed. Desirable features for such a promoter are summarized by Keasling to be: Tight control of expression at several levels and uniform induction of all cells in a culture (137). Several promoters for the use in metabolic engineering have been developed and examples are given in Table 8. As described above there is an increased need for tools to effectively control the expression of two or more genes simultaneously. Depending on the situation the needed level of control is different. For some applications the only necessary criterium is to achieve increased expression of two genes. The two genes of interest can then be cloned in a tandem fashion behind a single inducible promoter, exemplified by the dual expression of two enzymes from a heat inducible prophage promoter system developed in *Bacillus subtilis* (32). The advantages of this system being that both genes can be expressed at the same time, but unfortunately the expression levels of the last enzyme in the tandem is lower than the first and the total expression from the promoter also is reduced. Another example of such an approach is the one mentioned in the chapter under the transcription profiling and included co-expression of two genes from the IGF I synthesis machinery cloned in tandem behind the Lac promoter increasing the total production of IGF I (38).

Table 8: Examples of promoters used in metabolic engineering.

Pro	Origin and inducer	Example of use in metabolic engineering	Ref.[b]
P_{lac}	Promoter derived from the Lac-operon. Induced by lactose[a] and IPTG.	PHBH formation from gamma-aminobutyrate and glutamate	(163, 272)
P_{tac}	Hybrid promoter of P_{lac} and P_{trp}. Induced by lactose[a] and IPTG.	Improvement of biotech indigo production.	(17, 48)
P_{BAD}	Promoter from the arabinose catabolic genes of *E. coli.* AraC act as both an activator and as a repressor. Induced by L-arabinose[a].	Enhancement of lycopene production in *E. coli.*	(101, 133, 229)
Pm	Promoter of the genes of the *meta*-cleavage pathway from the TOL plasmid. XylS act as activator. Induced by benzoate, toluate and some of their derivatives.	Alteration of carbon flow. Stable long-term indigo production	(27, 79, 80, 215)
P_u	Promoter of the upper-cleavage pathway from the TOL-plasmid. XylR act as activator. Induced by xylene or toluene.	Construction of *Pseudomonas* strains for biodegradation of 2-chlorotoluene.	(80, 104)
P_{sal}	Promoter of the *nahG* gene from the NAH plasmid. NahR act as activator. Induced by addition of salicylate.	Stable long-term indigo production	(215, 289)
P_{T7}	Promoter from Lambda, requieres the T7 RNA polymerase. Induced by increased temprature and addition of rifampicine.	Production of plant-specific flavanones by *E. coli.*	(46, 124, 261)

[a] Requieres upptake system

[b]References both for the origin of the promoter and the example of use.

Another way of inducing expression of several genes at the same time is to use multiple promoters of the same type and clone each gene of interest behind an individual promoter (137). One example of this is the use of P_{BAD} in enhancing the lycopene production in *E. coli* by increasing the expression of three genes in the lycopene biosynthetic pathway (133). Another example is the use of the P_{T7} in cooperation with the *Pm* promoter (113). The T7 gene 1 (encoding the T7 RNA polymerase) was cloned downstream of the *Pm* promoter and the genes of interest downstream of two P_{T7}-promoters. By inducing the *Pm* promoter expression from the two P_{T7}-promoters were turned on leading to the expression of two genes simultaneously. The two T7 promoters in this study were identical but the used promoters

could also vary in their levels of expression in systems where different amount of each gene product is needed. This variation can be used to fine-tune the expression of the genes to optimal levels and promoters for such a purpose have been generated for expression in *Lactococcus lactis.* Here screening of a library of synthetic constitutive promoters resulted in a set of promoters with varying levels of expression (From 0-1000%) (130, 131). These promoters have more recently been used to control 10 genes from the glycolytic pathway in *Lactococcus lactis* at independent levels. They were thereby able to control the expression of all the genes in this biochemical pathway at different levels according to the need for the different proteins in the pathway (175). The difficulties with using constitutive promoters for these experiments might be to construct and find the best suited promoters for each application and there is also the disadvantage of being unable to control the genes expression during the experiments (137). To individually control the expression of several genes independently at different stages of growth by cloning them downstream of different promoters is the easiest method. An example of this approach is the specific synthesis and degradation of polyphosphate in *E. coli*, where the gene controlling the synthesis was regulated by P_{tac} and the gene responsible for the degradation was independently under the control of P_{BAD}. This system was developed to study bacterial phosphate transport and energy metabolism (274).

2. Aims of the study

A. vinelandii produces alginate with a varying G-content depending on culture conditions and stage of life cycle (F_G varies between 0.05 and 0.80). This variation makes the bacterium a candidate for producing alginates with predetermined structure and the main motivation of this study has been to generate an understanding that will make such a production possible. A second motivation was to understand the biological function of the epimerases as the majority of previous studies have focused on their *in vitro* properties.

The individual contribution from each of the epimerases in *A. vinelandii* is largely unknown so one important factor in being able to control the degree of epimerization *in vivo* is to obtain a better understanding of the specific role of each individual epimerase. A part of the work in this study therefore concentrated on making individual disruptions of all the secreted epimerases genes. The corresponding mutant strains should then be characterized by their ability to produce alginate, the amount of G introduced and their ability to make desiccation resistant cysts.

It was realized from start that the development of *A. vinelandii* strains for production of alginates with predetermined structure might require that a strain lacking all secreted epimerase genes were constructed first. This was therefore considered to be a very important milestone in this Ph.D thesis work. If successfully generated, such a mutant should be tested for its ability to produce alginate, and the monomer composition of this alginate should be determined. Idealy, selected *algE* genes should then be individually expressed in such a strain.

The *in vivo* production of alginates with a predetermined structure requires tools for expressing proteins of interest in these strains. The fourth important goal of this thesis was therefore to develop broad-host-range genetic tools that could later be used to design *A. vinelandii* strains for alginate production. A broad-host-range promoter system from the AraC/XylS family of promoters (*Pm*/XylS) has been developed in our group and has been shown to function in *A. vinelandii*. Challenges concerning the use of this promoter include a high level of background expression, so a partial goal of the work was to develop a method for finding promoter-mutants with a low background expression. This screening system was first to be established in gram-negative alginate-producing bacteria. To deal with the complexity of the alginate epimerization or to seperatly control the alginate biosynthetic genes it is probable that independent expression of more than one gene product is needed. To accomplish this task a new broad-host-range promoter system was to be developed.

3. Summary of Results, and Discussion

3.1 AlgE epimerases are needed for cell diferentiation and to control alginate structurein vivo in A. vinelandii

3.1.1 *A. vinelandii* forms Ca^{2+}-dependent cyst-like structures during growth in RA1-medium

(Paper 1, 2 and results not described in papers)

When *A. vinelandii* is grown in the RA1 medium (Paper 1), used for alginate production in shakeflasks, an interesting change in cell morphology can be observed. This morphology becomes apparent approximately after 48 hours of growth and starts with a rounding of the cells (Figure 6, Paper 1), followed by development of a coating on the outside of the cell resembling the exine in encysted cells (Figure 11a and b). This can more easily be observed using electron microscopy where the cyst-like structures become even more apparent with both an exine and a developing intine (Figure 11 c).

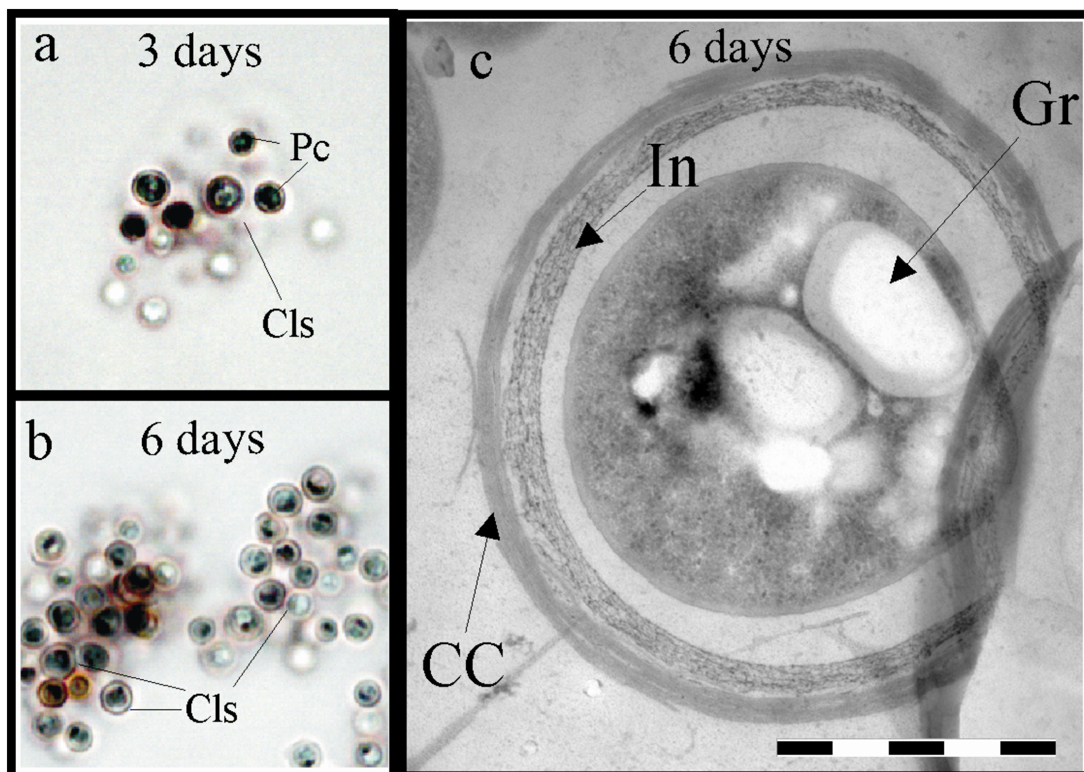


Figure 11: Light microscopy characterization of *A. vinelandii* strain E grown in RA1 medium for 3 and 6 days (a and b) and by electron microscopy (6 days, c). Pc, Precyst; Cls, cyst-like structure; In, intine; CC, cyst coating, exine; Gr, PHB granule. The bar represents 1µm.

This coating is believed to be partly composed of alginate and to attach the polymer to the cells (gelling) Ca^{2+} is believed to be important. To investigate if the calcium concentration is of importance for the observed process an experiment with different amounts of added Ca^{2+} was initiated. The cells rounded up in all cultures and started the encystment process. For the lowest Ca^{2+} concentration the cells seemed to have problems keeping the alginate in place and the capsule material is scattered around them (DB in Figure 12), probably leading to abortion of the encapsulation process.

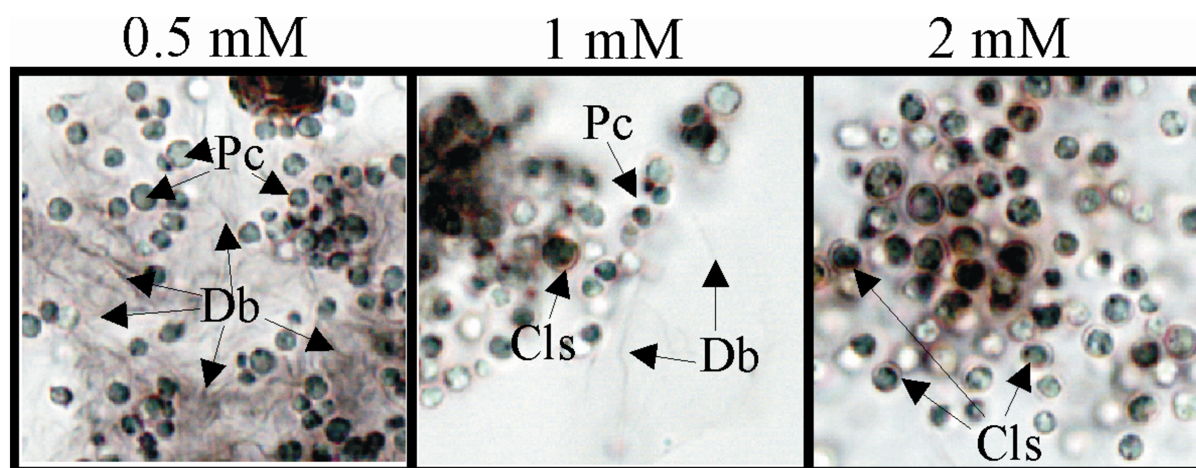


Figure 12: Cell morphology of *A. vinelandii* after 3 days of growth in RA1-medium with varying Ca^{2+}-concentrations. For cells grown with 0.5 mM only pre-cysts (Pc) were observed and the alginate was scattered around the cells as debris (Db). When the Ca^{2+}-concentration was increased to 1 mM some cyst-like structures (Cls) could be observed, but there were still some debris and pre-cysts. For the highest concentration (2 mM) only cyst-like structures could be observed.

For the cells grown in 1 mM Ca^{2+} less of these cell debris were observed and more of the cells were surrounded by a cyst-like structure. For the cells grown in 2 mM the debris was almost absent and all cells seemed to be encapsulated. These observations led to the investigation of the structures of the alginates that could be harvested from the various cultures, and results showed that the F_G for the samples increased with a decrease in Ca^{2+} concentration (Figure 13). Earlier results have shown that the maximum F_G is found around 0.5 mM Ca^{2+} with a decrease both at a lower and higher Ca^{2+} concentration (147). The decrease with lower concentration is probably due to the Ca^{2+}-dependency of the epimerases (60), while the decrease at higher concentration of Ca^{2+} is more difficult to explain. One posibility, based on the results shown here, could be that the alginate harvested and analyzed, is the polymer that has detached from the cells and is free in the supernatant. In situations with a high Ca^{2+}-concentration the G-rich alginates are probably attached to the cells and are thus not included in the harvested fraction.

Figure 13: ^{1}H-NMR of alginates harvested from *A. vinelandii* grown in RA1 with varying Ca^{2+} concentrations. The alginates were harvested and the ^{1}H-NMR was performed as described in Paper 2**.**

When the Ca^{2+}-concentration is low the alginate with high G-content is not trapped around the cells at the same degree and is harvested and included in the analysis. In addition the alginate is also probably more available to epimerization when it is more loosely attached to the cells. A decrease in degree of epimerization when the Ca^{2+}-concentration is raised has been shown for the AlgE epimerases *in vitro* (64, 260).

The cyst-like capsule structures observed in the RA1-medium, and the probable importance of alginates rich in G-blocks to make them, was used as a tool to observe changes when the proposed epimerase export system or the *algE* genes were interrupted genetically. To ensure that the observed effects were caused by changes in epimerization and not by lack of Ca^{2+} a 2.0 mM final concentration was used for these experiments.

3.1.2 Identification and characterization of the secretion apparatus for the AlgE-type mannuronan C5-epimerases in *A. vinelandii.*

(Paper 1)

The process of secreting the AlgE-epimerases has been proposed to happen by the use of a RTX-protein Type I secretion system (60). This is due to the InterPro hemolysin-type calcium-binding motif that all the secreted mannuronan C5-epimerases display. By using the genomic sequence of *A. vinelandii* OP putative Type I secretion systems were identified. A further analysis of this result left us with only one candidate and the three genes of the system were designated *eexD* (ABC-transporter), *eexE* (Membrane Fusion Protein, MFP) and *eexF* (Outer Membrane Pore, OMP). These genes from *A. vinelandii* E were cloned in *E. coli* and individually disrupted in *A. vinelandii* E by insertion of a tetracycline restance cassette. Characterizations of the resulting mutants showed that they all displayed greatly reduced G-content in the alginate produced in RA1-medium grown cells ($F_G < 0.02$) and that the alginate is without any G-blocks. The remaining G-content may originate from AlgG activity or from AlgE enzymes originating from lysed cells. These results therefore indicate that AlgG in *A.vinelandii* has a lower epimerase activity than its *Pseudomonas* homologue (84). AlgG may therefore have lost a functional role in *A. vinelandii*. Due to this the main reason for its precence may be related to its proposed role in formation of the biosynthetic complex. These hypotheses are also in agreement with those reported in Paper 2.

For the secretion system disruption mutants grown in RA1-medium another interesting property was discovered; these cells started the encystment process similar to the wild-type, but they were later in the process unable to generate the capsule surrounding the cell (Figure 6, paper 1). This morphological difference is probably the reason for another observed effect: When proteases were added to the medium the mutants produced alginates with a considerably higher molecular mass (> 1000 kDa) compared to the wild-type (146 kDa) under the same conditions. In the wild-type the coat around the cells probably serves as a protective harbor for the lyases, determining the molecular mass. When this protective coating collapses, as observed in the mutant, the lyases may become susceptible for protease attack. Consequently, without the lyases present the mean chain length of the alginates increase. This hypothesis is supported by similar observations in *P. fluorecens* which do not have this protective coating (83).

It has been hypothesized that the ability of *A. vinelandii* to make desiccation resistant cysts are connected to the presence of the secreted epimerases (119). Disruption in the export system of these enzymes should give mutants that are unable to survive desiccation. This proved to be the case and is a further indication that *eexD, eexE* and *eexF* are the main and probably only genes responsible for export of the epimerases through the periplasmic space.

3.1.3 Creating single disruption mutants of *algE1-7 in A. vinelandii*

- Effect on cell morphology and alginate production

(Paper 2 and unpublished results)

Secreted mannuronan C5-epimerases have been studied thoroughly *in vitro* in our group and a lot is therefore known about their properties. To better understand what role they play *in vivo* in *A. vinelandii* a study of the effect of their individual disruption was initiated. The genes encoding the epimerases have previously been cloned and these plasmids were used to make the disruption mutants in each individual epimerasegene by replacing a part of the sequence encoding the A-module with a tetracycline-resistance cassette. Plasmids from this process were subsequently used to disrupt the genes in the chromosome of *A. vinelandii* by linear transformation, and the disruptions were verified by Southern hybridization. A test on the growth in RA1-medium was initiated and the mutants seemed to be unaffected in their growth rates. The disruptions did not indicate any obvious morphological changes and all the mutants were still able to make the cystlike structures. Alginates were isolated from the strains and their structures were analyzed by ^{1}H-NMR. The results gave no indication of significant differences among them or compared to the wild-type structure. Recent findings have shown that even though the G-content is similar the distribution of the G-blocks could be different (120). This suggests that the use of these methods in determining the distribution of G could reveal differences not detectable by traditional ^{1}H-NMR.

To further test the phenotypes of these strains they were also grown in fermentors in PM1 medium (see Paper 2). In the beginning of the growth the alginate is surrounding the cells, making it difficult to centrifuge them, leaving behind a fluffy pellet (Figure 14, 12 hours Wt). Over time the alginate is released from the cells to the growth-medium and the cells could then easily be centrifuged to a compact pellet (Figure 14, Wt: 20 and 25 hours).

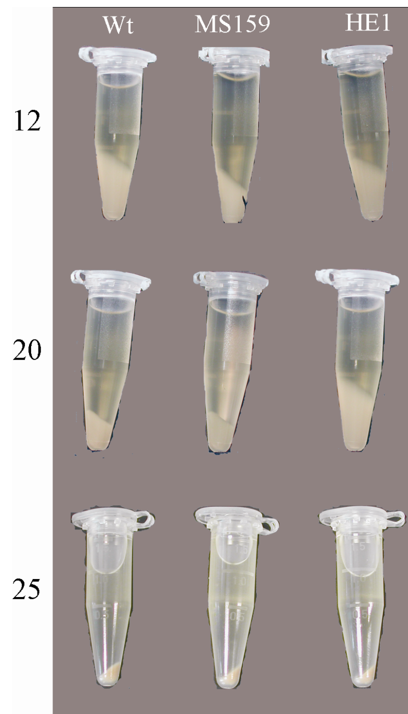


Fig 14: Time-course of cell pellet properties from fermentor grown cultures. The numbers at the left indicate hours after inoculation. Most mutants behaved like the wild type, exemplified by MS159 (*algE3* disrupted), except for HE1 (*algE7* disrupted) that displayed a later release of the alginate to the growth-medium (PM1).

The release of the alginate from the cells was similar for all the mutants (exemplified by MS159 in Figure 14), except for HE1 (*algE7* mutant). This strain released the alginate from the surface of the cells almost 5 hours after the other strains, but after 25 hours the cell pellets of HE1 displayed the same appearance as those from the other strains.

Figure 15 shows the amount of alginate accumulated during growth of the *algE* mutants. Initially, the disruption mutant of *algE7* seems to have a lower alginate production than the *algE1-6* mutants, but this is probably due to the reduced level of detachment of the alginate from the cells (Figure 14). Over time the fluffiness of the pellet from mutant HE1 was reduced to eventually disappear, and at this stage the alginate amount in the culture was similar to that of the rest of the mutants (and the wild-type). These observations indicate that the secreted epimerases do not influence the amount of alginate produced. They also indicate that AlgE7 is responsible for detachment of alginate from the cells at early stages of vegetative growth but that other factors, either enzymatic or mechanical, detach the alginate at later stages. New alginate lyases discovered recently might be responsible for this process (83).

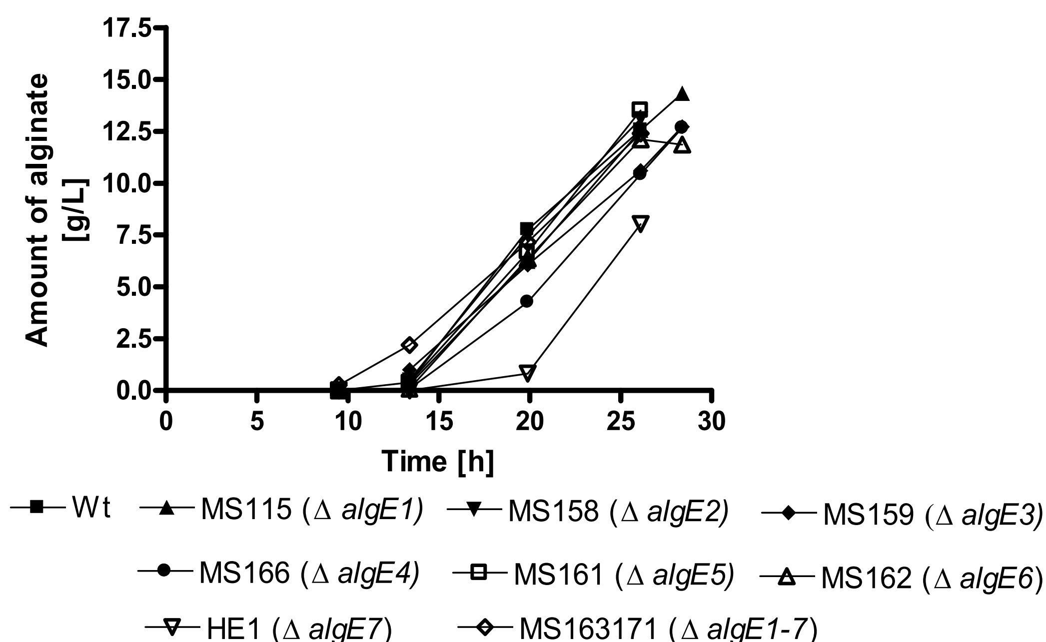


Figure 15: Alginate produced as a function of time in fermentations of *algE* mutants. The fermentations were run as described in Paper 2. The epimerase genedisrupted in each strain is indicated in brakcets behind the strain name.

- The effects on alginate structure are different in fermentor-grown (PM1-medium) cells compared to cells grown in shake-flasks (RA1-medium).

Alginate was isolated from all the single disruption strains grown in fermentor and the structures were analyzed with ^{1}H-NMR, giving the results shown in Table 2 of Paper 2 and in Figure 17. For most of the polymers there are no very significant differences with respect to the monomer composition (F_G between 0.23 and 0.29), compared to the wild-type ($F_G = 0.25$). The disruption mutant of *algE3* (strain MS159) is an exception, displaying a significant reduction in the G-content ($F_G = 0.08$). When the same strain was grown in RA1-medium it had only a moderately reduced G-content in its alginates compared to those of the wild-type (Figure 17).

This result was so unexpected that the experiment was repeated in triplicate with the same or very similar results. The strain was even made once more genetically and the fermentation was repeated with the same result for the new strain. These observations may mean that there is a difference in expression of the *algE* genes depending on the culture media used, and that in the fermentation medium (PM1) AlgE3 is the dominating epimerase. Another possible explanation would be that the physical conditions are different in a fermentor than in a shake-

flask. This explanation could be investigated by using the same growth-medium in both fermentor and shake-flask.

-The possible effects on cyst formation of individual *algE* gene disruptions were too small to be quantified.

(Tests of the ability to form cysts were performed by the group of Dr. Guadalupe Espìn, Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico) To further elucidate differences in the phenotype of single disruption strains their ability to make cysts and their resistance to desiccation was studied. After the induction of encystment by n-butanol the cells started to form aggregates, which were difficult to dissolve. It was therefore also difficult to determine the number of viable cells. On the other hand, all the mutated strains had viable cells after desiccation, indicating that they display at least some ability to make cysts. It is noteworthy that the mutant disrupted in *algE1* seemed to make larger aggregates and that they seemed more difficult to dissolve than the aggregates from the other strains. This might indicate that this disruption cause more significant effects on the surface of the cell than the remaining disruptions, and that AlgE1 might be more important for the cell phenotype under these conditions. Previous reported results on epimerase promoter activities indicated that *algE1* is the most expressed epimerase gene during encystment (18, 119), and this also supports the hypothesis that single AlgE epimerases play an important role under the growth conditions used for functional testing of encystment.

3.1.4 Generating deletion mutants of multiple *algE1-7* genes in *A. vinelandii*

- Dramatic effects on cell morphology and alginate structure in RA1-medium grown cells.

(Paper 2 and unpublished results)
To further investigate the role of the epimerases a strain containing disruptions in both *algE1* and *algE2* was generated. This strain was tested in RA1-medium and showed no apparent difference either in morphological characteristics or in the resulting alginate structure, relative to the wild-type. For this work a new version of the *algE1* disruption strain was needed, and this was made using a spectionmycin resistance gene as the disruptive element ($MS115_{sp}$). This strain was subsequently used to make a strain where the entire cluster of epimerases were deleted, leaving behind only *algE5* (strain MS163). Morphological testing of this strain in

RA1-medium showed a distinct change in the ability of the cells to make cyst-like structures (Figure 16 also shown in Paper 2 as Figure 2). The cells are rounded up as the wild-type, but it seemed like the cells have trouble keeping the alginates attached to the cell surface, and this became more apparent over time when more and more cells started to loose the structures around the cells, leaving behind only the central body of the cyst-like structure.

Analyses of the structures of the alginates produced indicated a reduction in G-content, but not as dramatic as one might predict ($F_G = 0.13$, a reduction from $F_G = 0.20$ for the wild-type). This result indicate that AlgE5 may be highly expressed under these conditions, or that the inactivation of the genes encoding the remaining secreted AlgE-epimerases leads to more favourable conditions for AlgE5 to act on its substrate.

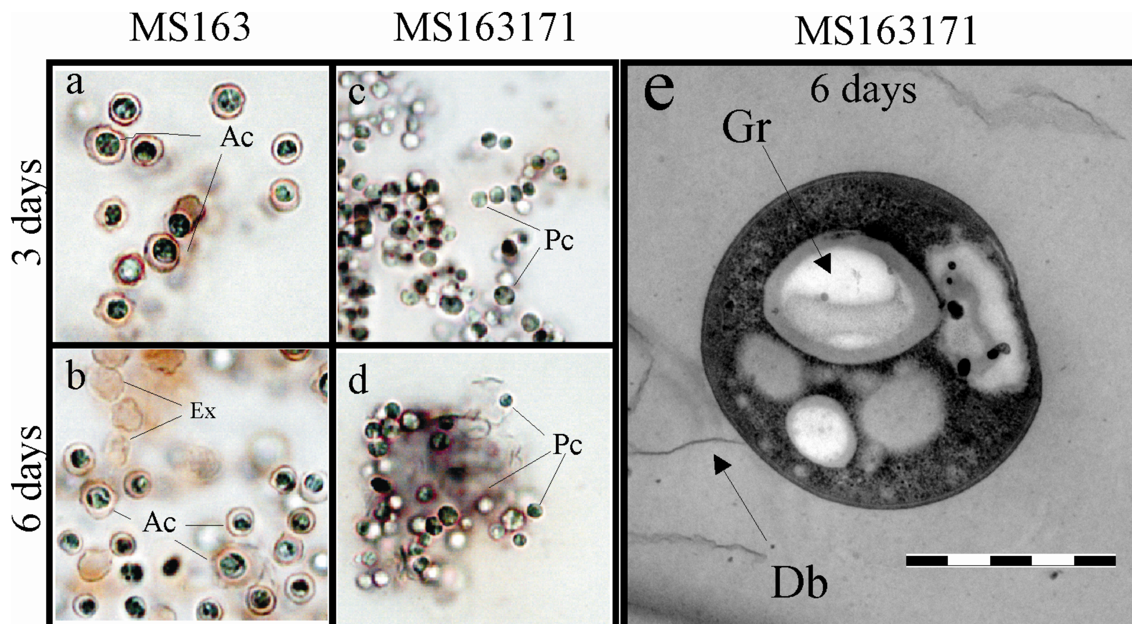


Figure 16: *A. vinelandii* mutants MS163 and MS163171 grown for 3 and 6 days in RA1 medium. Light microscopy of MS163 (a and b) and MS163171 (c and d**)**. The cells from MS163 are seen as structures resembling those previously described as abortive cysts (Ac) and the exine is seen floating around the cells (Ex). For the mutant MS163171 there is no coat development and the cells are seen as pre-cysts (Pc). To further see details an electron microscopy picture is included for cells of MS163171 grown for 6 days (e). Gr, Granules; Db, alginate debris; the bar represents 1 µm. These pictures are also found in Paper 2.

Another possible interpretation would be the same as for the Ca^{2+}-dependency of the observed change in F_G, where the analyzed alginate is the fraction dissolved in the medium, and not the alginate attached to the cells.

From MS163 a disruption mutant in *algE5* was created and the resultingr strain (MS163171) , thus has no active *algE* genes. Morphological studies on this strain revealed that it is completely unable to make the cyst coat seen in wild-type cells (Figure 16, also shown in Paper 2).

- Effect on the cells ability to make cysts

Strain MS163171 was tested for desiccation resistance by the method described for the single disruption mutations (experiments performed by the group of Dr. Guadalupe Espìn, as for the single disruption mutants). After cyst induction by n-butanol and subsequent desiccation of the resulting cysts for 5 days no cells survived, leading to the conclusion that *A. vinelandii* needs at least some of its secreted epimerases to make viable cysts under the conditions tested.

3.1.5 Strain MS163171 has a potential to be used for *in vivo* production of alginates with predetermined structures.

The strains with more than one epimerase gene disrupted or deleted were tested for ability to produce alginate during batch fermentation, and in addition the alginate compositions were determined. First the strain where the cluster of the secreted epimerases are deleted (Strain MS163) was investigated, and the results showed that alginate was produced at similar levels as in the wild-type, but there was a very significant decrease in the G-content and in the G-block content (Figure 17 and Table 2, and Figure 2 in Paper 2). An even more dramatic effect on the alginate structure is seen for strain MS163171, which the alginates from are almost lacking G-residues (F_G<0.02). This indicate that AlgG is responsible for the residual rest of the G residues in the alginate and that the AlgG activity can be altered by media composition (F_G varying between less than 0.02 in fermentors to 0.06 in RA1, Figure 17). This enzyme is thus much less active as an epimerase in *A. vinelandii* than in *Pseudomonas* species, where it introduces an F_G of up to 0.50 (273). Fortunately the alginate production is as high as in the wild-type strain indicating that the production level is independent of the secreted epimerases. This strain, with its low background of epimerization, represents a very interesting source for re-incorporation of the individual *algE* genes expressed under control of inducible promoters. This would potentially generate strains that are able to produce alginates with predetermined structure. Unfortunately, no specialized protein expression tools exist for *A. vinelandii,* but a broad-host-range system has been shown to function (*Pm/xylS*). For the production of bacterial alginates the expression of several genes probably need to be controlled

independently. For this reason the development of a new broad-host-range expression system is described in Chapter 3.2.

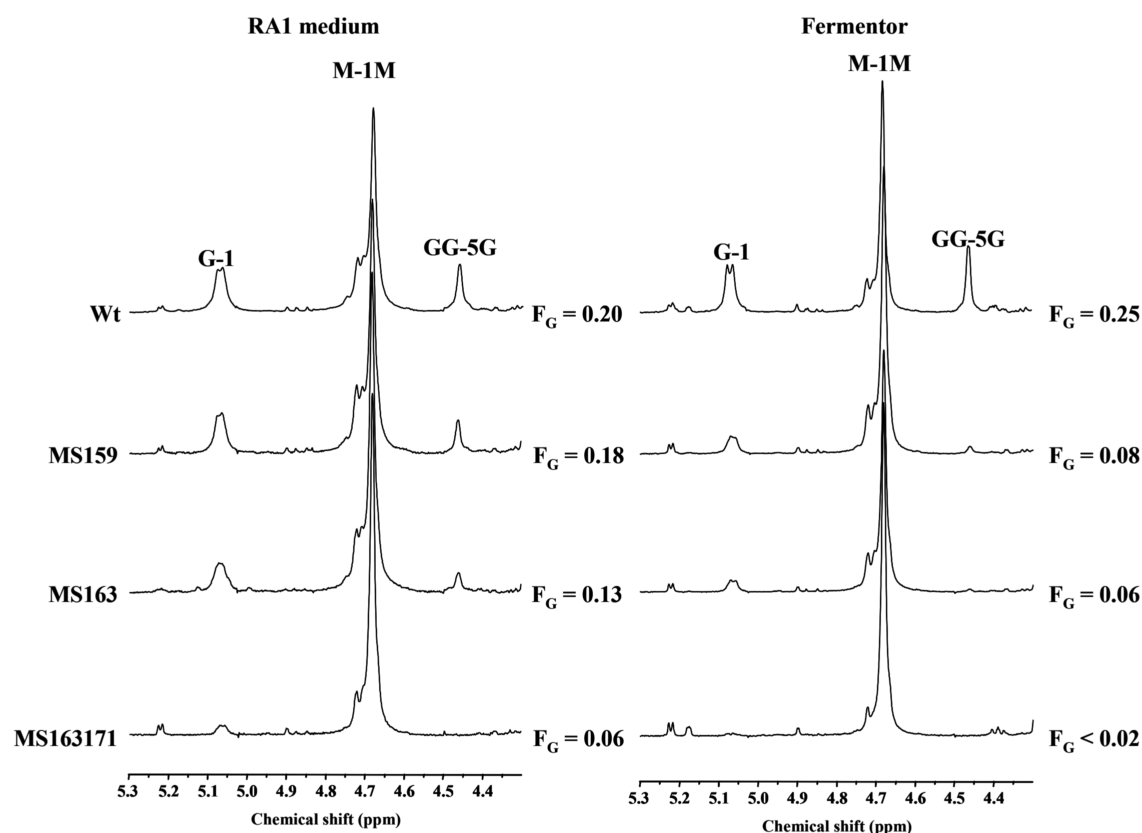


Figure 17: ^{1}H-NMR spectra recorded from alginate samples harvested from cultures grown in either shake flask (RA1 medium) or fermentor. The spectra illustrate the difference in G-content of the harvested alginate depending on both the mutant and the growth conditions. The ^{1}H-NMR spectra were recorded as described in Paper 2. The peaks at 5.25, ~5.17 and 4.85-4.9 are caused by the products resulting from alginate degradation, either enzymatically by lyases or during preparation of the samples for NMR.

3.2 Promoter systems for use in gram-negative bacteria

A probable need for several promoters to control the expression of several genes for metabolic engineering of alginate production was indicated in the previous chapter. For this purpose we wanted to develop a new promoter-system, as described in Chapter 3.2.1. In addition it was planned to take advantage of our extensive knowledge about the *Pm*/*xylS* system and to control the amount of G introduced into the alginate. Initial experiments of this work include an expression study of *algE6* which was cloned downstream of the *Pm* promoter in a medium copy-number vector (Figure 18 A). This vector was transferred from *E. coli* to *A. vinelandii* by conjugation and the resulting transconjugant was grown in Burks glucose medium (Burks medium described in Paper 2). To test whether *algE6* was expressed samples from the supernatant were subjected to the alginate epimerization assay on radioactive labeled alginate, as previously described (259). The results from this assay (Figure 18B) show that there is a very strong increase in the amount of epimerases in the medium for the strain harboring the plasmid containing *algE6,* indicating that the *Pm*/x*ylS* system functions for overproduction of this gene product in *A. vinelandii*.

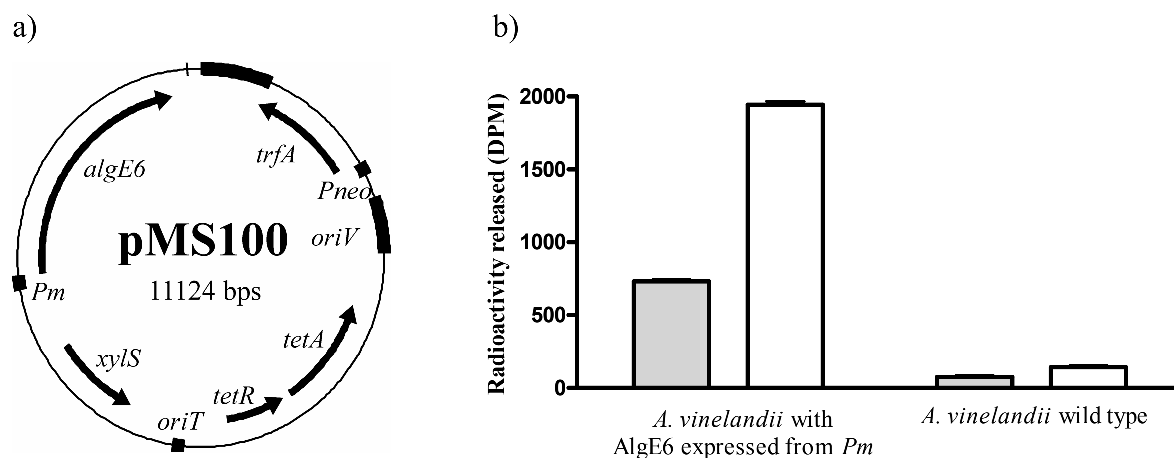


Figure 18: Over-expression of *algE6* in *A. vinelandii*. a) The construct of *algE6* under control of the *Pm* promoter in the broad host-range plasmid pJB866 (21, 22) b) Epimerase activities measured as the amount of radioactivity is released to the growth-medium after epimerization of radioactively labeled mannuronan (grey, induced; white, uninduced).

Interestingly, there was less activity from the culture samples where the inducer was added. This correlated with the observation that pMS100 was rapidly lost during growth of the cells, even when the cells were grown in the precence of tetracycline. This could possibly mean that the expression from *Pm* becomes a burden for the cells. Even the uninduced cells lost their plasmid after a few generations of growth. Plasmid loss or recombination with the epimerases

in the genome might relieve the cells, leading to enhanced growth-rate compared to cells containing pMS100. These results therefore implicate that the wild-type *Pm* promoter might have too high expression levels for this application, even in the absence of expression. This was the reason for developing a screening strategy for the discovery of low background and low expression mutants in the *Pm* promoter, described in chapter 3.2.2.

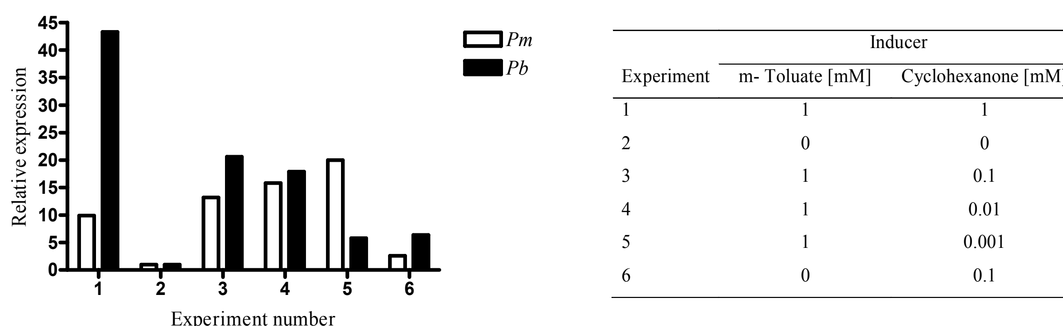
3.2.1 Construction of a new promoter system suitable for regulated gene expression in gram-negative bacteria.

(Paper 3)
Finding a new promoter-system was accomplished using four criteria: 1. A system that previously has been shown to work in at least two different organisms. 2. A system with an inducer that can not be metabolized by most species 3. A system with an inducer for which no uptake system is needed. 4. A system which can be used in combination with *Pm*/*xylS* in the same cell. The selected candidate was the system controlling the expression of *chnB* in *Acinetobacter sp.,* which is induced by cyclohexanone and where the regulator (ChnR) is a member of the AraC/XylS. This system was cloned in a plasmid as described in Paper 3 and tested for induction of its native gene, *chnB*, which encodes cyclohexanone mono-oxydase. This enzyme is part of the cyclohexanol degradation machinery in *Acinetobacter sp*. (35, 125). A clone with a functional copy of the promoter system (pMS101) was used as the starting point for the further studies, and *chnB* was replaced with a luciferase gene to facilitate the characterization of the system. Initially the system was tested on several different inducers based on the fact that *Acinetobacter sp.* can grow on several cyclic alcohols and ketones (34). The list of tested inducers are presented in Table 2 of Paper 3 and show that induction can be varied using a range of cyclic hydrocarbons and that cyclohexanone is the best inducer. Cyclohexanone was therefore used in the further studies, and the next goal was to find out if the system responded in correspondence to the amount of inducer added. At least in *E. coli* this appeared to be the case, as expression increased in proportion to the inducer concentration (Figure 2 a Paper 3). The expression was highest for 2 mM and further increases (10 and 20 mM) led to reduced growth, probably due to toxic effects of the inducer. *Pb* (designation of this promoter was made here) was also tested with respect to the time needed to reach maximum expression, and this was found to be 11 and 5 hours for cultures grown at 30 ºC and 37 ºC, respectively.

Broad-host-range promoter systems are useful in many applications, and since *Pb* worked both in *Acinetobacter sp.* and *E. coli* it might indicate that the system can be used in other gram-negative bacteria as well. At the stage of the project when this discovery was made the *A. vinelandii* strains, described in the previous chapters, had not been developed to a satisfactory level The system was therefore tested in the genetically more convenient *P. fluorescens.* The system was transferred by conjugation, and analyzed for luciferase expression. The results showed that the cassette can be induced also in this organism (Figure 2D Paper 3) and fortunately it seems like the background expression from theis system is lower than that of *Pm*/*xylS*.

Pb and its response-regulator ChnR could potentially be used in metabolic engineering applications together with the Pm/*xylS* system. AlgG (wild-type) and a mannuronan producing mutant (AlgG*) was used to test this potential. *algG* was cloned downstream of the *Pb* promoter, moved to a transposon vector (creating pMS186) and subsequently transferred to *P. fluorescens* Pf201 *ΔalgG*. *P. fluorescens* Pf201 *ΔalgG* is an *algG* deletion mutant of the alginate overproducing strain Pf201. The deletion makes the strain unable to produce alginate (84). The insertion of *algG* downstream of *Pb* led to restoration of the alginate production and an F_G in the alginate as for the alginate produced by the wild-type (Figure 3 Paper 3). *algG** was then cloned downstream of *Pm* and introduced into *P. fluorescens* Pf201 ΔalgG as well, restoring alginate synthesis, but producing alginate without G. When the two systems were introduced to the same strain (strain Pf201 ΔalgG::TnMS186::TnKB110) the alginate production was restored to similar levels as the wild type (based on amount of alginate harvested from the cultures) and without any inducer the alginates display a low G-content (F_G = 0.04) indicating that expression from the *Pm* system was dominating under these conditions. This is in agreement with the previous results, where *Pm* was shown to display a much higher background expression than *Pb*. When either of the systems were induced (1 mM inducer) the alginate was low in G-content when *Pm* is induced, and as in the wild-type when *Pb* was induced (Figure 3 Paper 3). When both systems were induced (1 mM of each inducer) the G-content of the alginates had an intermediate monomer composition (F_G = 0.16). Reduction of the *Pm* inducer concentration resulted in an increased G-content, while the opposite happened when the inducer concentration for *Pb* was reduced. These results indicate that the two systems can be controlled independently of each other in the same cell.

To further test whether the two systems really are independently controlled we also measured amounts from the two promoters for a set of different situations, shown in Figure 19 (Real time PCR, experiments performed by Ingrid Bakke). The cultures were inoculated (1 %) and induced at the same time and samples were harvested after 24 hours. Cell pellets from 1 ml culture (out of 10 ml cultures) were washed with cold NaCl (0.2 M 50 ml) and RNA was then isolated using the RNAqueous protocol (Ambion) with a bit less yield than expected. The samples are normalized against sample 2, which was similar for both promoters. This represents the uninduced or background levels of transcription from the promoters and indicates that the background transcription from *Pm* is not much higher in this situation even though the G-content in the alginate samples harvested from this strain indicate this (Figure 3 Paper 3). Another indication that the background from *Pm* is higher is the results from the *luc*-assay where the *Pb* had a significant lower background in *P. fluorescens.* For sample 1 it is clear that the relative expression from *Pb* is higher than for *Pm*, supporting the result from the alginate analyses. For samples 3-5 there is a decrease in the amount of transcript from the *Pb* promoter with a decrease in inducer added, indicating that the system is inducible at several levels. Sample 6, however, shows results in conflict with this and might indicate that m-Toluate or XylS in some way influence the expression from *Pb*. Due to the difficulties in the isolations of RNA these experiments have to be repeated for verification, and probably new and better ways of isolating RNA from these strains should be investigated. To further establish whether the promoters work in a satisfactory way in this organism it should be tested how they behave over time and also how the alginate structure change during the different stages of growth.



Experiment	Inducer	
	m- Toluate [mM]	Cyclohexanone [mM]
1	1	1
2	0	0
3	1	0.1
4	1	0.01
5	1	0.001
6	0	0.1

Figure 19: Real-time PCR quantification from cultures grown with different inducers and concentration for 24 hours. Experiment number refers to the Table on right. Results from the *Pm* promoter are represented by white bars and results from the *Pb* promoter by black bars. Results are normalized against sample 2 which had similar transcript amounts for both promoters and represent the background transcription levels (uninduced).

3.2.2 Development of a screening method for identification of *Pm* promoter mutants with a low background expression, based on a *Pm* promoter mutant library.

Earlier work in our laboratory resulted in the construction of a set of expression vectors based on the *Pm*/*xylS* expression system integrated into the RK2 replicon. These vectors have been demonstrated to work well in several gram-negative bacteria, both for the overexpression of heterolougus proteins and in metabolic engineering applications (11, 243). One of the negative aspects of this promoter system is its high uninduced expression level in some bacterial strains (e.g. *P. fluorescens* and *A. vinelandii*). The problem of plasmid-instability, shown earlier in this Thesis, was probably a result of such a high background expression (page 44-45). To deal with this challenge, a mutant library of the promoter was created based on previous work by Winther-Larsen et al. (281). In this method a promoter oligonucleotide mutant library with 12% errors in one strand of the promoter was designed, and the library was used to replace the wild-type promoter creating a library of more than 50000 mutants (Ingrid Bakke, Unpublished results). This library is cloned upstream of a β-lactamase resistance gene and in *E. coli* the library can be screened by using the level of β-lactam resistance to find promoters with new levels of activity. Unfortunatly, *P. fluorescens* and *A. vinelandii* strain E is already quite tolerant to this antibiotic, such that this procedure could not be used in these organisms. Therefore a new screening method for finding low-background mutants was developed. This work was performed in *P. fluorescens*, but it was believed that the screening method could be used in *A. vinelandii* as well. To screen the library in these bacteria the β-lactamase gene was replaced with the luciferace gene, and a new plasmid designated pHH100 was generated (Part of the work developing this screen was the Master thesis of Håvard Homberset, Department of Biotechnology, NTNU, supervised by Magnus Steigedal). The library generated from this plasmid contains 8000 independent clones and it was transferred by conjugation to *P. fluorescens.* Screening for reduced background expression of the luciferase gene (relative to the wild-type) was carried out by a modified procedure from a previously described method (283). To obtain reproducible results we found that the bacteria first had to be grown in microtiter plates containing 110 μl liquid PIA-medium (Described in Paper 3) with 40 μg/ml kanamycin (25 ^{o}C, 48 hours, shaking at 900 rpm). The cultures were then diluted in new medium, and then transferred onto two nylon filters that prior to the stamping were placed on two PIA-plates (Described in Paper 3) with and without inducer (1 mM m-Toluate). The plates with filters were incubated for 14 hours at

30 ^{o}C, and then placed in a Petri dish containing 3 ml luciferin (Promega, 1 mM in 0.1 M sodium citrate, pH 5.0), shaken until the liquid was distributed evenly, and incubated for 10 minutes. Filters were then blotted on a filter-paper to remove the liquid, and placed, face down, on transparent plastic film. A dry filter paper was placed on top of it to remove residual dampness. The nylon filter was then exposed for 10 min. using a Kodak 2000IR camera. 1200 colonies were inspected this way, and 84 showed significant lower background expression levels than the wild-type, while still retaining the ability to become induced. After a rescreening, 79 of these turned up as candidates for a low background system. Five of these candidates were further tested by growing the cells in shake-flasks both with and without inducer, and the samples were treated and prepared as described for the Luc-assay procedure in Paper 3. The results (Table 9) showed that the mutants display a significant reduction in the background level (4.2-0.67 % of the wild-type) but also that the induced level is decreased (0.5-11.4% of the wild-type).

Table 9 Promoter mutants, their induced- and uninduced-levels, and the ratios between them.

Promoter	Uninduced[1]	Ratio[2]
Wt	1	12
A2	0.01	8
B1	0.02	3
D6	0.02	8
D9	0.01	26
G5	0.03	33

[1]The results for the uninduced column are set as values relative to the wild type promoter.
[2]Ratio is fold induction that is observed between the uninduced and induced states.

Interestingly the ratios between induced and uninduced levels have increased for some of the mutants, G5 being the most interesting one, increasing more than 100%. This mutant was chosen for further investigations and was shown to have three point-mutations relative to the wild-type promoter. Because of its low background expression this mutant has been used in metabolic engineering applications (11) and also in a patented system for producing alginates (85). It will probably be useful for a range of other applications as well. Recent results have shown that promoter mutants only to a limited extent will diplay the same characteristics in different species (Ingrid Bakke, personal communication). Because of this we believe that the discovered low background mutant of the *Pm* promoter probably can not be used directly in

A. vinelandii as a low background expression mutant. But the screening method developed here can be used for identifying similar mutants in *A. vinelandii*.

4. Concluding remarks

Much is known about properties of the AlgE epimerases *in vitro* but little was known about their role *in vivo* prior to this thesis work. It was known before that the AlgE epimerases are expressed and that there is a change in the patterns of expression during the process of encystment (18, 119). A part of this thesis has elucidated more on what effect individual disruption of the *algE1-7* genes has on the phenotype of the organism. Especially the effect on encystment and on the composition of the produced alginate has been investigated. For mutants grown in RA1-medium (shake-flasks) there were no apparent phenotypic change, including alginate composition. The mutants were also tested for ability to produce viable cysts but problems with the method of establishing the correct number of survivors led us only to conclude that these strains had retained at least some ability to produce desiccation resistant cells. These results indicate that the different epimerases can compensate for each other to some extent and also strengthens the hypothesis that they are individually expressed. When the same strains were grown under fermentors the changes in alginate structure was very significant for one of the mutants (disruption of *algE3*). This result highlights how growth conditions can influence the composition and structure of the alginates and this should be taken into consideration when designing future work on bacterial production of alginates.

Deletion of several epimerasegenes resulted in significant alterations in morphology of the cells in RA1-medium, rendering them unable to form a stable alginate coating. The deletion mutant where all the epimerasegenes were deleted produced alginate with an extremely low G-content, indicating that the AlgG enzyme has a low epimerase activity in *A. vinelandii* compared to that of *P. fluorecens* (84). That *A. vinelandii* still has this enzyme is most likely explained by the probable structural role AlgG was recently shown to have in the biosynthesis of alginate in *P. fluorecens* and *P. aeruginosa* (84, 126), and most probably have in *A. vinelandii* as well. The strain without any of the AlgE epimerases was unable to make desiccation resistant cysts and this is probably caused by the lack of G-blocks in the produced alginate, proving that the AlgE epimerases are essential for the ability to make desiccation resistant cysts. This is in agreement with the findings for the mutant where the proposed export system was deleted, and strengthens the hypothesis that epimerase transport is the function of the gene products of the deleted genes in this mutant.

The strain without any exported epimerases have such a low G-content originating from AlgG that it has a great potential for reintroduction of *algE* genes under a controllable promoter. Such a strain would potentially be a source of alginate with a predetermined structure. For further work with this strain *algG* should also be inactivated, as described for *P. fluorescens* (84) to have a strain producing pure mannuronan. Furthermore, it has been shown that the genes for acetylation can be deleted from *P. fluorescens* (83), and we believe this could be done in *A. vinelandii*, generating a strain that produce un-acetylated alginate. This may be important in *A. vinelandii* if an increased G content is a goal, since it is known that acetyl groups block epimerization (44, 239). An additional way of increasing the G-content in the alginate is to increase the export of AlgE epimerases out of the cell. This could be done by genetically manipulations of the secretion system identified as part of this thesis.

Another important factor for success in these attempts is the availability of genetic tools for manipulating the expression of genes in *A. vinelandii*, either in the alginate biosynthesis machinery, the epimerases or the secretion system of the epimerases. For this organism one must probably rely on broad-host-range systems like *Pm*/x*ylS*. In this work we experienced problems with too high background expression from this system. To deal with this a system for identification of inducible promoter mutants with decreased background expression was developed. This screening identified such promoters in *P. fluorescens,* which have been used in metabolic engineering processes and a patent (11,85), and we believe that the same screening strategy could be applied to find suitable promotors for *A. vinelandii*.

Increased knowledge on several proteins involment in cellular processes generates a need to control the expression of several genes independently of each other in metabolic engineering. This is most easily achieved by using different promoters whose activities can be individually controlled and in this thesis the development of such a new promoter system is described. This system can be used in several gram-negative bacteria, as shown in this thesis, and was also found to most likely function together with the *Pm*/*xylS* system when presented in the same cell.

5. References

1. **Albrecht, M. T., and N. L. Schiller.** 2005. Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **187:**3869-3872.
2. **Aldor, I. S., D. C. Krawitz, W. Forrest, C. Chen, J. C. Nishihara, J. C. Joly, and K. M. Champion.** 2005. Proteomic profiling of recombinant *Escherichia coli* in high-cell-density fermentations for improved production of an antibody fragment biopharmaceutical. Apl Environ Microbiol **71:**1717-1728.
3. **Allen, R. M., R. Chatterjee, M. S. Madden, P. W. Ludden, and V. K. Shah.** 1994. Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. Critical Reviews in Biotechnology **14:**225-249.
4. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. Microbiological Reviews **54:**450-472.
5. **Annison, G., and I. Couperwhite.** 1986. Influence of Calcium on Alginate Production and Composition in Continuous Cultures of *Azotobacter vinelandii.* Appl microbiol and biotech **25:**55-61.
6. **Askenazi, M., E. M. Driggers, D. A. Holtzman, T. C. Norman, S. Iverson, D. P. Zimmer, M. E. Boers, P. R. Blomquist, E. J. Martinez, A. W. Monreal, T. P. Feibelman, M. E. Mayorga, M. E. Maxon, K. Sykes, J. V. Tobin, E. Cordero, S. R. Salama, J. Trueheart, J. C. Royer, and K. T. Madden.** 2003. Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nature Biotechnology **21:**150-156.
7. **Atkins, E. D., I. A. Nieduszynski, W. Mackie, K. D. Parker, and E. E. Smolko.** 1973. Structural components of alginic acid. I. The crystalline structure of poly-beta-D-mannuronic acid. Results of x-ray diffraction and polarized infrared studies. Biopolymers **12:**1865-78.
8. **Atkins, E. D. T., W. Mackie, K. D. Parker, and E. E. Smolko.** 1971. Crystalline Structures of Poly-D-Mannuronic and Poly-L-Guluronic Acids. Journal of Polymer Science Part B-Polymer Letters **9:**311-316.
9. **Bagdasarian, M., and K. N. Timmis.** 1981. Host-vector systems for gene-cloning in Pseudomonas. Current Topics in Microbiology and Immunology **96:**47-67.

10. **Bailey, J. E.** 1991. Toward a science of metabolic engineering. Science **252:**1668-1675.

11. **Bakkevig, K., H. Sletta, M. Gimmestad, R. Aune, H. Ertesvåg, K. Degnes, B. E. Christensen, T. E. Ellingsen, and S. Valla.** 2005. Role of the applied and environmental microbiology alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J Bacteriol **187:**8375-84.

12. **Ballesteros, F., J. Gonzalezlopez, T. Larubia, J. Moreno, J. Aneiros, and A. Ramoscormenzana.** 1986. Growth of *Azotobacter vinelandii* in dialyzed soil medium - studies upon the life-cycle. Annales De L Institut Pasteur-Microbiologie **137A:**55-64.

13. **Barnett, M. J., V. Oke, and S. R. Long.** 2000. New genetic tools for use in the *Rhizobiaceae* and other bacteria. Biotechniques **29:**240-2, 244-5.

14. **Baynham, P. J., A. L. Brown, L. L. Hall, and D. J. Wozniak.** 1999. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and algD transcriptional activation. Mol Microbiol **33:**1069-80.

15. **Baynham, P. J., and D. J. Wozniak.** 1996. Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa* algD transcription. Mol Microbiol **22:**97-108.

16. **Beale, J. M., Jr., and J. L. Foster.** 1996. Carbohydrate fluxes into alginate biosynthesis in *Azotobacter vinelandii* NCIB 8789: NMR investigations of the triose pools. Biochemistry **35:**4492-501.

17. **Berry, A., T. C. Dodge, M. Pepsin, and W. Weyler.** 2002. Application of metabolic engineering to improve both the production and use of biotech indigo. **28:**127-133.

18. **Bjerkan, T. M.** 2004. Structure - function analyses of mannuronan C-5-epimerases. Ph.D. Thesis, NTNU, Trondheim.

19. **Bjerkan, T. M., C. L. Bender, H. Ertesvåg, F. Drabløs, M. K. Fakhr, L. A. Preston, G. Skjåk-Bræk, and S. Valla.** 2004. The *Pseudomonas syringae* genome encodes a combined mannuronan C-5-epimerase and O-acetylhydrolase, which strongly enhances the predicted gel-forming properties of alginates. J Biol Chem **279:**28920-9.

20. **Bjerkan, T. M., B. E. Lillehov, W. I. Strand, G. Skjåk-Bræk, S. Valla, and H. Ertesvåg.** 2004. Construction and analyses of hybrid *Azotobacter vinelandii* mannuronan C-5 epimerases with new epimerization pattern characteristics. Biochem J **381:**813-21.

21. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, and S. Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Apl Environ Microbiol **63:**370-379.

22. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, P. Karunakaran, and S. Valla.** 1997. Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. Plasmid **38:**35-51.

23. **Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J Bacteriol **178:**511-23.

24. **Boucher, J. C., M. J. Schurr, and V. Deretic.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol Microbiol **36:**341-51.

25. **Boucher, J. C., M. J. Schurr, H. Yu, D. W. Rowen, and V. Deretic.** 1997. *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. Microbiology **143 (Pt 11):**3473-80.

26. **Boyd, A., M. Ghosh, T. B. May, D. Shinabarger, R. Keogh, and A. M. Chakrabarty.** 1993. Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. Gene **131:**1-8.

27. **Brautaset, T., S. Petersen, and S. Valla.** 1998. An experimental study on carbon flow in Escherichia coli as a function of kinetic properties and expression levels of the enzyme phosphoglucomutase. **58:**299-302.

28. **Brivonese, A. C., and I. W. Sutherland.** 1989. Polymer Production by a Mucoid Stain of *Azotobacter vinelandii* in Batch Culture. Appl microbiol and biotech **30:**97-102.

29. **Bro, C., and J. Nielsen.** 2004. Impact of 'ome' analyses on inverse metabolic engineering. Metabolic Engineering **6:**204-211.

30. **Campa, C., S. Holtan, N. Nilsen, T. M. Bjerkan, B. T. Stokke, and G. Skjåk-Bræk.** 2004. Biochemical analysis of the processive mechanism for epimerization of alginate by mannuronan C-5 epimerase AlgE4. Biochemical Journal **381:**155-164.

31. **Campos, M., J. M. Martìnez-Salazar, L. Lloret, S. Moreno, C. Nunez, G. Espín, and G. Sòberon-Chàvez.** 1996. Characterization of the gene coding for GDP-mannose dehydrogenase (*algD*) from *Azotobacter vinelandii*. J Bacteriol **178:**1793-9.

32. **Chan, A. Y., M. M. Chan, H. M. Lo, Y. C. Leung, and B. L. Lim.** 2002. A dual protein expression system in *Bacillus subtilis*. Protein Expression and Purification **26:**337-342.

33. **Chen, W. P., J. Y. Chen, S. C. Chang, and C. L. Su.** 1985. Bacterial Alginate Produced by a Mutant of *Azotobacter vinelandii*. Apl Environ Microbiol **49:**543-546.

34. **Cheng, Q., S. M. Thomas, K. Kostichka, J. R. Valentine, and V. Nagarajan.** 2000. Genetic analysis of a gene cluster for cyclohexanol oxidation in *Acinetobacter sp*. Strain SE19 by in vitro transposition. J Bacteriol **182:**4744-51.

35. **Cheng, Q., S. M. Thomas, K. Kostichka, J. R. Valentine, and V. Nagarajan.** 2000. Genetic analysis of a gene cluster for cyclohexanol oxidation in *Acinetobacter sp*. Strain SE19 by *in vitro* transposition. J. Bacteriology **182:**4744-51.

36. **Chitnis, C. E., and D. E. Ohman.** 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J Bacteriol **172:**2894-2900.

37. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol Microbiol **8:**583-93.

38. **Choi, J. H., S. J. Lee, S. J. Lee, and S. Y. Lee.** 2003. Enhanced production of insulin-like growth factor I fusion protein in *Escherichia coli* by coexpression of the down-regulated genes identified by transcriptome profiling. Apl Environ Microbiol **69:**4737-4742.

39. **Chu, L., T. B. May, A. M. Chakrabarty, and T. K. Misra.** 1991. Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. Gene **107:**1-10.

40. **Clementi, F., M. A. Crudele, E. Parente, M. Mancini, and M. Moresi.** 1999. Production and characterisation of alginate from *Azotobacter vinelandii*. Journal of the Science of Food and Agriculture **79:**602-610.

41. **Clementi, F., P. Fantozzi, F. Mancini, and M. Moresi.** 1995. Optimal conditions for alginate production by *Azotobacter vinelandii*. Enzyme and Microbial Technology **17:**983-988.

42. **Cote, G. L., and L. H. Krull.** 1988. Characterization of the exocellular polysaccharides from *Azotobacter chroococcum*. Carbohydrate Research **181:**143-152.

43. **Dalton, H., and J. R. Postgate.** 1968. Effect of Oxygen on Growth of *Azotobacter chroococcum* in Batch and Continuous Cultures. J gen microbiol **54:**463-471.

44. **Davidson, I. W., I. W. Sutherland, and C. J. Lawson.** 1977. Localization of O-acetyl groups of bacterial alginate. J gen microbiol **98:**603-606.

45. **Davison, J.** 2002. Genetic tools for pseudomonads, rhizobia, and other gram-negative bacteria. Biotechniques **32:**386-401.

46. **Davison, J., N. Chevalier, and F. Brunel.** 1989. Bacteriophage-T7 RNA polymerase-controlled specific gene-expression in pseudomonas. Gene **83:**371-375.

47. **de Vos, P., B. J. de Haan, A. de Haan, J. van Zanten, and M. M. Faas.** 2004. Factors influencing functional survival of microencapsulated islet grafts. Cell Transplant **13:**515-24.

48. **Deboer, H. A., L. J. Comstock, and M. Vasser.** 1983. The Tac Promoter - A functional hybrid derived from the Trp and Lac promoters. **80:**21-25.

49. **Defeyter, R., C. I. Kado, and D. W. Gabriel.** 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene **88:**65-72.

50. **Delepelaire, P.** 2004. Type I secretion in gram-negative bacteria. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1694:**149-161.

51. **DelicAttree, I., B. Toussaint, J. Garin, and P. M. Vignais.** 1997. Cloning, sequence and mutagenesis of the structural gene of *Pseudomonas aeruginosa* CysB, which can activate *algD* transcription. Mol microbiol **24:**1275-1284.

52. **Delorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacteriol **172:**6568-6572.

53. **Deretic, V., R. Dikshit, W. M. Konyecsni, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J Bacteriol **171:**1278-83.

54. **Deretic, V., J. H. Leveau, C. D. Mohr, and N. S. Hibler.** 1992. In vitro phosphorylation of AlgR, a regulator of mucoidy in *Pseudomonas aeruginosa*, by a histidine protein kinase and effects of small phospho-donor molecules. Mol Microbiol **6:**2761-7.

55. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J Bacteriol **176:**6677-87.

56. **Dingler, C., and J. Oelze.** 1987. Superoxide-dismutase and catalase in *Azotobacter vinelandii* grown in continuous culture at different dissolved-oxygen concentrations. Archives of Microbiology **147:**291-294.

57. **Donati, I., S. Holtan, Y. A. Mørch, M. Borgogna, M. Dentini, and G. Skjåk-Bræk.** 2005. New hypothesis on the role of alternating sequences in calcium-alginate gels. Biomacromolecules **6:**1031-1040.

58. **Draget, K. I., G. Skjåk-Bræk, and O. Smidsrød.** 1994. Alginic acid gels: the effect of alginate chemical composition and molecular weight. Carbohydrate Polymers **25:**31-38.

59. **Eady, R. R.** 1995. A review of the structure and function of vanadium nitrogenase of Azotobacter. Journal of Inorganic Biochemistry **59:**583.

60. **Ertesvåg, H., B. Doseth, B. Larsen, G. Skjåk-Bræk, and S. Valla.** 1994. Cloning and expression of an *Azotobacter vinelandii* mannuronan C-5-epimerase gene. J Bacteriol **176:**2846-2853.

61. **Ertesvåg, H., F. Erlien, G. Skjåk-Bræk, B. H. A. Rehm, and S. Valla.** 1998. Biochemical properties and substrate specificities of a recombinantly produced *Azotobacter vinelandii* alginate lyase. J Bacteriol **180:**3779-3784.

62. **Ertesvåg, H., H. K. Høidal, I. K. Hals, A. Rian, B. Doseth, and S. Valla.** 1995. A Family of modular type mannuronan C-5-epimerase genes controls alginate structure in *Azotobacter vinelandii*. Mol microbiol **16:**719-731.

63. **Ertesvåg, H., H. K. Høidal, H. Schjerven, B. I. Svanem, and S. Valla.** 1999. Mannuronan C-5-epimerases and their application for in vitro and in vivo design of new alginates useful in biotechnology. Metab Eng **1:**262-9.

64. **Ertesvåg, H., H. K. Høidal, G. Skjåk-Bræk, and S. Valla.** 1998. The *Azotobacter vinelandii* mannuronan C-5-epimerase AlgE1 consists of two separate catalytic domains. Journal of Biological Chemistry **273:**30927-30932.

65. **Fell, D. A.** 1998. Increasing the flux in metabolic pathways: A metabolic control analysis perspective. Biotechnology and Bioengineering **58:**121-124.

66. **Ferrala, N. F., A. K. Champlin, and F. A. Fekete.** 1986. Morphological differences in the capsular polysaccharide of nitrogen-fixing Azotobacter-Chroococcum-B-8 as a function of iron and molybdenum starvation. FEMS Microbiology Letters **33:**137-142.

67. **Fett, W. F., S. F. Osman, M. L. Fishman, and T. S. I. Siebles.** 1986. Alginate production by plant-pathogen *Pseudomonads*. Appl. Environ. Microbiol. **52:**466-473.

68. **Fett, W. F., S. F. Osman, C. Wijey, S. Singh, and B. Koehler.** 1992. Synthesis and regulation of alginate production by plant-associated bacteria. Abstracts of Papers of the American Chemical Society **204:**59-Carb.
69. **Fialho, A. M., N. A. Zielinski, W. F. Fett, A. M. Chakrabarty, and A. Berry.** 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily B procaryotes. Appl. Environ. Microbiol. **56:**436-443.
70. **Fischer, F. G., and H. Dörfel.** 1955. Die Polyuronsauren Der Braunalgen - (Kohlenhydrate Der Algen-I). Hoppe-Seylers Zeitschrift Fur Physiologische Chemie **302:**186-203.
71. **Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman.** 1994. *Pseudomonas aeruginos*a AlgG Is a Polymer Level Alginate C5-Mannuronan Epimerase. J Bacteriol **176:**1821-1830.
72. **Franklin, M. J., and D. E. Ohman.** 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J Bacteriol **175:**5057-65.
73. **Franklin, M. J., and D. E. Ohman.** 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J Bacteriol **178:**2186-95.
74. **Franklin, M. J., and D. E. Ohman.** 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J Bacteriol **184:**3000-7.
75. **Fujihara, M., and T. Nagumo.** 1992. The effect of the content of D-mannuronic acid and L-guluronic acid blocks in alginates on antitumor-activity. Carbohydrate Research **224:**343-347.
76. **Fujiwara, S., N. A. Zielinski, and A. M. Chakrabarty.** 1993. Enhancer-like activity of A1gR1-binding site in alginate gene activation: positional, orientational, and sequence specificity. J Bacteriol **175:**5452-9.
77. **Gacesa, P.** 1987. Alginate-modifying enzymes: A proposed unified mechanism of action for the lyases and epimerases. FEBS Letters **212:**199-202.
78. **Gacesa, P.** 1992. Enzymatic degradation of alginates. International Journal of Biochemistry **24:**545-552.

79. **Gallegos, M. T., R. Schleif, A. Bairoch, K. Hofmann, and J. L. Ramos.** 1997. AraC/XylS family of transcriptional regulators. Microbiology and Molecular Biology Reviews **61:**393-410.

80. **Gallegos, M. T., P. A. Williams, and J. L. Ramos.** 1997. Transcriptional control of the multiple catabolic pathways encoded on the TOL plasmid pWW53 of *Pseudomonas putida* MT53. J Bacteriol **179:**5024-5029.

81. **Gama-Castro, S., C. Nunez, D. Segura, S. Moreno, J. Guzman, and G. Espìn.** 2001. *Azotobacter vinelandii* aldehyde dehydrogenase regulated by sigma(54): Role in alcohol catabolism and encystment. J Bacteriol **183:**6169-6174.

82. **Gaona, G., C. Nunez, J. B. Goldberg, A. S. Linford, R. Najera, M. Castaneda, J. Guzman, G. Espìn, and G. Soberon-Chavez.** 2004. Characterization of the *Azotobacter vinelandii algC* gene involved in alginate and lipopolysaccharide production. FEMS Microbiol Lett **238:**199-206.

83. **Gimmestad, M.** 2005. A Study of bacterial genes involved in post-polymerization control of alginate structure, Ph.D. thesis, NTNU, Trondheim.

84. **Gimmestad, M., H. Sletta, H. Ertesvåg, K. Bakkevig, S. Jain, S. Suh, G. Skjåk-Bræk, T. E. Ellingsen, D. E. Ohman, and S. Valla.** 2003. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J Bacteriol **185:**3515-3523.

85. **Gimmestad, M., H. Sletta, P. Karunakaran, K. Bakkevig, H. Ertesvåg, T. Ellingsen, G. Skjåk-Bræk, and S. Valla.** February 2004. Patent WO 2004/011628 A1. New mutant strains of *Pseudomonas fluorescens* and variants thereof, methods for their production, and uses thereof in alginate production.

86. **Goldberg, J. B., and T. Dahnke.** 1992. *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. Mol Microbiol **6:**59-66.

87. **Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman.** 1993. A mutation in *algN* permits trans activation of alginate production by algT in Pseudomonas species. J Bacteriol **175:**1303-8.

88. **Gonzales-Lopez, J., and G. R. Vela.** 1981. True morphology of the Azotobacteraceae-filterable bacteria. Nature **289:**588-590.

89. **Gorin, P. A. J., and J. F. T. Spencer.** 1966. Exocellular alginic acid from *Azotobacter vinelandii*. Canadian Journal of Chemistry **44:**993-998.

90. **Govan, J. R., J. A. Fyfe, and T. R. Jarman.** 1981. Isolation of alginate-producing mutants of *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas mendocina*. J Gen Microbiol **125:**217-20.

91. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiological Reviews **60:**539-574.

92. **Govan, J. R. W., J. A. M. Fyfe, and T. R. Jarman.** 1981. Isolation of alginate-producing mutants of *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas mendocina*. J gen microbiol **125:**217-220.

93. **Grabert, E., J. Wingender, and U. K. Winkler.** 1990. An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa*. FEMS Microbiol Lett **56:**83-7.

94. **Grant, G. T., E. R. Morris, D. A. Rees, P. J. C. Smith, and D. Thom.** 1973. Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Lett. **32:**195-198.

95. **Grasdalen, H.** 1983. High-Field, ^{1}H-nmr Spectroscopy of Alginate - Sequential Structure and Linkage Conformations. Carbohydrate Research **118:**255-260.

96. **Grasdalen, H., B. Larsen, and O. Smidsrød.** 1977. C-13-NMR Studies of Alginate. Carbohydrate Research **56:**C11-C15.

97. **Grasdalen, H., B. Larsen, and O. Smidsrød.** 1981. C-13-NMR Studies of Monomeric Composition and Sequence in Alginate. Carbohydrate Research **89:**179-191.

98. **Grasdalen, H., B. Larsen, and O. Smidsrød.** 1979. A Pmr Study of the Composition and Sequence of Uronate Residues in Alginates. Carbohydrate Research **68:**23-31.

99. **Gross, M., and K. Rudolph.** 1987. Demonstration of levan and alginate in bean-plants (*Phaseolus vulgaris*) Infected by *Pseudomonas syringae* pv Phaseolicola. Journal of Phytopathology-Phytopathologische Zeitschrift **120:**9-19.

100. **Gross, M., and K. Rudolph.** 1987. Studies on the extracellular polysaccharides (Eps) produced *in vitro* by *Pseudomonas phaseolicola*. 3. Kinetics of levan and alginate formation in batch culture and demonstration of levansucrase activity in crude eps. Journal of Phytopathology-Phytopathologische Zeitschrift **119:**289-297.

101. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P-bad Promoter. J Bacteriol **177:**4121-4130.

102. **Hammad, A. M. M.** 1998. Evaluation of alginate-encapsulated *Azotobacter chroococcum* as a phage-resistant and an effective inoculum. Journal of Basic Microbiology **38:**9-16.

103. **Han, M. J., K. J. Jeong, J. S. Yoo, and S. Y. Lee.** 2003. Engineering *Escherichia coli* for increased productivity of serine-rich proteins based on proteome profiling. Apl Environ Microbiol **69:**5772-5781.

104. **Haro, M. A., and V. de Lorenzo.** 2001. Metabolic engineering of bacteria for environmental applications: construction of *Pseudomonas* strains for biodegradation of 2-chlorotoluene. J. biotechnol **85:**103-113.

105. **Hartmann, M., O. B. Holm, G. A. B. Johansen, G. Skjåk-Bræk, and B. T. Stokke.** 2002. Mode of action of recombinant *Azotobacter vinelandii* mannuronan C-5 epimerases AlgE2 and AlgE4. Biopolymers **63:**77-88.

106. **Hashimoto, T., Y. Suzuki, K. Suzuki, T. Nakashima, M. Tanihara, and C. Ide.** 2005. Review: peripheral nerve regeneration using non-tubular alginate gel crosslinked with covalent bonds. J Mater Sci Mater Med **16:**503-9.

107. **Hasse, C., T. Böhrer, P. Barth, B. Stinner, R. Cohen, H. Cramer, U. Zimmermann, and M. Rothmund.** 2000. Parathyroid xenotransplantation without immunosuppression in experimental hypoparathyroidism: Long-term in vivo function following microencapsulation with a clinically suitable alginate. World Journal of Surgery **24:**1361-1366.

108. **Haug, A.** 1964. Composition and properties of alginates. Norsk institutt for tang og tareforskning, report no 30.

109. **Haug, A., and B. Larsen.** 1971. Biosynthesis of alginate.2. Polymannuronic acid C-5-epimerase from *Azotobacter vinelandii* (Lipman). Carbohydrate Research **17:**297-308.

110. **Haug, A., and B. Larsen.** 1969. Biosynthesis of alginate. epimerisation of D-mannuronic to L-guluronic acid residues in polymer chain. Biochimica Et Biophysica Acta **192:**557-559.

111. **Haug, A., B. Larsen, and O. Smidsrød.** 1967. Studies on sequence of uronic acid residues in alginic acid. Acta Chemica Scandinavica **21:**691-704.

112. **Haug, A., B. Larsen, and O. Smidsrød.** 1966. A study of constitution of alginic acid by partial acid hydrolysis. Acta Chemica Scandinavica **20:**183-190.

113. **Herrero, M., V. Delorenzo, B. Ensley, and K. N. Timmis.** 1993. A T7 Rna polymerase-based system for the construction of pseudomonas strains with phenotypes dependent on Tol-meta pathway effectors. Gene **134:**103-106.

114. **Herrero, M., V. Delorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol **172:**6557-6567.

115. **Hershberger, C. D., R. W. Ye, M. R. Parsek, Z. D. Xie, and A. M. Chakrabarty.** 1995. The *algT* (*algU*) Gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative sigma-factor (Sigma(E)). Proceedings of the National Academy of Sciences of the United States of America **92:**7941-7945.

116. **Hitchins, V. M., and H. L. Sadoff.** 1970. Morphogenesis of cysts in *Azotobacter vinelandii*. J Bacteriol **104:**492-498.

117. **Hitchins, V. M., and H. L. Sadoff.** 1973. Sequential metabolic events during encystment of *Azotobacter vinelandii*. J Bacteriol **113:**1273-1279.

118. **Høidal, H. K., H. Ertesvåg, G. Skjåk-Bræk, B. T. Stokke, and S. Valla.** 1999. The recombinant *Azotobacter vinelandii* mannuronan C-5-epimerase AlgE4 epimerizes alginate by a nonrandom attack mechanism. Journal of Biological Chemistry **274:**12316-12322.

119. **Høidal, H. K., B. I. G. Svanem, M. Gimmestad, and S. Valla.** 2000. Mannuronan C-5 epimerases and cellular differentiation of *Azotobacter vinelandii*. Environ Microbiol **2:**27-38.

120. **Holtan, S., P. Bruheim, and G. Skjåk-Bræk.** 2006. Mode of action and subsite studies of the guluronan block forming mannuronan C-5 epimerases AlgE1 and AlgE6. Biochem J.

121. **Horan, N. J., T. R. Jarman, and E. A. Dawes.** 1981. Effects of carbon cource and inorganic-phosphate concentration on the production of alginic acid by a mutant of *Azotobacter vinelandii* and on the enzymes involved in its biosynthesis. J gen microbiol **127:**185-191.

122. **Horan, N. J., T. R. Jarman, and E. A. Dawes.** 1983. Studies on some enzymes of alginic acid biosynthesis in *Azotobacter vinelandii* grown in continuous culture. J gen microbiol **129:**2985-2990.

123. **Hortelano, G., N. Xu, A. Vandenberg, J. Solera, P. L. Chang, and F. A. Ofosu.** 1999. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. Hum Gene Ther **10:**1281-1288.

124. **Hwang, E. I., M. Kaneko, Y. Ohnishi, and S. Horinouchi.** 2003. Production of plant-specific flavanones by Escherichia coli containing an artificial gene cluster. **69:**2699-2706.

125. **Iwaki, H., Y. Hasegawa, M. Teraoka, T. Tokuyama, H. Bergeron, and P. C. Lau.** 1999. Identification of a transcriptional activator (ChnR) and a 6-oxohexanoate dehydrogenase (ChnE) in the cyclohexanol catabolic pathway in *Acinetobacter sp.* Strain NCIMB 9871 and localization of the genes that encode them. Appl Environ Microbiol **65:**5158-62.

126. **Jain, S., M. J. Franklin, H. Ertesvåg, S. Valla, and D. E. Ohman.** 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol Microbiol **47:**1123-33.

127. **Jain, S., and D. E. Ohman.** 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J Bacteriol **180:**634-41.

128. **Jain, S., and D. E. Ohman.** 2005. Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infection and Immunity **73:**6429-6436.

129. **Jarman, T. R., L. Deavin, S. Slocombe, and R. C. Righelato.** 1978. Investigation of effect of environmental-conditions on rate of exopolysaccharide synthesis in *Azotobacter vinelandii*. J gen microbiol **107:**59-64.

130. **Jensen, P. R., and K. Hammer.** 1998. Artificial promoters for metabolic optimization. Biotechnology and Bioengineering **58:**191-195.

131. **Jensen, P. R., and K. Hammer.** 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Apl Environ Microbiol **64:**82-87.

132. **Kamal-Eldin, A., A. Pouru, C. Eliasson, and P. Åman.** 2001. Alkylresorcinols as antioxidants: hydrogen donation and peroxyl radical-scavenging effects. J. Sci. Food Agric. **81:**353-356.

133. **Kang, M. J., S. H. Yoon, Y. M. Lee, S. H. Lee, J. E. Kim, K. H. Jung, Y. C. Shin, and S. W. Kim.** 2005. Enhancement of lycopene production in *Escherichia coli* by optimization of the lycopene synthetic pathway. **15:**880-886.

134. **Karunakaran, P., D. T. Endresen, H. Ertesvåg, J. M. Blatny, and S. Valla.** 1999. A small derivative of the broad-host-range plasmid RK2 which can be switched from a replicating to a non-replicating state as a response to an externally added inducer. FEMS Microbiol Lett **180:**221-7.

135. **Kato, J., L. Chu, K. Kitano, J. D. DeVault, K. Kimbara, A. M. Chakrabarty, and T. K. Misra.** 1989. Nucleotide sequence of a regulatory region controlling alginate synthesis in *Pseudomonas aeruginosa*: characterization of the algR2 gene. Gene **84:**31-8.

136. **Kato, J., T. K. Misra, and A. M. Chakrabarty.** 1990. AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **87:**2887-91.

137. **Keasling, J. D.** 1999. Gene-expression tools for the metabolic engineering of bacteria. Trends in Biotechnology **17:**452-460.

138. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191-197.

139. **Kennedy, L., K. McDowell, and I. W. Sutherland.** 1992. Alginases from Azotobacter species. J. Gen. Microbiol. **138:**2465-2471.

140. **Kidambi, S. P., G. W. Sundin, D. A. Palmer, A. M. Chakrabarty, and C. L. Bender.** 1995. Copper as a signal for alginate synthesis in *Pseudomonas syringae* pv. syringae. Appl Environ Microbiol **61:**2172-9.

141. **Konyecsni, W. M., and V. Deretic.** 1990. DNA sequence and expression analysis of *algP* and *algQ*, components of the multigene system transcriptionally regulating mucoidy in *Pseudomonas aeruginosa*: algP contains multiple direct repeats. J Bacteriol **172:**2511-20.

142. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, 2nd, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-6.

143. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop, 2nd, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques **16:**800-2.

144. **Kozubek, A., and J. H. Tyman.** 1999. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem Rev **99:**1-26.

145. **Kraiwattanapong, J., K. Motomura, T. Ooi, and S. Kinoshita.** 1999. Characterization of alginate lyase (ALYII) from Pseudomonas sp OS-ALG-9

expressed in recombinant *Escherichia coli*. World Journal of Microbiology & Biotechnology **15:**117-122.

146. **Lange, B., J. Wingender, and U. K. Winkler.** 1989. Isolation and characterization of an alginate lyase from *Klebsiella aerogenes*. Archives of Microbiology **152:**302-308.

147. **Larsen, B., and A. Haug.** 1971. Biosynthesis of alginate.1. composition and structure of alginate produced by *Azotobacter vinelandii* (Lipman). Carbohydrate Research **17:**287-296.

148. **Lin, L. P., S. Pankratz, and H. L. Sadoff.** 1978. Ultrastructural and physiological changes occurring upon germination and outgrowth of *Azotobacter vinelandii* cysts. J Bacteriol **135:**641-646.

149. **Lin, L. P., and H. L. Sadoff.** 1969. Chemical composition of *Azotobacter vinelandii* cysts. J Bacteriol **100:**480-6.

150. **Lin, L. P., and H. L. Sadoff.** 1968. Encystment and polymer production by *Azotobacter vinelandii* in the presence of beta-hydroxybutyrate. J Bacteriol **95:**2336-43.

151. **Lin, L. P., and H. L. Sadoff.** 1969. Preparation and ultrastructure of outer coats of *Azotobacter vinelandii* cysts. J Bacteriol **98:**1335-1341.

152. **Lin, T. Y., and W. Z. Hassid.** 1966. Pathway of algnic acid synthesis in the marine brown alga, Fucus gardneri Silva. J Biol Chem **241:**5284-97.

153. **Linker, A., and R. S. Jones.** 1966. A new Polysaccharide resembling alginic acid isolated from Pseudomonads. J. Biol. Chem. **241:**3845-3851.

154. **Linker, A., and R. S. Jones.** 1964. A Polysaccharide resembling alginic acid from a Pseudomonas micro-organism. Nature **204:**187-8.

155. **Lloret, L., R. Barreto, R. Leon, S. Moreno, J. Martinez-Salazar, G. Espìn, and G. Soberon-Chavez.** 1996. Genetic analysis of the transcriptional arrangement of *Azotobacter vinelandii* alginate biosynthetic genes: identification of two independent promoters. Mol Microbiol **21:**449-57.

156. **Loperfid.B, and H. L. Sadoff.** 1973. Germination of *Azotobacter vinelandii* cysts - sequence of macromolecular synthesis and nitrogen-fixation. J Bacteriol **113:**841-846.

157. **Lopez, J. G., and G. R. Vela.** 1981. True morphology of the Azotobacteraceae - filterable bacteria. Nature **289:**588-590.

158. **Lynn, A. R., and J. R. Sokatch.** 1984. Incorporation of isotope from specifically labeled glucose into alginates of *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. J Bacteriol **158:**1161-2.

159. **Ma, S., D. J. Wozniak, and D. E. Ohman.** 1997. Identification of the histidine protein kinase KinB in *Pseudomonas aeruginosa* and its phosphorylation of the alginate regulator algB. J Biol Chem **272:**17952-60.

160. **Machluf, M., A. Orsola, S. Boorjian, R. Kershen, and A. Atala.** 2003. Microencapsulation of Leydig Cells: A System for Testosterone Supplementation. Endocrinology **144:**4975-4979.

161. **Maharaj, R., T. B. May, S. K. Wang, and A. M. Chakrabarty.** 1993. Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene **136:**267-9.

162. **Mai, G., N. T. Huy, P. Morel, J. Mei, D. Bosco, T. Berney, P. Majno, G. Mentha, D. Trono, and L. H. Buhler.** 2005. Treatment of fulminant liver failure by transplantation of microencapsulated primary or immortalized xenogeneic hepatocytes. Transplantation Proceedings **37:**527-529.

163. **Maloney, P. C., and B. Rotman.** 1973. Distribution of suboptimally induced beta-D-galactosidase in *Escherichia coli* - Enzyme content of individual cells. Journal of Molecular Biology **73:**77-91.

164. **Manchak, J., and W. J. Page.** 1994. Control of polyhydroxyalkanoate synthesis in *Azotobacter vinelandii* strain uwd. Microbiology-Uk **140:**953-963.

165. **Martin, D. W., B. W. Holloway, and V. Deretic.** 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a Bacillus sigma factor. J Bacteriol **175:**1153-64.

166. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of mucB causes conversion to mucoidy. Mol Microbiol **9:**497-506.

167. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc Natl Acad Sci U S A **90:**8377-81.

168. **Martìnez-Salazar, J. M., S. Moreno, R. Nàjera, J. C. Boucher, G. Espìn, G. Soberòn-Chàvez, and V. Deretic.** 1996. Characterization of the genes coding for the putative sigma factor AlgU and its regulators MucA, MucB, MucC, and MucD in

Azotobacter vinelandii and evaluation of their roles in alginate biosynthesis. J Bacteriol **178:**1800-8.

169. **Martinez, P., J. Guzman, and G. Espìn.** 1997. A mutation impairing alginate production increased accumulation of poly-beta-hydroxybutyrate in *Azotobacter vinelandii*. Biotechnology Letters **19:**909-911.

170. **Mathee, K., C. J. McPherson, and D. E. Ohman.** 1997. Posttranslational control of the algT (algU)-encoded sigma22 for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). J Bacteriol **179:**3711-20.

171. **May, T. B., D. Shinabarger, A. Boyd, and A. M. Chakrabarty.** 1994. Identification of amino acid residues involved in the activity of phosphomannose isomerase-guanosine 5'-diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem **269:**4872-7.

172. **Mejìa-Ruìz, H., J. Guzmàn, S. Moreno, G. Soberòn-Chàvez, and G. Espìn.** 1997. The *Azotobacter vinelandii alg8* and *alg44* genes are essential for alginate synthesis and can be transcribed from an algD-independent promoter. Gene **199:**271-7.

173. **Mejia-Ruìz, H., S. Moreno, J. Guzmàn, R. Najera, R. Leon, G. Soberòn-Chàvez, and G. Espìn.** 1997. Isolation and characterization of an *Azotobacter vinelandii algK* mutant. FEMS Microbiol Lett **156:**101-6.

174. **Mermod, N., J. L. Ramos, P. R. Lehrbach, and K. N. Timmis.** 1986. Vector for regulated expression of cloned genes in a wide-range of gram-negative Bacteria. J Bacteriol **167:**447-454.

175. **Mijakovic, I., D. Petranovic, and P. R. Jensen.** 2005. Tunable promoters in systems biology. **16:**329-335.

176. **Mohr, C. D., J. H. Leveau, D. P. Krieg, N. S. Hibler, and V. Deretic.** 1992. AlgR-binding sites within the algD promoter make up a set of inverted repeats separated by a large intervening segment of DNA. J Bacteriol **174:**6624-33.

177. **Monday, S. R., and N. L. Schiller.** 1996. Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. J Bacteriol **178:**625-32.

178. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39-47.

179. **Narbad, A., M. J. Hewlins, P. Gacesa, and N. J. Russell.** 1990. The use of 13C-n.m.r. spectroscopy to monitor alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. Biochem J **267:**579-84.

180. **Narbad, A., N. J. Russell, and P. Gacesa.** 1988. Radiolabelling patterns in alginate of *Pseudomonas aeruginosa* synthesized from specifically-labelled 14C-monosaccharide precursors. Microbios **54:**171-9.

181. **Newman, J. R., and C. Fuqua.** 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. Gene **227:**197-203.

182. **Nielsen, J.** 2001. Metabolic engineering. Appl microbiol and biotech **55:**263-283.

183. **Nikolskaya, A. N., and M. Y. Galperin.** 2002. A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. Nucleic Acids Research **30:**2453-2459.

184. **Nùñez, C., R. Leon, J. Guzmàn, G. Espìn, and G. Soberòn-Chàvez.** 2000. Role of *Azotobacter vinelandii mucA* and *mucC* gene products in alginate production. J Bacteriol **182:**6550-6.

185. **Nùñez, C., S. Moreno, G. Soberòn-Chàvez, and G. Espìn.** 1999. The *Azotobacter vinelandii* response regulator AlgR is essential for cyst formation. J Bacteriol **181:**141-8.

186. **Nyvall, P., E. Corre, C. Boisset, T. Barbeyron, S. Rousvoal, D. Scornet, B. Kloareg, and C. Boyen.** 2003. Characterization of mannuronan C-5-epimerase genes from the brown alga *Laminaria digitata*. Plant Physiology **133:**726-735.

187. **Obika, H., J. Sakakibara, and Y. Kobayashi.** 1993. Optimization of cultivation conditions in alginate production by *Azotobacter vinelandii*. Seibutsu-Kogaku Kaishi-Journal of the Society for Fermentation and Bioengineering **71:**23-28.

188. **Oelze, J.** 2000. Respiratory protection of nitrogenase in Azotobacter species: is a widely held hypothesis unequivocally supported by experimental evidence? Fems Microbiology Reviews **24:**321-333.

189. **Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai, and M. Ikeda.** 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl microbiol and biotech **58:**217-223.

190. **Okabe, E., M. Nakajima, H. Murooka, and K. Nisizawa.** 1981. Investigation of carbon and phosphorus sources in cultural media of a selected strain of alginate-producing *Azotobacter vinelandii*. Journal of Fermentation Technology **59:**1-7.

191. **Orive, G., R. M. Hernandez, A. Rodriguez Gascon, R. Calafiore, T. M. Chang, P. de Vos, G. Hortelano, D. Hunkeler, I. Lacik, and J. L. Pedraz.** 2004. History, challenges and perspectives of cell microencapsulation. Trends Biotechnol **22:**87-92.

192. **Otterlei, M., K. Ostgaard, G. Skjåk-Bræk, O. Smidsrød, P. Soon-Shiong, and T. Espevik.** 1991. Induction of cytokine production from human monocytes stimulated with alginate. J Immunother **10:**286-91.

193. **Page, W. J., and H. L. Sadoff.** 1975. Relationship between calcium and uroinic acids in the encystment of *Azotobacter vinelandii*. J Bacteriol **122:**145-51.

194. **Pal, S., A. Manna, and A. K. Paul.** 1997. Induction of encystment and poly-beta-hydroxybutyric acid production by *Azotobacter chroococcum* MAL-201. Current Microbiology **35:**327-330.

195. **Parekh, S., V. A. Vinci, and R. J. Strobel.** 2000. Improvement of microbial strains and fermentation processes. Appl microbiol and biotech **54:**287-301.

196. **Parente, E., M. A. Crudele, M. Aquino, and F. Clementi.** 1998. Alginate production by *Azotobacter vinelandii* DSM576 in batch fermentation. Journal of Industrial Microbiology & Biotechnology **20:**171-176.

197. **Peciña, A., A. Pascual, and A. Paneque.** 1999. Cloning and expression of the *algL* gene, encoding the *Azotobacter chroococcum* alginate lyase: purification and characterization of the enzyme. J Bacteriol **181:**1409-14.

198. **Peña, C., N. Campos, and E. Galindo.** 1997. Changes in alginate molecular mass distributions, broth viscosity and morphology of *Azotobacter vinelandii* cultured in shake flasks. Appl microbiol and biotech **48:**510-515.

199. **Peña, C., L. Miranda, D. Segura, C. Nùñez, G. Espìn, and E. Galindo.** 2002. Alginate production by *Azotobacter vinelandii* mutants altered in poly-beta-hydroxybutyrate and alginate biosynthesis. J Ind Microbiol Biotechnol **29:**209-13.

200. **Peñaloza-Vàzquez, A., S. P. Kidambi, A. M. Chakrabarty, and C. L. Bender.** 1997. Characterization of the alginate biosynthetic gene cluster in Pseudomonas syringae pv. syringae. J Bacteriol **179:**4464-72.

201. **Piggott, N. H., I. W. Sutherland, and T. R. Jarman.** 1981. Enzymes involved in the biosynthesis of alginate by *Pseudomonas aeruginosa*. European Journal of Appl microbiol and biotech **13:**179-183.

202. **Pindar, D. F., and C. Bucke.** 1975. Biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochemical Journal **152:**617-622.

203. **Ramsey, D. M., and D. J. Wozniak.** 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol Microbiol **56:**309-22.

204. **Ramstad, M. V.** 1999. Expression of mannuronan C-5-epimerases in *Azotobacter vinelandii*, and biochemical properties of the recombinant epimerase AlgE2. PhD. NTNU, Trondheim.

205. **Read, T. A., V. Stensvaag, H. Vindenes, E. Ulvestad, R. Bjerkvig, and F. Thorsen.** 1999. Cells encapsulated in alginate: A potential system for delivery of recombinant proteins to malignant brain tumours. International Journal of Developmental Neuroscience **17:**653-663.

206. **Regni, C., L. Naught, P. A. Tipton, and L. J. Beamer.** 2004. Structural basis of diverse substrate recognition by the enzyme PMM/PGM from P. aeruginosa. Structure (Camb) **12:**55-63.

207. **Rehm, B. H.** 1996. The *Azotobacter vinelandii* gene *algJ* encodes an outer-membrane protein presumably involved in export of alginate. Microbiology **142 (Pt 4):**873-80.

208. **Rehm, B. H., H. Ertesvåg, and S. Valla.** 1996. A new *Azotobacter vinelandii* mannuronan C-5-epimerase gene (*algG*) is part of an alg gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. J Bacteriol **178:**5884-9.

209. **Rehm, B. H. A., and S. Valla.** 1997. Bacterial alginates: biosynthesis and applications. Appl microbiol and biotech **48:**281-288.

210. **Reusch, R. N., and H. L. Sadoff.** 1979. 5-n-Alkylresorcinols from encysting *Azotobacter vinelandii*: isolation and characterization. J Bacteriol. **139:**448-453.

211. **Robles-Price, A., T. Y. Wong, H. Sletta, S. Valla, and N. L. Schiller.** 2004. AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **186:**7369-77.

212. **Rødde, R. S. H., K. Østgaard, and B. A. Larsen.** 1993. Mannuronan C-5 epimerase activity in protoplasts of *Laminaria digitata*. Hydrobiologia **261:**577-581.

213. **Rokstad, A. M., B. Strand, K. Rian, B. Steinkjær, B. Kulseng, G. Skjåk-Bræk, and T. Espevik.** 2003. Evaluation of different types of alginate microcapsules as bioreactors for producing endostatin. Cell Transplantation **12:**351-364.

214. **Rowley, J. A., G. Madlambayan, and D. J. Mooney.** 1999. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials **20:**45-53.

215. **Royo, J. L., E. Moreno-Ruiz, A. Cebolla, and E. Santero.** 2005. Stable long-term indigo production by overexpression of dioxygenase genes using a chromosomal integrated cascade expression circuit. **116:**113-124.

216. **Ruckert, C., A. Puhler, and J. Kalinowski.** 2003. Genome-wide analysis of the L-methionine biosynthetic pathway in Corynebacterium glutamicum by targeted gene deletion and homologous complementation. Journal of Biotechnology **104:**213-228.

217. **Raab, M. R., Tyo, K., Stephanopoulos.** 2005. Metabolic Engineering, p. 1-17. *In* J. Nielsen (ed.), Biotechnology for the Future, vol. 100. Springer.

218. **Sabra, W., A. P. Zeng, and W. D. Deckwer.** 2001. Bacterial alginate: physiology, product quality and process aspects. Appl microbiol and biotech **56:**315-325.

219. **Sabra, W., A. P. Zeng, H. Lunsdorf, and W. D. Deckwer.** 2000. Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase. Apl Environ Microbiol **66:**4037-4044.

220. **Sabra, W., A. P. Zeng, S. Sabry, S. Omar, and W. D. Deckwer.** 1999. Effect of phosphate and oxygen concentrations on alginate production and stoichiometry of metabolism of *Azotobacter vinelandii* under microaerobic conditions. Appl microbiol and biotech **52:**773-780.

221. **Sadoff, H. L.** 1975. Encystment and Germination in *Azotobacter vinelandii*. Bacteriological Reviews **39:**516-539.

222. **Saito, T., Kobayashi, T.** 2002. Intracellular degradation of PHAs, p. 23-39. *In* Doi, Y., Steinbüchel, A. (ed.), Polyesters II, vol. 3b. Wiley-VCH, Weinheim.

223. **Sasakawa, C., and M. Yoshikawa.** 1987. A Series of Tn5 Variants with Various Drug-Resistance Markers and Suicide Vector for Transposon Mutagenesis. Gene **56:**283-288.

224. **Savalgi, V., and V. Savalgi.** 1992. Alginate Production by *Azotobacter vinelandii* in Batch Culture. Journal of General and Applied Microbiology **38:**641-645.

225. **Sawabe, T., Y. Ezura, and T. Kimura.** 1992. Purification and Characterization of an Alginate Lyase from Marine Alteromonas-Sp. Nippon Suisan Gakkaishi **58:**521-527.

226. **Sawabe, T., M. Ohtsuka, and Y. Ezura.** 1997. Novel alginate lyases from marine bacterium Alteromonas sp. strain H-4. Carbohydrate Research **304:**69-76.

227. **Sawabe, T., C. Sawada, E. Suzuki, and Y. Ezura.** 1998. Intracellular alginate-oligosaccharide degrading enzyme activity that is incapable of degrading intact

sodium alginate from a marine bacterium Alteromonas sp. Fisheries Science **64:**320-324.

228. **Schiller, N. L., S. R. Monday, C. M. Boyd, N. T. Keen, and D. E. Ohman.** 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (algL): cloning, sequencing, and expression in Escherichia coli. J Bacteriol **175:**4780-9.
229. **Schleif, R.** 1992. DNA Looping. Annual Review of Biochemistry **61:**199-223.
230. **Schlictman, D., M. Kubo, S. Shankar, and A. M. Chakrabarty.** 1995. Regulation of nucleoside diphosphate kinase and secretable virulence factors in *Pseudomonas aeruginosa*: roles of algR2 and algH. J Bacteriol **177:**2469-74.
231. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J Bacteriol **178:**4997-5004.
232. **Segura, D., T. Cruz, and G. Espín.** 2003. Encystment and alkylresorcinol production by *Azotobacter vinelandii* strains impaired in poly-beta-hydroxybutyrate synthesis. Archives of Microbiology **179:**437-443.
233. **Segura, D., J. Guzmaan, and G. Espín.** 2003. *Azotobacter vinelandii* mutants that overproduce poly-beta-hydroxybutyrate or alginate. Appl microbiol and biotech **63:**159-163.
234. **Selden, C., and H. Hodgson.** 2004. Cellular therapies for liver replacement. Transplant Immunology **12:**273-288.
235. **Shinabarger, D., A. Berry, T. B. May, R. Rothmel, A. Fialho, and A. M. Chakrabarty.** 1991. Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem **266:**2080-8.
236. **Shinabarger, D., T. B. May, A. Boyd, M. Ghosh, and A. M. Chakrabarty.** 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling acetylation of alginate. Mol Microbiol **9:**1027-35.
237. **Simon, R., M. Oconnell, M. Labes, and A. Puhler.** 1986. Plasmid vectors for the genetic-analysis and manipulation of *Rhizobia* and other gram-negative bacteria. Methods in Enzymology **118:**640-659.
238. **Skjåk-Bræk, G., H. Grasdalen, and B. Larsen.** 1986. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydr Res **154:**239-50.

239. **Skjåk-Bræk, G., B. Larsen, and H. Grasdalen.** 1985. The Role of O-Acetyl Groups in the Biosynthesis of Alginate by *Azotobacter vinelandii*. Carbohydrate Research **145:**169-174.

240. **Skjåk-Bræk, G., and T. Espevik.** 1996. Application of alginate gels in biotechnology and biomedicine. Carbohydr Eur **14:**19-25.

241. **Skjåk-Bræk, G., and A. Martinsen.** 1991. Application of some algal polysaccharides in Biotechnology, p. 219-257. *In* M. D. Guiry and G. Blunden (ed.), Seaweed resources in Europe: uses and potential. John Wiley & Sons, NY.

242. **Sletmoen, M., G. Skjåk-Bræk, and B. T. Stokke.** 2004. Single-molecular pair unbinding studies of mannuronan C-5 epimerase AlgE4 and its polymer substrate. Biomacromolecules **5:**1288-1295.

243. **Sletta, H., A. Nedal, T. E. Aune, H. Hellebust, S. Hakvåg, R. Aune, T. E. Ellingsen, S. Valla, and T. Brautaset.** 2004. Broad-host-range plasmid pJB658 can be used for industrial-level production of a secreted host-toxic single-chain antibody fragment in Escherichia coli. Appl Environ Microbiol **70:**7033-9.

244. **Smidsrød, O.** 1975. Molecular-basis for some physical-properties of alginates in gel state. Faraday Discussions **1974:**263-275.

245. **Smidsrød, O.** 1970. Solution properties of alginate. Carbohydrate Research **13:**359-372.

246. **Smidsrød, O., and K. I. Draget.** 1996. Chemistry and physical properties of alginates. Carbyhydr Eur **14:**6-13.

247. **Smidsrød, O., and T. Painter.** 1973. Effect of periodate oxidation upon stiffness of alginate molecule in solution. Carbohydrate Research **26:**125-132.

248. **Smidsrød, O., Whitting.Sg, and A. Haug.** 1972. Molecular basis for some physical properties of polyuronides. Acta Chemica Scandinavica **26:**2563-2566.

249. **Snook, C. F., P. A. Tipton, and L. J. Beamer.** 2003. Crystal structure of GDP-mannose dehydrogenase: a key enzyme of alginate biosynthesis in *Pseudomonas aeruginosa*. Biochemistry **42:**4658-68.

250. **Socolofsky, M. D., and O. Wyss.** 1962. Resistance of *Azotobacter c*yst. J Bacteriol **84:**119-124.

251. **Soon-Shiong, P.** 1999. Treatment of type I diabetes using encapsulated islets. Advanced Drug Delivery Reviews **35:**259-270.

252. **Soon-Shiong, P., R. E. Heintz, N. Roswitha, Q. X. Yao, Z. Yao, T. Zheng, M. Murphy, M. Moloney, M. Schmehl, M. Harris, R. Mendez, R. Mendez, and P. A.**

Sandford. 1994. Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. Lancet **343:**950-951.

253. **Sousa, C., V. de Lorenzo, and A. Cebolla.** 1997. Modulation of gene expression through chromosomal positioning in Escherichia coli. Microbiology-Uk **143:**2071-2078.
254. **Stanford, E. C. C.** 1881. Patent 142. London.
255. **Stephanopoulos, G., and T. W. Simpson.** 1997. Flux amplification in complex metabolic networks. Chemical Engineering Science **52:**2607-2627.
256. **Stevenso.Lh, and Socolofs.Md.** 1966. Cyst formation and poly-beta-hydroxybutyric acid accumulation in *Azotobacter*. J Bacteriol **91:**304-310.
257. **Stevenso.Lh, and Socolofs.Md.** 1972. Encystment of *Azotobacter vinelandii* in Liquid Culture. Antonie Van Leeuwenhoek Journal of Microbiology **38:**605-616.
258. **Stokke, B. T., O. Smidsrød, P. Bruheim, and G. Skjåk-bræk.** 1991. Distribution of uronate residues in alginate chains in relation to alginate gelling properties. Macromolecules **24:**4637-4645.
259. **Svanem, B. I. G., G. Skjåk-Bræk, H. Ertesvåg, and S. Valla.** 1999. Cloning and expression of three new *Azotobacter vinelandii* genes closely related to a previously described gene family encoding mannuronan C-5-epimerases. J Bacteriol **181:**68-77.
260. **Svanem, B. I. G., W. I. Strand, H. Ertesvåg, G. Skjåk-Bræk, M. Hartmann, T. Barbeyron, and S. Valla.** 2001. The catalytic activities of the bifunctional *Azotobacter vinelandii* mannuronan C-5-epimerase and alginate lyase AlgE7 probably originate from the same active site in the enzyme. Journal of Biological Chemistry **276:**31542-31550.
261. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage-T7 RNA-polymerase promoter system for controlled exclusive expression of specific genes. Proceedings of the National Academy of Sciences of the United States of America **82:**1074-1078.
262. **Takeshita, S., T. Oda, and T. Muramatsu.** 1995. Spectroscopic studies on denaturants and guluronate lyase from a marine bacterium. Bioscience Biotechnology and Biochemistry **59:**881-885.
263. **Takeshita, S., N. Sato, M. Igarashi, and T. Muramatsu.** 1993. A highly denaturant-durable alginate lyase from a marine bacterium - Purification and Properties. Bioscience Biotechnology and Biochemistry **57:**1125-1128.

264. **Tatnell, P. J., N. J. Russell, and P. Gacesa.** 1994. GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. Microbiology **140 (Pt 7):**1745-54.

265. **Tcan, Y. T.** 1984. Family II: Azotobacteraceae. p. 219-233. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.

266. **Thorneley, R. N. F., and G. A. Ashby.** 1989. Oxidation of nitrogenase iron protein by dioxygen without inactivation could contribute to high respiration rates of *Azotobacter* species and facilitate nitrogen-fixation in other aerobic environments. Biochemical Journal **261:**181-187.

267. **Thorsen, F., T. A. Read, M. Lund-Johansen, B. B. Tysnes, and R. Bjerkvig.** 2000. Alginate-encapsulated producer cells: A potential new approach for the treatment of malignant brain tumors. Cell Transplantation **9:**773-783.

268. **Tielen, P., M. Strathmann, K. E. Jæger, H. C. Flemming, and J. Wingender.** 2005. Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. Microbiological Research **160:**165-176.

269. **Titok, M. A., J. Chapuis, Y. V. Selezneva, A. V. Lagodich, V. A. Prokulevich, S. D. Ehrlich, and L. Janniere.** 2003. *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector. Plasmid **49:**53-62.

270. **Tokiwa, Y., and B. P. Calabia.** 2004. Degradation of microbial polyesters. Biotechnol Lett **26:**1181-9.

271. **Toussaint, B., I. Delicattree, and P. M. Vignais.** 1993. *Pseudomonas aeruginosa* Contains an Ihf-Like Protein That Binds to the *algD* Promoter. Biochemical and Biophysical Research Communications **196:**416-421.

272. **Valentin, H. E., S. Reiser, and K. J. Gruys.** 2000. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) formation from gamma-aminobutyrate and glutamate. **67:**291-299.

273. **Valla, S., and G. Skjåk-Bræk.** 1996. Alginate - A target molecule for genetic engineers and a versatile material for the biotechnologist. Agro Food Industry Hi-Tech **7:**38-41.

274. **VanDien, S. J., S. Keyhani, C. Yang, and J. D. Keasling.** 1997. Manipulation of independent synthesis and degradation of polyphosphate in Escherichia coli for investigation of phosphate secretion from the cell. Apl Environ Microbiol **63:**1689-1695.

275. **Vazquez, A., S. Moreno, J. Guzman, A. Alvarado, and G. Espìn.** 1999. Transcriptional organization of the *Azotobacter vinelandii algGXLVIFA* genes: characterization of *algF* mutants. Gene **232:**217-22.

276. **Vela, G. R., G. D. Cagle, and P. R. Holmgren.** 1970. Ultrastructure of *Azotobacter vinelandii*. J Bacteriol **104:**933-940.

277. **Vermeulen, H., D. Ubbink, A. Goossens, R. de Vos, and D. Legemate.** 2004. Dressings and topical agents for surgical wounds healing by secondary intention. Cochrane Database Syst Rev**:**CD003554.

278. **Visted, T., and M. Lund-Johansen.** 2003. Progress and challenges for cell encapsulation in brain tumour therapy. Expert Opinion on Biological Therapy **3:**551-561.

279. **Wang, L., R. M. Shelton, P. R. Cooper, M. Lawson, J. T. Triffitt, and J. E. Barralet.** 2003. Evaluation of sodium alginate for bone marrow cell tissue engineering. Biomaterials **24:**3475-3481.

280. **Whitchurch, C. B., R. A. Alm, and J. S. Mattick.** 1996. The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **93:**9839-43.

281. **Winther-Larsen, H. C., J. M. Blatny, B. Valand, T. Brautaset, and S. Valla.** 2000. Pm promoter expression mutants and their use in broad-host-range RK2 plasmid vectors. Metab Eng **2:**92-103.

282. **Wong, T. Y., L. A. Preston, and N. L. Schiller.** 2000. Alginate lyase: Review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. Annual Review of Microbiology **54:**289-340.

283. **Wood, K. V., and M. DeLuca.** 1987. Photographic detection of luminescence in *Escherichia coli* containing the gene for firefly luciferase. Anal Biochem **161:**501-7.

284. **Wozniak, D. J., and D. E. Ohman.** 1993. Involvement of the alginate algT gene and integration host factor in the regulation of the *Pseudomonas aeruginosa* algB gene. J Bacteriol **175:**4145-53.

285. **Wozniak, D. J., and D. E. Ohman.** 1991. *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for algD transcription. J Bacteriol **173:**1406-13.

286. **Wozniak, D. J., and D. E. Ohman.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes algR, algB, and algD reveals a hierarchy of alginate gene expression which is modulated by algT. J Bacteriol **176:**6007-14.

287. **Wyss, O., M. G. Neumann, and Socolofs.Md.** 1961. Development and germination of *Azotobacter* cyst. Journal of Biophysical and Biochemical Cytology **10:**555-565.

288. **Wyss, O., D. D. Smith, L. M. Pope, and K. E. Olson.** 1969. Endogenous Encystment of *Azotobacter vinelandii*. J Bacteriol **100:**475-479.

289. **Yen, K. M., and I. C. Gunsalus.** 1985. Regulation of Naphthalene Catabolic Genes of Plasmid Nah7. J Bacteriol **162:**1008-1013.

290. **Yu, H., M. Mudd, J. C. Boucher, M. J. Schurr, and V. Deretic.** 1997. Identification of the *algZ* gene upstream of the response regulator *algR* and its participation in control of alginate production in *Pseudomonas aeruginosa*. J Bacteriol **179:**187-193.

291. **Zheng, S., Z. X. Xiao, Y. L. Pan, M. Y. Han, and Q. Dong.** 2003. Continuous release of interleukin 12 from microencapsulated engineered cells for colon cancer therapy. World Journal of Gastroenterology **9:**951-955.

292. **Zielinski, N. A., A. M. Chakrabarty, and A. Berry.** 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. J Biol Chem **266:**9754-63.

293. **Aachmann, F. L., B. I. Svanem, P. Guntert, S. B. Petersen, S. Valla, and R. Wimmer.** 2006. NMR structure of the R-module -A parallel beta -roll subunit from an *Azotobacter vinelandii* mannuronan C-5 epimerase. J Biol Chem.

294. **Aarons, S. J., I. W. Sutherland, A. M. Chakrabarty, and M. P. Gallagher.** 1997. A novel gene, algK, from the alginate biosynthesis cluster of *Pseudomonas aeruginosa*. Microbiology **143 (Pt 2):**641-52.