Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Anita Solem

Investigation of Encystment and Characterization of Exopolysaccharides in *Azotobacter* Species

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Helga Ertesvåg
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Preface

This thesis marks the completion of the five year Master's Degree programme in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology in Trondheim. It is a continuation of a specialization project carried out the fall of 2021. The work was carried out at the Department of Biotechnology and Food Science, spring 2022, with guidance from Professor Helga Ertesvåg.

I want to express my gratitude to my supervisor Helga Ertesvåg for giving me the opportunity to explore on my own, and for the invaluable guidance and support when facing challenges. It has been a pleasure to learn from her. I would also like to thank the rest of the lab group and my study mates at the reading room for encouragements and inspiring discussions. I want to give a special thank you to Nikalet for her proofreading efforts. Lastly, i want to thank my family for their continued support during this work.

I am very grateful for the opportunity this work has given me to dive into the world of microbiology and molecular genetics. It has inspired me to continue exploring, and I am committed to continue searching for answers to the mysteries of this fields, as well as the opportunities they hold.

Anita Solem
Trondheim, June 2022

Summary

Species of the genus *Azotobacter* are able to form a dormant cell type known as cysts. Cysts are much more resistant than vegetative cells towards environmental stressors such as desiccation, irradiation and sonication. Understanding the mechanisms underlying encystment may aid in the understanding of the universal mechanisms underlying dormancy. Alginate is essential for encystment and additionally has many areas of use, such as a stabilizing, gelling, and thickening agent in foods, as well as in cell immobilization, drug delivery and wound dressings. While a lot is known about *Azotobacter vinelandii* (*A. vinelandii*), little is known about the alginate as well as other polysaccharides synthesized by *Azotobacter chroococcum* (*A. chroococcum*). This work has focused on mechanisms underlying encystment, and the composition and structure of polysaccharides in *A. vinelandii* and *A. chroococcum*.

To study mechanisms underlying encystment, it was planned to study mutants of *Avin05390*, putatively encoding a polysaccharide biosynthesis protein, *Avin05750*, putatively encoding an alanine dehydrogenase, and *Avin17310* putatively encoding a regulatory protein of the MarR family, and to assess the encystment capabilities of these strains. The mutant strain *A. vinelandii* ATCC 12518Δ05390 was constructed previously, ATCC 12518Δ05750 was constructed in this work, while ATCC 12518Δ17310 could not be because the necessary sequences were unclonable in *E. coli*. When assessing the encystment capabilities of the wild-type strain *A. vinelandi* ATCC 12518 it was found that this strain was unable to, or only rarely could form desiccation resistant cysts. Compared to two other wild-type strains of *A. vinelandii*, strain ATCC 12837 and strain E, it was clearly inferior. It was therefore not possible to assess the importance of *Avin05390* and *Avin05750* for the encystment process.

During encystment, the cells of *A. vinelandii* introduces alkylresorcinols into the cell membrane and the outer layer of the cyst coat. The knockout of either of the three genes *arsF*, *arsG*, or *arsH* resulted in cells where these lipids could no longer be detected on the cell surface. In this work it was shown that the strains carrying a mutation of either of the three genes still synthesized alkylresorcinols. Complementation of ATCC 12518 ΔarsH with the wild type *arsH* gene restored transport of alkylresorcinols to the cell surface. This confirms that *arsH* is involved in the transport of alkylresorcinols to the cell surface, and strongly suggests that *arsF* and *arsG* are also involved in this transport.

The resistance of cysts to harmful conditions is to a large extent warranted by the cyst coat forming around the cells. Since alginate and other polysaccharides are important constituents of the cyst coat, it was of interest to characterise the monomer composition of the carbohydrates of the "intine" and "exine", two separate fractions of the cyst coat. This was, however, not possible as the isolated intine and exine fractions were found to be insoluble in both water and DMSO.

Exopolysaccharides of *A. chroococcum* were partly characterized by ^{1}H-NMR, HSQC, and HPAEC-PAD. It was found that the exopolysaccharides of *A. chroococcum* probably constitutes one high molecular weight M-block alginate and one polymer consisting of rhamnose, galactose, arabinose, glucose, mannose, and three unidentified monosaccharides. AcAlgE1 is a mannuronan C-5 epimerase, and hypothesized to affect the compo-

sition of the alginate, as well as the capability to form cysts. To study the function and importance of AcAlgE1 *in vivo*, it was planned to knockout this gene. The plasmid containing a mutated *AcalgE1* could not be transferred to *A. chroococcum*, and the specific function of AcAlgE1 *in vivo* could therefore not be studied. However, the low proportion of G (6%), and no detection of consecutive G-residues in the exocellular alginate of *A. chroococcum* suggests that AcAlgE1 is inactive during vegetative growth.

Sammendrag

Bakterier tilhørende slekten *Azotobacter* kan danne en hvilene celletype, kalt cyster. Cyster er mer motstandsdyktige enn vegetative celler mot skadelige effekter som tørking, stråling og sonikering. En forståelse av hvilke mekanismer som ligger til grunn for cystedannelsen kan øke forståelsen av de universelle mekanismene som ligger til grunn for dannelsen av hvileceller. Alginat er essensielt for dannelse av cyster, og har i tillegg flere bruksområder. Dette inkluderer stabilisering, gelering og fortykking av matvarer, i tillegg til celleimmobilisering, medisiner og sårbandasjer. Mye er kjent om *Azotobacter vinelandii* (*A. vinelandii*), mens relativt lite er kjent om alginat og andre polysakkarider syntetisert av *Azotobacter chroococcum* (*A. chroococcum*). Dette arbeidet har fokusert på mekanismer som ligger til grunn for cystedanning hos *A. vinelandii* og *A. chroococcum*, og på komposisjonen og strukturen til polysakkarider syntetisert av *A. chroococcum*.

For å undersøke mekanismene som ligger til grunn for cystedanning var det planlagt å konstruere stammer med mutasjoner i gener, som er antatt viktige for cystedannelse, og å analysere evnen til cystedannelse hos disse stammene. De aktuelle genene var *Avin05390*, som er antatt å kode for et polysakkarid biosynteseprotein, *Avin05750*, som er antatt å kode for en alanin dehydrogenase, og *Avin17310*, som er antatt å kode for et regulatorprotein tilhørende MarR-familien. Stammen *A. vinelandii* ATCC 12518Δ05390 har blitt konstruert tidligere, ATCC 12518Δ05750 ble konstruert i dette arbeidet, mens ATCC 12518Δ17310 ikke kunne konstrueres fordi de nødvendige sekvensene ikke lot seg klone i *E. coli*. Ved undersøkelse av kapasiteten til å danne cyster hos villtypestammen *A. vinelandii* ATCC 12518, ble det funnet at denne stammen ikke dannet tørke-resistente cyster. Når denne stammen ble sammenlignet med to andre villtypestammer av *A. vinelandii*, stamme ATCC 12837 og stamme E, overlevde klart færre celler tørking. Siden villtypen ikke dannet, eller bare svært sjeldent dannet tørke-resistente cyster, kunne ikke viktigheten av genene *Avin05390* og *Avin05750* for dannelsen av slike cyster avgjøres.

Under cystedannelse innkorporeres alkylresorcinoler i cellemembranen og i det ytterste laget av cystekapselen. Inaktivering av hvert enkelt av genene *arsF*, *arsG* og *arsH* resulterer i celler, hvor disse lipidene ikke lenger kan detekteres på overflaten av cellene. I dette arbeidet ble det vist at disse cellene likevel syntetiserer alkylresocinoler. Komplementering av ATCC 12518ΔarsH med villtypen av *arsH* gjenopprettet transporten av alkylresorcinoler til celleoverflaten. Dette bekrefter at *arsH* er involvert i transport av alkylresorcinoler til celleoverflaten og antyder sterkt at også *arsF* og *arsG* er involvert i denne transporten.

Cystenes resistens mot destruktive forhold kan i stor grad forklares av cystekapselen som dannes rundt cellene. Alginat og andre polysakkarider er viktige konstituenter, og det var derfor av interesse å karakterisere monomerkomposisjonen av karbohydratene i cystekapselens to lag, "intine" og "exine". Dette viste seg å bli vanskelig å gjennomføre fordi de isolerte fraksjonene av intine og exine var uløselige både i vann og i DMSO.

Exopolysakkarider syntetisert av *A. chroococcum* ble analysert ved bruk av ^{1}H-NMR, HSQC og HPAEC-PAD. Det ble funnet at *A. chroococcum* mest sannsynlig syntetiserer et M-blokk alginat av høy molekylvekt og en polymer bestående av rhamnose, galaktose, arabinose, glukose, mannose og tre uidentifiserte monosakkarider. AcAlgE1 er en man-

nuronan C-5 epimerase og vil derfor kunne påvirke alginatets sammensetning, i tillegg til evnen til å danne cyster. For å studere funksjonen og viktigheten av AcAlgE1 *in vivo*, var det planlagt å inaktivere dette genet. Plasmidet som skulle brukes til dette kunne ikke overføres til *A. chroococcum*, og den spesifikke funksjonen til AcAlgE1 *in vivo* kunne derfor ikke bestemmes. På grunn av den lave andelen av G (6 %), og ingen funn av G-blokker i alginatet, var det likevell mulig å si med stor sannsynlighet, at AcAlgE1 er inaktiv ved vegetativ vekst.

Contents

Appendices

Chapter 1

Introduction

The species within the bacterial genus *Azotobacter* are of great interest in medical, industrial as well as agricultural fields. This is due to their unique characteristics, which includes production of alginate, the formation of dormant cysts and aerobic nitrogen-fixation. Their characteristics makes the azotobacters a useful tool when seeking to understand biochemical processes, as well as potentially serving as producers of specific biochemical compounds. Understanding the processes underlying their characteristics is therefore of great value.

1.1 Characteristics of *A. vinelandii* and *A. chroococcum*

Azotobacter species are gram-negative and encapsulated bacteria that are able to fix nitrogen. They are strictly aerobic and exists in soils worldwide [1]. Their ability to fix nitrogen, that is to reduce N_2 to NH_3, under aerobic conditions, makes them useful as biofertilizers, providing crops with fixed nitrogen [2]. In addition to their nitrogen-fixing abilities, most *Azotobacter* species produce diffusable exopolysaccharides such as alginates, which has numerous medical and industrial areas of use [3]. Another feature that all species within the *Azotobacter* genus displays, is the ability to differentiate into a dormant cell type, called cysts, when growth conditions decline, or upon chemical induction [1]. Under such conditions, they also synthesize poly-β-hydroxybutyrate, which has potential uses as a degradable bioplastic [4].

Because of their remarkable traits, the Azotobacters, with *Azotobacter vinelandii* and *Azotobacter chroococcum* in the forefront, has been extensively studied. This has lead to numerous important discoveries, including the mechanisms of aerobic nitrogen fixation, the synthesis of alginate and the process of encystment and germination [5, 6, 7]. The conversion of N_2 to ammonium is carried out by oxygen-sensitive nitrogenase enzymes [5]. Several mechanisms has been proposed to function as a protection of the nitrogenase enzymes from oxidative damage in *Azotobacter* cells. Both *A. vinelandii* and *A. chroococcum* is found to increase the respiration rate under hyperbaric oxygen conditions [8, 9]. This keeps the intracellular concentration of oxygen at a minimum even at high oxygen

concentrations and limits the exposure of the nitrogenases and other oxygen-sensitive enzymes [9]. It has also been suggested that the production of alginate plays a central role in protection of oxygen sensitive enzymes by hampering oxygen entrance into the cell [10].

Both *Azotobacter vinelandii* and *Azotobacter chroococcum* are easily cultivable and can utilize a range of compounds as carbon sources, including hexose sugars, sugar alcohols, alcohols, and organic acids. While they fix nitrogen, growth rates are enhanced when other nitrogen sources are provided [11]. *A. vinelandii* and *A. chroococcum* are difficult to distinguish by the naked eye or in the microscope. However, when grown in nitrogen-free medium with high aeration rates, *A. chroococcum* forms melanin, which is visible in the culture as a brown to black, insoluble pigment [12]. Genetically, the two species also differ. Although their genomes are comparable in size and show high levels of synteny, there are significant differences in both gene content and organization of the genomes. *A. vinelandii* carries a single chromosome, while *A. chroococcum* also carries six megaplasmids [13]. Both species exhibits polyploidy, and exponential-phase cells may contain over 40 copies of the chromosome [14, 15]. This poses a challenge for genetic modification as obtaining pure mutants may be difficult. The genome of *A. chroococcum* does also encode restriction modification systems [13], a feature that adds to the challenge of genetic modification. Such restriction modification systems has not yet been identified in *A. vinelandii* [13]. In addition to this, the production of alginate, both capsular and exocellular, may complicate the introduction of DNA, and has been shown to decrease transformation efficiencies in *A. vinelandii* [16].

1.2 *Azotobacter* cysts

The differentiation of vegetative cells into cysts in *Azotobacter* species follows nutrient exhaustion, and leaves the cell in a dormant and protected state [7, 17]. It appears to be a natural part of the life cycle of *Azotobacter* cells [7] . *Azotobacter* cysts exhibit enhanced tolerance towards environmental stressors such as desiccation, irradiation and sonication [18]. It has been demonstrated that these features are mostly attributed to the multilayered capsule forming around the cell during encystment (Figure 1.1)[19]. The capsule comprise two distinct layers: the intine and the exine. The intine presents as a single layer of disorganised material, mainly consisting of carbohydrates and lipids. The exine appears as a multi-layered structure and is composed of approximately equal amounts of carbohydrates, lipids and proteins [20]. Alginate (section 1.4) is a major component of both the exine and the intine [21], and is in fact essential for the formation of desiccation resistant cysts [22]. The central body of the cyst, encapsulated by the intine and exine, resembles the vegetative cell in that it has a cell wall and a plasma membrane [19]. They are however differentiated from the vegetative cell, by containing large granules of poly-β-hydroxybutyrate (PHB). An increase in the PHB content of the cell during early stages of encystment, followed by a decrease in the PHB content suggests that this polymer provides the cell with carbon and energy during the encystment process [23]. A great part of the acculmulated PHB is actually incorporated into the plasmamembrane and the exine in the form of alkylresorcinols during encystment [24, 25].

A **B**

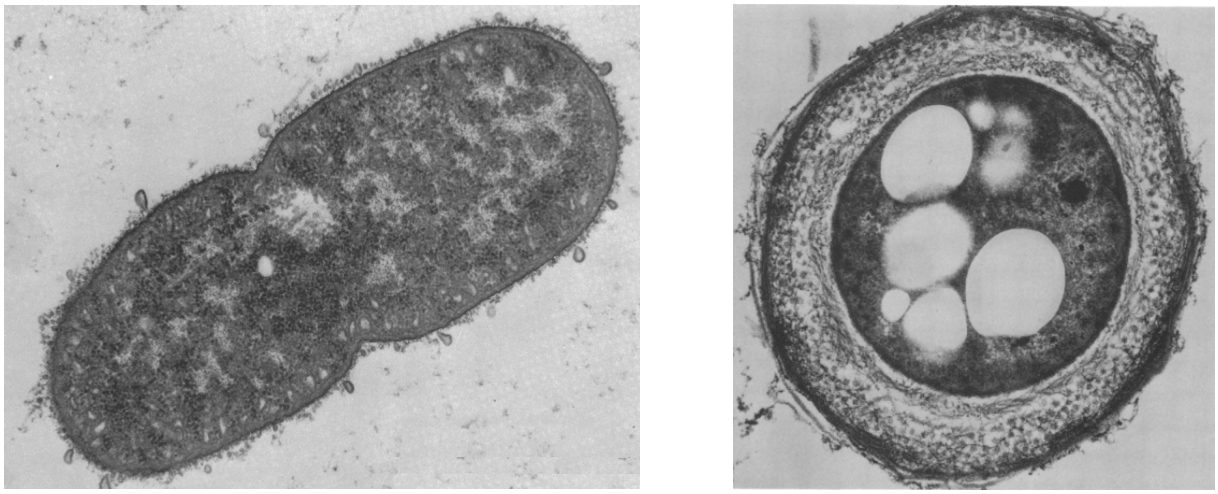


Figure 1.1: (A) Vegetative cell of *A. vinelandii* (copied from Wyss et al. [26]). (B) Cyst of *A. vinelandii*. Exine is the outermost dark, multilayered structure. The intine is located between the exine and the central body. The white PHB granules are readily visible in the central body. (copied from Lin et al. [27])

The encystment process may be induced by adding n-butanol, or it's metabolites crotonate or β-hydroxybutyrate (BHB) as the only carbon source in the growth medium. It is important that glucose is properly removed from the cells before induction as reminant glucose will result in abortive encystment, in which the exine appears disorganised and a viscous matter is released. Additionally, the encystment is significantly increased in nitrogen-free media, compared to media supplied with a nitrogen source [28]. Under the described conditions, close to 100% encysted cells can be observed within 120 hours after induction [28, 29]. The encystment is a sequential process, beginning with the cell losing its mobility and condense into a smaller and more round-shaped form. A thick layer of a disorganised material is formed around the cell as it undergoes a last division, resulting in two pre-cysts [7]. While nitrogen-fixation is halted during the early stages of encystment, PHB is accumulating in the cell continously from induction and becomes visible in the pre-cyst [30]. After division, vesicles originating from the cell wall wanders into the material around the cell and fuse to gradually form the multilayered structure of the exine (Figure 1.1). The central body and the exine is compacted concurrently with a decrease in the PHB content and an enlargement of the intine [20, 30]. The compaction of the central body and the exine is the last stage of the differentiation of a vegetative cell into a mature cyst.

The cyst germinates into vegetative cells when conditions yet again supports growth. In the laboratory, this corresponds to incubating the cysts in a medium containing an exogenous carbon source such as glucose, sucrose or acetate [31]. Cysts germinates even after over 10 years in dry soil, showing that encystment is an effective survival strategy of *Azotobacter* species [32]. Although the strategy of encystmeny-and germination seems to be of great importance for these bacteria, the knowledge on the biochemical processes and the genes governing these process are limited. It is however known that genes affecting alginate synthesis and structure also affects the desiccation resistance of cysts [22,

33, 34], which is of no surprise as alginate is essential for the formation of desiccation resistant cysts. It is more surprising that PHB accumulation and alkylresorcinol synthesis is found to be non-essential for cyst formation [35, 36]. Up until now, a few mechanisms have been shown to be involved in the regulation of encystment. This includes expression of the alternative sigma factor RpoS, and the GacS/GacA system [37, 38]. Recently, a transcriptome of *A. vinelandii* during encystment was constructed (Ertesvåg, unpublished). Among the genes upregulated during encystment are *Avin05390*, *Avin05750*, and *Avin17310*. Based on the sequence obtained by Setubal et al. with accession number CP001157 in GenBank, *Avin05390* presumably encodes an exopolysaccharide biosynthesis protein, *Avin05750* presumably encodes an alanine dehydrogenase, and *Avin17310* presumably encodes a regulatory protein of the MarR family [39]. Gaining knowledge on the function and importance of these genes and other mechanisms underlying the encystment and germination processes may aid in the understanding of the universal mechanisms governing dormant and resistant cells.

1.3 Alkylresorcinols during encystment

During encystment, the relative lipid content of the cells increases to almost the double of what is found in exponentially growing cells. Mature cysts also contain a larger variety of fatty acids [20]. The increase in lipid content is mainly due to synthesis of novel 5-*n*-alkylresorcinols and 6-*n*-alkylpyrones (Figure 1.2), which are not found in vegetative cells [40]. The main phenolic lipids of the *A. vinelandii* cyst are 5-*n*-heneicosylresorcinol, 5-*n*-tricosylresorcinol, and the galactose derivates of these, followed by 6-heneicosyl-4-hydroxy-2- pyrone and 6-tricosyl-4-hydroxy-2- pyrone. These compounds consists of a polar aromatic ring and a hydrophobic alkyl chain, making them amphiphatic. Amphiphatic lipid compounds are main components of plasma membranes, and a great part of the alkylresorcinols and alkylpyrones synthesized during encystment are shown to be incorporated into the plasma membrane of the encysting cells, at the expense of phospholipids [25]. They also constitute an appreciable amount of the exine [24]. Segura et al. showed that mutants unable to synthesize alkylresorcinols formed cysts with disorganized exines. These mutants still resisted dessication, leading to the hypothesis that alkylresorcinols have a structural role in the cysts [35].

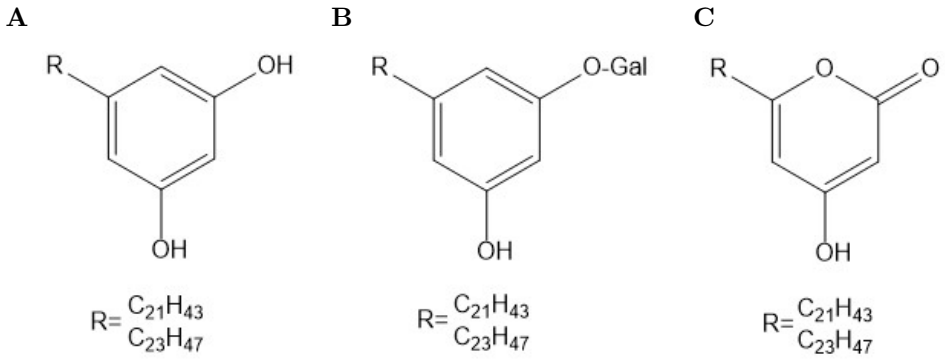


Figure 1.2: The most abundant alkylresorcinols and alkylpyrones in *A. vinelandii* cysts [40] (A) 5-*n*-alkylresorcinol, (B) 5-*n*-alkylresorcinol galactoside, (C) 6-*n*-alkylpyrone.

The synthesis of alkylresorcinols and alkylpyrones in *Azotobacter* cells, is governed by the genes in the *arsABCD* operon. Together, the four enzymes ArsA, ArsB, ArsC and ArsD synthesizes alkylresorcinols and alkylpyrones from malonyl-CoA [41]. ArsA and ArsD are fatty acid synthases, which cooperatively synthesizes C_{22}-C_{26} fatty acids from malonyl-CoA. The fatty acids, together with malonyl-CoA, serves as starting substrates for ArsB and arsC, which synthesizes alkylresorcinols and alkylpyrones, respectively [42, 41]. The introduction of these phenolic lipids into the plasmamembrane and the exine of the encysting cells of *Azotobacter* is still to be disclosed, but it is likely that the genes *arsF*, *arsG*, and *arsH*, located immediately downstream of *arsABCD* (Figure 1.3) is involved in this task. These genes are upregulated during encystment (Ertesvåg, unpublished), and are annotated as ABC transporter ATP-binding protein, FtsX-like permease family protein and outer membrane lipoprotein-sorting protein, respectively. Dalland [43] performed separate knockouts of *arsF*, *arsG*, and *arsH*, resulting in three mutant strains that were unable to incorporate alkylresorcinols in their exines during encystment. This strengthens the hypothesis of the genes being involved in transport of alkylresorcinols to the cell surface.

Figure 1.3: Organization of the alkylresorcinol synthesis operon (arsABCD) and the putative alkylresorcinol transport operon (arsFGH).

1.4 Alginate

The group of polysaccharides known as alginates are naturally occuring biopolymers found i brown and red algae [44, 45], and bacteria of the *Pseudomonas* and *Azotobacter* genera [46, 47]. Alginates have several industrial and medical applications. They are frequently used as stabilizing, gelling or thickening agents in food production [48], as well as in textile dyes [49]. Because of their compatibility, their use in the medical field includes cell immobilization, drug delivery and wound dressings [50]. Studies also reveal great potentials for the use of alginate in several other areas, including tissue regeneration [51, 52], treatment of obesity [53] and wastewater treatment [54].

Alginates are linear, anionic copolymers consisting of (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The monomers are arranged in blocks, with distinct structural organizations and properties [55]. Three block structures are found: G-blocks with consecutive G residues, M-blocks with consecutive M residues, and MG-blocks with alternating M and G residues (Figure 1.4)[56]. Bacterial alginates may also be O-acetylated at C-2 and C-3 of the M residues [57].

The properties of alginates predominantly depends on their monomer composition and sequence, as well as their molecular weight. In addition to these structural elements, the pH, ionic strength, and the presence of divalent ions also affects the properties [55]. While alginic acid is insoluble in water, alginate readily dissolves to form viscous solutions. The

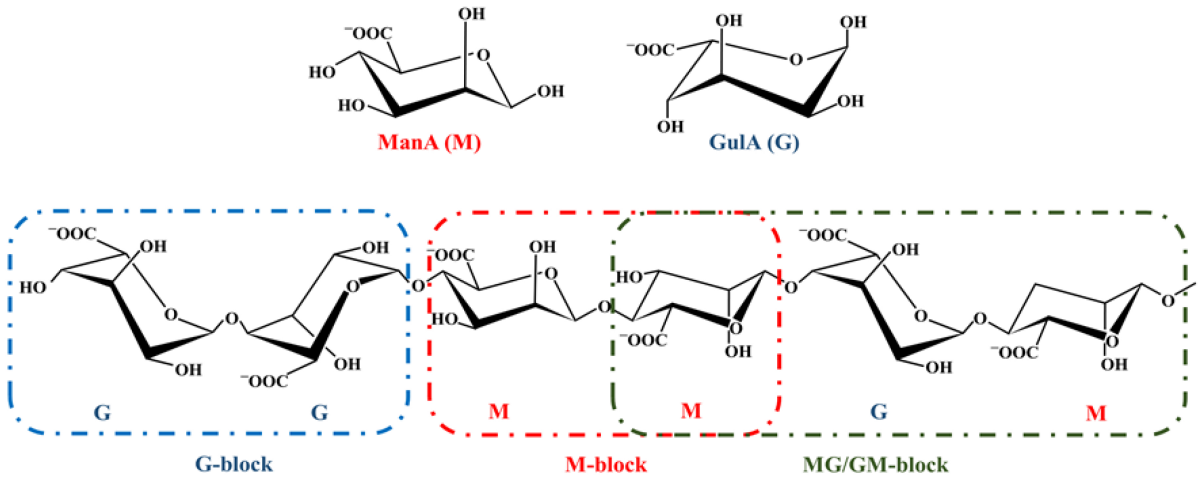


Figure 1.4: The monomer components of alginate, mannuronic acid (M) and guluronic acid (G) and the three block structures of alginate: G-block, M-block, and MG/GM-block. Copied from Martau et al [58].

pH of the solution must therefore be kept above the pKa of alginate. This value depends on the molecular weight, the monomer composition, and the ionic strength of the solution [55]. However, all alginates are insoluble at pH below the pKa of the uronic acid monomers, which is 3.38 and 3.65 in 0.1 M NaCl, for mannuronic acid and guluronic acid respectively [59]. The intrinsic viscosity of alginate in solution increases with increasing molecular weight due to a larger hydrodynamic volume. Additionaly, the viscosity increases with decreasing strength and decreasing pH [60].

When the pH of an alginate solution is decreased below the pKa value of the uronic acids, the solution may solidify and form an acid gel [55]. It is suggested that such gels are stabilized by intra- and intermolecular hydrogen bonds [61]. Higher strength gels are formed by interactions between alginate and divalent cations, such as Ca^{2+} [62]. These ionic gels are thought to be formed when divalent cations bind to G-blocks and form junction zones between G-blocks in adjacent alginate molecules [63, 64]. Both acid gels and ionic gels can be formed at low temperatures, as opposed to gels formed by many other polymers [55]. Because of the stabilizing meachanisms, the properties of both acid gels and ionic gels are highly dependent on the block structures of the alginate. High proportions of long G-blocks are correlated with increased gel strength [65, 66]. Higher proportions of M-residues are correlated with more elastic gels [55]. Acetylated alginates, produced by bacteria, exhibits higher water-holding capacity [67]. In addition to gel strength and elasticity, the water-holding capacity of alginate gels is of great importance for many applications.

The number of applications of alginate is increasing. Moreover, many applications require alginates with specific compositions, and properties. G-block containing alginates are of high interest as they are necessary for many applications. Such requirements are difficult to meet with today's production of alginate, which is mainly based on extraction from brown algae. The composition and monomer sequence may vary substantially within single species of brown algae [68, 44]. This renders a mixture of alginates with different properties upon harvesting. Alginate producing bacteria may facilitate pro-

duction of alginates with more defined and consistent compositions. Moreover, genetic engineering allows for easy modification of alginate-producing bacteria. This may enable the production of custom-made alginates, specifically suited for its uses. In addition to production of such alginates *in vivo*, bacterial enzymes may also provide a means of *in vitro* modification of alginates from both algal and bacterial origin [69, 70].

1.5 Alginate synthesis and function in *Azotobacter*

Alginate synthesized by bacteria serves functional roles in the bacterial cell, and the structure and properties reflects this role. *Pseudomonas* produces alginate with high proportions of acetylated M residues, and only single G-residues, e.g. no G-blocks [55]. This gives a polymer with high water-holding capacity, excellent for forming biofilms [6]. On the other hand, *Azotobacter* is able to synthesize alginates of various composition and block-structures. During vegetative growth, *A. vinelandii* synthesizes alginate with relatively low G content, both for inclusion in the cell capsule and for excretion. The outer coat of the cyst, however, contains a larger proportion of G-blocks [21]. Considering the rigid structure of G-blocks, this may provide the strength and robustness needed for the cyst to survive adverse conditions. While the alginates of *A. vinelandii* has been extensively studied, few studies on the alginate of the other species of *Azotobacter* has been carried out. Cote and Krull found that the exocellular alginate of *A. chroococcum* B8 mostly consisted of mannuronic acid [71], but no studies quantifying the M and G contents of its cyst capsule has been found.

Independent of its final structure and function in the cell, bacterial alginates are first synthesized as mannuronan by polymerization of GDP-mannuronic acid (Figure 1.5) [6]. This reaction is catalyzed by two of enzymez that is a part of a large polymerase complex. This complex is located in the cytoplasmic membrane of the cell, and does also comprise proteins and enzymes that translocate the mannuronan chain across the membrane, as well as adding acetyl groups [6]. During translocation, the periplasmic enzyme AlgG introduces single G-residues by the epimerization of M-residues [72, 33]. The epimerization of M-residues by AlgG is however diminishingly low in *A. vinelandii*. The composition and sequence of monomers in alginates from *A. vinelandii* is controlled by a group of secreted mannuronan C-5 epimerase enzymes [33]. *A. vinelandii* encodes seven such enzymes, namely AlgE1-7 [73]. These enzymes introduces G-residues in different patterns, and it is proposed that they together can optimize the alginate in response to the growth conditions and the specific needs of the cell [73, 33].

In addition to the epimerase function, AlgE7 also presents with alginate lyase activity, cleaving the glycosidic linkages of alginate molecules [74]. The precise effect of it's lyase activity *in vivo* is not known, but it is suggested to be involved in the release of alginate from the cell [75]. Further three alginate lyases are synthesized by *A. vinelandii*: AlyA1, AlyA2 and AlyA3. Of these, AlyA3 has proved to be essential for the cysts ability to germinate [75]. Finally, the periplasmic lyase AlgL is thought to degrade alginate that erroneously has ended up in the periplasm, preventing an increase of the osmotic pressure [57].

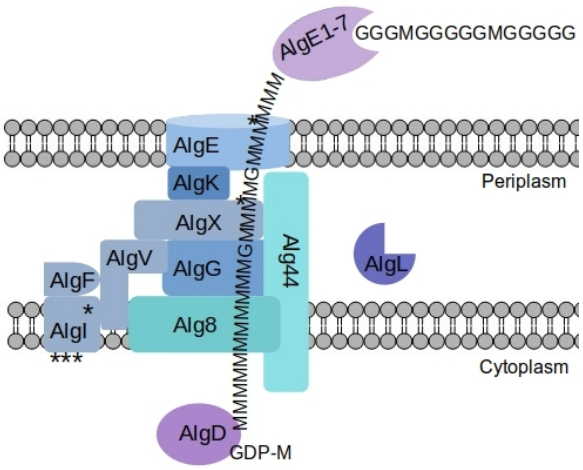


Figure 1.5: Alginate synthesis-, export-, and modification machinery in *Azotobacter*. *:acetyl group. Modified from Hay et al. [6].

As all known alginate producing organisms, *A. chroococcum* also encodes the AlgL lyase [57]. Other parts of the alginate synthesis and modifications in this species has been relatively little studied until Robson et al. released the genome of *A. chroococcum* NCIMB 8003 in 2015 [13]. Not surprising, they found that *A. chroococcum* encodes a 12 gene operon homologous to the alginate biosynthesis operon in *A. vinelandii*. A more interesting observation is that *A. chroococcum* only encodes three enzymes with homology to the *A. vinelandii* epimerases [57]. This is in contrast to the 7 mannuronan C-5 epimerases expressed by *A. vinelandii*. Two of the genes showed high similarity to *algE7*, encoding a bifunctional enzyme in *A. vinelandii*, while the third (*AcAlgE1*) showed high similarity to *algE2*, encoding a unifunctional mannuronan C-5 epimerase. The epimerase activity of AcAlgE1, as well as the combined epimerase/lyase activity of AcAlgE2 and AcAlgE3 were later confirmed [76]. AcAlgE1 was also found to be very effective in introducing G-residues and forming long G-blocks *in vitro*.

1.6 Bacterial Plasmids

Plasmids are extrachromosomal genetic elements existing in prokaryotes, and a few eukaryotes [77]. They are most often circular, but linear plasmids do also exist [78]. The plasmids replicate independently of the chromosome, and carry genetic elements that under most circumstances are inessential for the survival of the host cell. Often, the plasmids give the host genetic advantages in certain environmental conditions, such as providing antibiotic resistance genes that will enable the host to survive when grown in the presence of the relevant antibiotic. A cell can carry several plasmids of distinct origins. At the same time a specific plasmid can exist in numerous copies in the same cell [79], a property of the plasmid known as "copy number". The copy number of a plasmid is maintained by control systems encoded within the plasmid. These systems controls initiation of replication, and only allows replication when the number of plasmids deviates from the characteristic copy number of the plasmid [80].

Carrying a plasmid impose a metabolic burden on the cell, and if the cost of maintaining the plasmid is greater than the benefit, the plasmid might be cured from the cell. The stability of a plasmid depends on several factors including plasmid size, copy number, the effect on growth rate, mode of replication, and the presence of toxin/antitoxin systems [81, 82]. Plasmids may be lost from cells, but can also enter cells by horizontal gene transfer. This may in some cases counteract segregational losses. Horizontal gene transfer is a parasexual mechanism allowing for genetic exchange in bacteria. Three such processes exists: transformation, conjugation and transduction. Transformation involves the uptake of naked DNA from the environment, while conjugation involves transfer of DNA from a host cell to a recipient through a physical cell-to-cell connection. Transduction involves the transfer of DNA by the help of a phage [77].

1.6.1 Vectors

The ability of plasmids to carry specific genes and to be transferred into cells by transformation, conjugation, or transduction makes them excellent vectors for carrying and transporting DNA. Cloning vectors are DNA molecules that can carry foreign DNA and introduce this into cells. For a plasmid to function as a cloning vector, three elements must be present: an origin of replication, a selectable marker, and a minmum of one restriction endonuclease recognition site [77]. Vectors may also carry additional elements, enabling conjugation, expression of genes, or transposition. Today, plasmids with specific elements, and hence specific properties are easily constructed in the lab.

It is essential that the vector can be introduced and replicated in the host of interest. Since *E. coli* is often preferred as a host for general cloning purposes, the vector must be able to replicate in *E. coli*, as well as the bacterial strain one wants to introduce the plasmid into. Several such broad-host-range plasmids exists, and are based on a few replicons able to transfer between and replicate in several species. One important example is plasmids based on the RK2 replicon, which can be stably maintained in many gram-negative bacteria, including *E. coli* [83]. These plasmids belongs to the incompatibility group IncPα. Plasmids of the same incompatibility group share similar replication and partitioning systems, and are therefore unable to be stably maintained over many generations [84].

Suicide- and conditional suicide vectors are two types of plasmids that are especially useful when performing manipulations of the chromosome. Suicide vectors are of various reasons, including the lack of proteins necessary for replication, unable to replicate in the strain of interest. This makes them useful as vectors for transposon mutagenesis. A transposon is a genetic element that can move from one genetic location to another, and hence can be inserted at random in the chromosome [77]. As the suicide vector does not replicate in the host, repeated transposition events are effectively blocked. An example of suicide vectors are those based on the R6K replicon, which requires the π-protein for replication and may only be replicated in strains encoding this protein [85]. Conditional suicide vectors (Figure 1.6) are able to replicate in the strain of interest under certain conditions. Plasmids carrying the XylS/Pm expression system in relation to the plasmid replication system are conditional suicide vectors that requires the addition of benzoic-acid derivates for replication [86]. The use of these plasmid in site-directed mutagenesis

provides an easy method for the isolation of cells with allelic exchanges, carried out by homologous recombination between the chromosome and the plasmid [87].

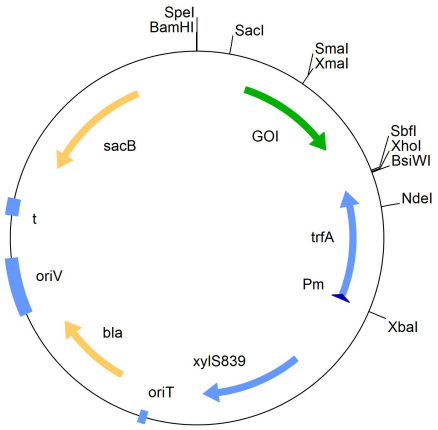


Figure 1.6: Example of a conditional suicide vector carrying a gene of interest (GOI). The XylS/Pm promoter system is coupled to the replication system of the plasmid and therefore controls it's replication. Selection markers are marked in yellow. OriT is origin of transfer, necessary for conjugal transfer, and oriV is origin of vegetative replication, necessary for the replication of the plasmid. t is a bidirectional transcription terminator. Restriction sites are marked with the names of the respective restriction endonucleases.

The XylS/Pm system was isolated from *Pseudomonas putida* and consists of the promotor *Pm*, which is an inducible promoter. It is induced by the protein XylS, which is constitutvely expressed at low levels. The expression of XylS can, however, be induced by several different benzoic-acid derivates including m-toluate [86]. The RK2 replicon requires the protein TrfA for replication, and placing the *trfA* gene under the control of the *Pm* promoter confer a conditional suicide plasmid, depending on the presence of a benzoic acid derivate for replication. The XylS/Pm system can also be used for controlled gene expression. As long as the gene is under the control of the *Pm* promoter, its expression will be controlled by XylS, and hence by the presence of an *XylS* inducer such as m-toluate.

Chapter 2

Aim of the study

Encystment and alginate synthesis are both characteristics of *Azotobacter* cells. These processes works as a protection of the cell and cellular machinery in different conditions. Since the encysted cells are dormant, gaining knowledge on this process not only increases the understanding of encysting cells, but may also aid in understanding the universal processes governing dormancy. In the medical field this is of great interest as dormant cells may survive medical treatments and sterilizations [88]. Being essential for the encystment, alginate is itself a target of study in this context. Additionally, the interest of alginate in the medical field and the industry adds to this. In relation to encystment and alginate production, this project aimed to investigate four areas.

The expression of the genes *Avin05390*, *Avin05750*, and *Avin17310*, is upregulated during encystment (Ertesvåg, unpublished). These genes presumably encode an exopolysaccharide biosynthesis protein, an alanine dehydrogenase, and a regulator of the MarR family, respectively. Since the genes are upregulated during encystment, they have previously been suggested to be important for the formation of desiccation resistant cysts in *A. vinelandii* [89, 90]. To test this hypothesis, separate mutants with a disruption in each of these genes had to be tested for encystment and the desiccation resistance of the cysts. Before testing the desiccation resistance, the mutant strains had to be constructed. For *Avin05390*, the mutant was constructed in a previous work [89].

The synthesis of alkylresorcinols is upregulated during encystment, and these lipids are incorporated into the plasmamembrane and the exine. Dalland showed that disruption of either of the three genes *arsF*, *arsG*, or *arsH*, which are thought to be responsible for the transport of alkylresorcinols out of the cell, leads to phenotypes without detectable alkylresorcinols on the cell surface [43]. To confirm that the changes in phenotype was a result of the mutation of these genes, it is necessary to complement the mutated strain with the corresponding wild-type genes. Additionally, it was a question if these genes are only essential for the transport of alkylresorcinols out of the cell, or if they also affect the synthesis.

What is known about cysts and encystment is mostly learned from studies done on *A. vinelandii*. As *A. chroococcum* differs from *A. vinelandii* on certain areas, such as the composition of their exopolysaccharides, it is tempting to believe that the composition

of the cyst coat may differ as well. Carbohydrates, being an important structural components, was of main interest in this question. The composition of the carbohydrates in the intine and exine of *A. chroococcum* was therefore a target of this work. Additionally, it was intended to analyze the expolysaccharides of *A. chroococcum* and compare the composition and structure to the exopolysaccharides of *A. vinelandii*.

Lastly, the recent discovery by Gawin et al., that *A. chroococcum* only encodes a single secreted unifunctional C5-epimerase (AcAlgE1), as opposed to *A. vinelandi*, which encodes six unifunctional C5-epimerases [86], prompted the investigation of the function and importance of this enzyme *in vivo*. A characterization of the excreted alginate of the wild type could give some clues. In addition to the effects it might have on the composition of the exocellular alginate, it may also have essential functions in the formation of desiccation resistant cysts. Construction of a mutant strain with inactivated *AcAlgE1*, and subsequent characterization of excreted alginate, as well as the ability to form desiccation resistant could answer these questions.

In summary, the aim of this study was to answer the following questions:

- Do *Avin05390*, *Avin05750*, and *Avin17310* affect the encystment, and the desiccation resistance of cysts in *A. vinelandii*?
- Are the genes *arsF*, *arsG*, and *arsH* essential for the transport of alkylresorcinols out of the cell during encystment in *A. vinelandii*, and do they affect the synthesis of alkylresorcinols?
- What is the composition of the carbohydrates of the intine and exine of *A. chroococcum* cysts?
- Which polysaccharides do *A. chroococcum* excrete, and how does this compare to *A. vinelandii*?
- How does the mannuronan C5-epimerase AcAlgE1 affect alginate composition and the encystment process in *A. chroococcum*?

Chapter 3

Materials and Methods

The materials and methods used in this work are described in the following sections. General methods are described in section 3.1, methods of genetic modifications are described in section 3.2, the transfer of DNA are described in section 3.3, section 3.5 and section 3.6 describes extraction and analysis of alginate, and cyst induction and analysis of cysts are given in section 3.4. This work builds on a report written december 2021 [90], and parts of this chapter is reproduced from this report.

3.1 General methods

3.1.1 Bacterial Strains and cultivation

The bacterial strains used in this study are given in Table 3.1.

Both *A. vinelandii* and *A. chroococcum* were kept on Burk's agar (BA) plates for up to 6 weeks. The plates were stored at room temperature and covered in parafilm to avoid drying of the plates. Liquid cultures were prepared by inoculating the media with a colony from an agar plate. Burk's medium (BM) was routinously used for growth of the *Azotobacter* strains. For cyst induction, cells were grown in liquid BM with βHB as the only carbon source, or on BA plates with n-butanol as the only carbon source. For alginate extraction, cultures were grown in RA1 medium. Incubations were done at 30°C with shaking at 225 rpm for liquid cultures and without shaking for plates.

E. coli strains were kept on Luria Agar (LA) plates at 4°C for up to 6 weeks. If the strain possessed antibiotic resistance genes, the agar plate contained the relevant antibiotic. Liquid cultures were prepared by inoculating the media with either a colony from the plates or directly from frozen stocks. *E. coli* was usually grown in Luria Broth (LB). The appropriate antibiotic was added when neccessary. Incubations of *E. coli* strains were done at 37°C with shaking at 225 rpm for liquid cultures and without shaking for agar plates.

The compositions of all growth media, together with description of antibiotic solutions, and other solutions are given in Appendix A and Appendix B.

Table 3.1: Bacterial strains used in this work, together with relevant characteristics and their source. *BRL=Bethesda Research Laboratories, ATCC=American Type Culture Collection

Strain	Characteristics	Source
A. vinelandii		
ATCC 12518	wild-type	ATCC
ATCC 12837	wild-type	ATCC
E	wild-type	[91]
ATCC 12518Δ05390	*avin05390*, Am^r	[89]
ATCC 12518Δ05750	*avin05750*, Tc^r	This work
ATCC 12518ΔarsF	*arsF*, Tc^r	[43]
ATCC 12518ΔarsG	*arsG*, Tc^r	[43]
ATCC 12518ΔarsH	*arsH*, Tc^r	[43]
ATCC 12518ΔarsHTnOG115	*arsH*, complemented with transposon from pOG115, Tc^r, Am^r	This work
A. chroococcum		
B (ATCC 4412)	wild-type	ATCC
E. coli		
DH5α	*recA1*, *endA*	BRL*
S17.1	$trfA^+$, integrated RP4	[92]
S17.1λpir	*trfA*, integrated RP4, encodes λpir	[93]

Plasmids, used and created in this work, are given in Table 3.2 together with descriptions and sources. Plasmid maps can be found in Appendix D.

Table 3.2: Plasmids used and constructed in this work. Descriptions and sources are also given. Up=unpublished

Plasmid	Description	Source
pAGL5-AcAlgE1	RK2-derived plasmid with replication controlled by the XylS/Pm system; *AcAlgE1*::am^r; Ap^r	[Gawin, up]
pAS02	Derivative of pCR-Blunt II-TOPO. Contains a 1.7 kb product from overlap extension PCR with the downstream element of *Avin17310*; Km^r	[90]
pAS03	RK2-derived plasmid with replication controlled by the XylS/Pm system. Contains *Avin05750*; km^r, Zeo^r	[90]
pAS05	Derivative of pAS03, where *Avin05750* was inactivated by SmaI digestion and insertion of Tc^r from pHE608 (BamHI digested and blunted with Klenow); *Avin05750*::tc^r; Ap^r	This work
pAS06	Derivative of pCR-Blunt II-TOPO with the 1.2 kb PCR product, obtained from *A. vinelandii* gDNA with the primer pair ADH NdeI/ADH R2, inserted; Km^r, Zeo^r	This work

pAS07	Derivative of pHE539 where the 1.2 kb fragment containing *Avin05750* was inserted (NdeI-SpeI digested); Km^r, Zeo^r	This work
pAS10	Derivative of pHE539 with the 2.0 kb fragment from pHH106 inserted (NotI-XhoI digested). Contains $P_{constitutive}$; Km^r	This work
pAS11	Derivative of pAS10 with the 0.9 kb fragment from pMD116 inserted (NdeI-NotI digested). Carries *arsF* under the control of $P_{constitutive}$	This work
pAS12	Derivative of pAS10 with the 1.3 kb fragment from pMD117 inserted (NdeI-NotI digested). Carries *arsG* under the control of $P_{constitutive}$	This work
pAS13	Derivative of pAS10 with the 1.1 kb fragment from pMD118 inserted (NdeI-NotI digested). Carries *arsH* under the control of $P_{constitutive}$	This work
pHE216	RK-derived plasmid with replication controlled by the XylS/Pm system. COntains *OriT*; Am^r, Tc^r	[Ertesvåg, up]
pHE539	RK2-derived expression vector with *algB* under the control of Pm. Contains *OriT*; Km^r	[Ertesvåg up]
pHE608	Cloning vector with *M13ori*. Contains the XylS/Pm system; Ap^r, Tc^r	[Ertesvåg, up]
pHH106	RK2-derived expression vector with the constitutive promoter $P_{constitutive}$. Contains *OriT*; Km^r	[Haaland, up]
pMD116	pUC128-derived cloning vector carrying *arsF*	[43]
pMD117	pUC128-derived cloning vector carrying *arsG*	[43]
pMD118	pUC128-derived cloning vector carrying *arsH*	[43]
pOG115	RK6-derived transposon vector with *Tn5*-based transposon carrying *arsH*. Ap^r, Ap^r	[94]

3.1.2 Preparation of glycerol stocks and revival of frozen cells

For storage of bacterial cultures over longer time periods, glycerol-stocks can be made and stored in an ultrafreezer. Glycerol protects the cells from damage by decreasing the amount of ice forming and it follows that the amount of ions being concentrated during freezing is lowered [95]. The osmotic pressure will therefore be lowered compared to only using culture media. Bacteria can be stored like this for several years [96].

Procedure

1 ml of liquid culture in late exponential phase or stationary phase was mixed with 300µl 60% glycerol in a cryotube. The tube was stored in a -80°C ultrafreezer. Revival of the cells were done by streaking directly from the cryotube on the appropriate agar plate or by inoculating a small amount directly into liquid media and incubating at the appropriate conditions for the bacteria.

3.1.3 Measurement of bacterial growth by spectrophotometry

In certain cases it is important to know the cell density of a culture, as well their physiological state. A culture growing in a closed environment such as a shake flask, exhibits different growth phases. Furthermore, the condition of the cells depends on the growth phase of the culture. When starting a new culture, the cells first go through a lag phase in which no increase in cell density is detected. In this phase, the cells adapt to the environment and synthesize necessary proteins and metabolites. The cells then enter the exponential phase, where the cells grow exponentially. This is also the stage with the highest number if dividing cells. Eventually, the nutrients will be exhausted, waste builds up, spacing becomes scarce, and the growth slows down. This marks the entry into the stationary phase. In this phase the growth rate is zero due to equal numbers of dividing and dying cells. Finally the culture enters the death phase and the growth rate becomes negative [97].

Growth of bacterial cultures can be estimated by measuring the optical density (OD). Such measurements are based on the scattering of light from the bacterial cells in the culture. The concentration of cells is then deduced from Beer-Lamberts law, which relates absorbance to concentration [29]. Since the cells do not absorb, but rather scatter light, this only gives an estimation of cell density in a limited span of cell densities. When the concentration of cells rises above a certain level, light might be scattered several times and end up outside the detector. In such cases the absorbance will be overestimated [98]. Hence, the estimation of cell culture densities should only be done in dilute culture. The cut-off is usually set at OD=0.4 when measuring at 600 nm.

Procedure

Measurements of optical density was done with a Unicam Helios ϵ spectrophotometer. All measurements were done at a wavelength of 600 nm. Growth media or water was used as blanks. If the reading was above 0.4, the sample was diluted with growth media to obtain a value below 0.4.

3.2 Genetic modification of genes

The construction of recombination plasmids containing mutated versions of selected genes involves several steps. The methods used to accomplish these steps are described in the following sections.

3.2.1 Bioinformatic tools

Bioinformatic tools are helpful in planning, performing and analyzing results of biological experiments. The bioinformatic tools used in this work were CloneManager[1] and Benchling[2].

CloneManager was used to plan and visualize the cloning procedures. This included de-

[1]Can be found on https://www.scied.com/
[2]https://benchling.com

signing primers and planning of restriction digests, ligations and TOPO cloning. Benchling is an online tool made for molecular biology experiments. In this work, Benchling was used to visualize plasmids, restriction digests and ligations. It was also used for alignments of sequenced DNA fragments to database sequences. In this work, sequences were retrieved from the genetic sequence database of National Health Institute, GenBank.

3.2.2 Isolation of genomic DNA

To isolate genomic DNA from cells, the cells must first be lysed to release the cell contents. This can be done by the usage of enzymes, detergents or mechanical force. Centrifugation sediments the DNA, together with other large molecules, such as RNA and proteins. The sediment is resolubilized creating a solution from which proteins can be removed by organic extraction, precipitation or the usage of affinity chromatography columns that binds DNA before washing[99, 100]. A ribonuclease (RNase) enzyme , which hydrolyses RNA, may be added to the solution if removal of RNA is necessary [99]. After purification, the DNA molecules can be precipitated by the addition of alcohols such as isopropanol or 70% ethanol, which binds large macromolecules and causes precipitation [101].

In recent years, many kits for DNA extraction and purification have become commercially available. The MasterPure Complete DNA and RNA Purification Kit from Lucigen was utilized in this work for extraction and purification of genomic DNA [102].

Procedure

1.5 ml of an overnight (ON) culture of A.Vinelandii was centrifuged at 13.000 rpm for 10 minutes to harvest the cells. The supernatant was removed and 1 ml 0.9% NaCl was added to dissolve alginate. The cells were pelleted again and the supernatant removed. 300 µl Tissue and Cell Lysis solution was mixed with 2.5 µl proteinase K, before it was added to the pellet and the pellet was dissolved. The solution was incubated at 65 ℃ for 15 minutes, with 10 seconds vortexing every 5 minutes to achieve cell lysis. The sample was cooled to 37 ℃ and 1 µl of 5 µg/ml RNAse A was added. The solution was mixed and then incubated for 30 min at 37 °C. The sample was then placed on ice for 5 minutes before 150 µl MPC Protein Precipitation Reagent was added. This was vortexed for 10 seconds before it was centrifuged to pellet the proteins. The supernatant was tranferred to a clean eppendorftube and 500 µl isopropanol was added. This was mixed by inverting the tube 40 times before centrifugation. The isopropanol was then removed and the final pellet was rinsed twice with 70% ethanol by carefully suspending the pellet end then removing the ethanol. As much as possible of the ethanol was removed before the pellet was left to dry with the lid open for 60 min. The DNA pellet was resuspended in 10 mM tris buffer and incubated ON in a shake incubator at 30 °C. Storage was done at -20°C.

3.2.3 Isolation of plasmid DNA

As with chromosomal DNA isolation, plasmid isolation begins with lysing of the cells. To achieve separation between chromosomal DNA and plasmid DNA, alkaline lysis, originally described by Birnboim and Doly, is often used [103]. This procedure lyses the cells and denatures the DNA in one step by adding sodium dodecyl sulfate (SDS) and sodium

hydroxide (NaOH). For gram-positive bacteria, the cell wall is first weakened by lysozyme. SDS denatures proteins and NaOH increases the pH to a point where chromosomal DNA is denatured, while supercoiled plasmid DNA stays intact. Denaturation of plasmid DNA can occur if the pH is raised above 12.40. Control of pH is therefore essential for good plasmid recovery when preparing isolates [104, 105]. After denaturation of chromosomal DNA, sodium acetate is added to neutralize the solution, causing chromosomal DNA to renature into insoluble aggregates, which precipitates together SDS-protein complexes [103]. Plasmid DNA is precipitated by ethanol, or may be further purified by methods such as solid-phase extraction. In this method, the plasmid DNA is bound to a silica-membrane in the presence of chaotropic agents, which disrupt the hydrogen-bonding in DNA. This allows binding to the silica. When DNA is bound to the membrane, other molecules in the solution can be washed away, before bound DNA is released by a low-salt buffer or water [106].

In this work, plasmid DNA was isolated using ZR Plasmid Miniprep kit from Zymo Research. This kit uses a modified alkaline lysis protocol and purifies the plasmid DNA by by the use of a silica-based column [107].

Procedure

1.5 ml of an ON culture, carrying the plasmid of interest, was centrifuged for 2 minutes at 13 000 rpm to pellet the cells. The supernatant was removed and the cells were resuspended in 200 µl buffer P1. 200 µl buffer P2 was added and the tube was inverted 4 times and incubated for 1-2 minutes before 400 µl buffer P3 was added. The tube was inverted 4 times and incubated for 4-5 minutes, periodically inverting the tube. When the color had changed to yellow and a white precipitate was visible, this was centrifuged for 5 minutes at 13 000 rpm. The supernatant was transferred to a Zymo-Spin IIN column, placed in a collection-tube, and centrifuged for one minute. The flow-through was discarded and the column was reinserted into the collection-tube. 200 µl Endo-Wash buffer was added before a 1-minute centrifugation. After this, 400 µl Plasmid wash buffer was added and this was centrifuged for 2 minutes at 13 000 rpm. The column was then transferred to a clean 1.5 ml eppendorf tube and 50 µl DNA Elution Buffer was added to the center of the column. This was incubated for 1-5 minutes, followed by a 1-minute centrifugation in order to elute the plasmid DNA.

3.2.4 Measurement of DNA concentration in solution

The concentration of DNA in a solution is critical for the success of many procedures in molecular genetics. For example when performing restriction digests or ligations, the result can be optimized by using specific ratios of enzyme to DNA or vector to insert. DNA concentrations in a solution can be quantified by spectrophotometric methods, and UV-visible spectroscopy has become a common tool for quantification of biochemical molecules in many biology and chemical laboratories [108]. UV-visible spectroscopy measures absorbance of light at the UV-visible wavelengths and correlates them to concentrations of absorbing species in the sample through the Beer-Lamberts law [109].

The development of microvolume UV-visible spectrometry has enabled quantification of sub-µl concentrations of analyte. This is convenient for the measurements of DNA in small

sample sizes. The NanoDrop One UV-Vis spectrophotometer from ThermoScientific uses surface tension to hold a small volume of sample between two surfaces in the optical path of the instrument and measures absorbance at 260 nm. In addition to concentration measurements, it also measures the purity of the sample by measuring absorbance at other wavelengths, representing different types of contamination [110].

Procedure

Both pedestals of the instrument was cleaned with 2µl MQ-water. The NanoDrop Spectrophotometer was set to dsDNA (double-stranded DNA) measurements and blanked with 1µl of the same elution buffer as in the sample. 1 µl of the sample was loaded on to the pedestal and the measurement was carried out. The pedestal was wiped off with lens paper between each measurement.

3.2.5 Preparation of competent *E. coli*

Competence is the ability of bacteria take up DNA from the environment through the process known as transformation. Since the discovery of natural competence of *E. coli* in 1928 [111], several methods have been developed to induce competency. Two methods are generally used: electroporation and chemical treatment of the cells. During electroporation, the cells are subjected to a high-voltage electric pulse. This creates transient pores in the membrane, enabeling the entry of DNA [112]. Chemical competency was first induced by treating the cells with an ice-cold medium containing $CaCl_2$, before heat-shocking [113]. Later, different additions to the original treatment have been made in order to further increase the transformation efficiency of the cells. This includes modifications of the transformation media, such as adding Mg2+, Mn2+, Rb+, DMSO or PEG to the original $CaCl_2$-medium [114].

Procedure

The *E. coli* strain to be made competent was grown in 10 ml Psi medium ON. 1 ml of the culture was transferred to 100 ml fresh medium and incubated at 37°C until the optical density (OD_{600}) was measured to 0.4. The culture was immediately put on ice, to halt growth, for 15 minutes before pelleting the cells by centrifugation (4000 rpm) at 4°C for 5 minutes. The supernatant was removed and 40 ml cooled (4°C) TFB1 was added. The cells were resuspended by gently mixing before a new round of centrifugation. The supernatant was removed and the cells were resusbended in 3 ml TFB2 by very gently mixing. The competent cells were aliquoted in 100 µl amounts to pre-chilled eppendorf tubes. The aliquotations were snap-frozen in liquid N_2 before storing them at -80°C.

3.2.6 Polymerase chain reaction

Amplification of specific nucleotide sequences can be done by the polymerase chain reaction (PCR). The reaction is carried out *in vitro* and is a rapid way of sequence amplification. The reaction is based on the *in vivo* mechanism of DNA replication, where a new DNA-strand is synthesized by DNA-polymerase in the 3'-5' direction by incorporating dNTPs using the complementary strand as a template [115]. The amplifica-

tion of a DNA-sequence by PCR requires a thermo-stable DNA polymerase and mono-deoxyribonucleoside triphosphates, in addition to designed oligonucleotides that flank the sequence to be amplified. These oligonucleotides, also known as primers, complements the sequences flanking the target sequence, on opposite strands and has their free 3'-OH group facing towards each other. The primers function as a starting-point for the elongation by the polymerase [116].

The reaction takes place in three repeating steps: denaturation, annealing, and elongation (Figure 3.1). During denaturation, the two DNA-strands separate due to heat. During annealing, the temperature is decreased to allow the primers to bind to the separated strands. In the elongation step, the polymerase binds to the free 3'-OH of the primers and extends the strand complementary to the template. These three steps are repeated a number of times, resulting in numerous copies of the DNA-sequence of interest [115]. PCR can also be used to introduce insertion or deletion mutations in genes (Figure 3.1). This is done by first generating separate PCR products of the upstram and downstream elements of the gene to be mutated. Using primers that overlap will produce two fragments with complementary ends. These can then be fused in a new PCR, using the forward primer of the upstream element and the reverse primer of the downstream element. This can create a mutation by either inserting or deleting an oligonucleotide [117].

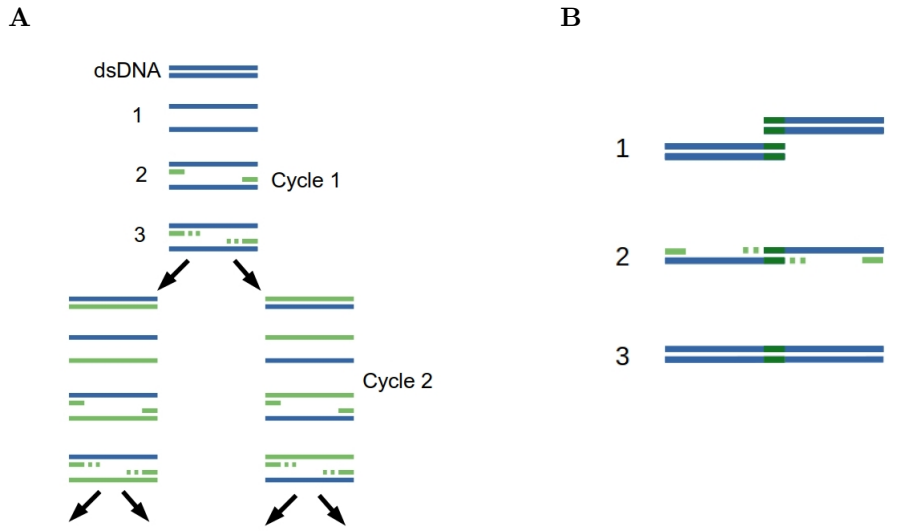


Figure 3.1: (A) Amplification by PCR. The three repeated steps in PCR are 1. denaturation, 2. annealing, and 3. elongation. (B) Mutation by fusion PCR. Mutation by fusion PCR is achieved by three steps: 1. construction of upstream and downstream element, 2. annealing of the complementary sequences, and 3. elongation. Primers are colored light green and complementary sequences are colored dark green. Elongation direction is denoted by dots.

Different polymerases have been used for the amplification of DNA by PCR. The first enzyme used was the Klenow fragment of *E. coli* Polymerase I [118]. This enzyme was however not thermostable and was inactivated by heat during the denaturation step. Therefore, new enzymes had to be added for every cycle. A thermostable polymerase from the thermophillic bacterium, *Thermus aquaticus* (*Taq*) simplified the reaction when it was implemented. It also allowed higher temperatures during the annealing step, which

increased the binding specificity of the primers and the yield of the wanted DNA fragment [116]. In later years, other polymerases with 3'-5' exonuclease activity have been discovered. The exonuclease activity makes the polymerase able to proofread the newly synthesized DNA and correct wrongly inserted nucleotides. Such polymerases, with a low error-rate are abbrievated "high-fidelity". The use of these high-fidelity polymerases increases the accuracy of the amplified sequences [119, 120]. The different DNA polymerases available for PCR reactions create PCR-products with different ends. The Q5 high-fidelity DNA polymerase creates PCR-products with blunt ends [121].

Procedure

The components neccessary for a 50 µl PCR reaction (Table 3.3) were mixed together in a 0.2 µl PCR-tube on ice. The Q5 high-fidelity DNA polymerase was added just before starting the reaction. If a smaller reaction volume was used, the quantity of the reaction components were adjusted accordingly. Primers used in this work are presented in appendix E.

Table 3.3: Components added to the 50µl PCR reaction mixture.

Component	**quantity [µl]**
5x NEB Q5 Reaction Buffer	10
10 mM dNTP mix	1
10 µM Forward Primer	2.5
10 µM Reverse Primer	2.5
Template DNA	1
5X Q5 High GC Enhancer	10
NEB Q5 High-fidelity DNA polymerase	0.5
Autoclaved RO-water	22.50

The DNA used as template was prepared either by isolation of plasmid- or chromosome DNA, or by boiling liquid cultures or colonies from agar plates. When using colonies, one colony was picked from an agar plate and resuspended in 200 µl water. The cell suspension was boiled at 100 °C for 15 minutes. The boiled suspension was used directly in the PCR mix.

To carry out the reaction, Eppendorf thermocyclers Mastercycler gradient or the Mastercycler personal was used. The program was adjusted to each reaction's requirements(Table 3.4). The machine was started and allowed to reach 72°C before inserting the PCR tubes. The PCR reaction, given by step 2, was run for 35 cycles in total. When boiled cell suspensions was used to provide template DNA, the initial denaturation time was increased to 3 minutes.

Table 3.4: Program used for the PCR amplification of DNA sequences.

Step		Reaction	Temperature [°C]	Time[s]
1		Initial denaturation	98	30
2	a.	Denaturation	98	10
	b.	Annealing	Lowest T_m-3	30
	c.	Elongation	72	45 per kb
3		Final extension	72	120
4		Hold	4	Infinite

3.2.7 TOPO cloning

Cloning of blunt end DNA-fragments into a vector in a one-step procedure is made possible by the utilization of a topoisomerase I enzyme isolated from the *Vaccina* virus [122]. Topoisomerases are enzymes that increase or decrease the degree of supercoiling in DNA by one of two methods. Type I topoisomerases cut one of the DNA strands and passes the other strand through the break before religation. Type II topoisomerases cut both strands and rotate the strands before religation [115]. The *Vaccina* topoisomerase is a type I topoisomerase that recognizes the sequence 5'-(C/T)CCTT-3', to which it can covalently bind and introduce a single strand break. The enzyme is released when a DNA molecule with a free 5'-OH group binds to the phosphate and joins the two DNA molecules [122].

TOPO cloning was performed using the Zero Blunt TOPO PCR cloning kit from ThermoFisher. This kit utilizes a linearized version of the plasmid vector pCR-Blunt II-TOPO, which has the topoisomerase I from *Vaccina* covalently bound to each 3' end. The vector has the lethal *ccd*B gene fused to the C-terminus of the lacZα fragment. In plasmid containing an insert, the lacZα fragment will be disrupted and *ccd*B will not be transcribed. Plasmids not containing an insert will transcribe the lethal *ccd*B and these cells will die. Hence, only colonies containing an insert will grow. In addition, the vector also contains resistance genes for Zeocine and kanamycin for selection purposes [123].

Procedure

2 µl PCR-product was mixed with 0.5 µl TOPO vector and 0.5 µl salt solution. This was incubated at room temperature for 30 minutes before transforming to competent *E. coli* cells.

3.2.8 DNA sequencing

To confirm that the correct sequence has been amplified during PCR, the amplified PCR-fragment must be sequenced. A range of sequencing methods are available, and many of them can sequence great amounts of DNA in short time [77]. However, for the sequencing of single sequences containing only one or a few genes, an automated version of Sanger Sequencing is often preferred. The original Sanger Sequencing was invented in 1977 by Frederick Sanger and has since contributed to a lot of todays knowledge

about genes and related molecules [124]. The method is based on DNA polymerization in the presence of both deoxynucleotide triphospahtes (dNTPs) and 2',3'-dideoxynucleotide triphosphates (ddNTPs). ddNTPs do not have a free 3'-hydroxyl group and when incorporated by DNA polymerase, subsequent extension is blocked. This produces a collection of DNA molecules, each with identical 5'-ends and a 3'-termini at every position along the DNA-sequence. The ddNTPs are each labeled with a specific fluorescent dye, enabeling differentiation between the nucleotides at the 3'-termini. Separation of the different sized fragments is done by acrylamide gel electrophoresis in a thin capillary tube. A scanning laser and a fluorescence detector is used for the detection of the four different fluorescent dyes, representing the four ddNTPs at the end of the fragments. As the length is directly coupled to the sequence, the sequence can be directly recorded when the fragments pass the fluorescence detector [77].

The sequencing in this work was done by the company Eurofins Genomics. Their LightRun service utilize the automated version of the Sanger sequencing method [125]. The results are given as a sequence together with a chromatogram. The chromatogram shows fluorescent peaks at each position along the sequence. Each peak corresponds to a specific nucleotide. The quality of the sequencing can often be read from the chromatogram. A chromatogram from a high-quality sequencing should give a single, strong signal for each position[126]

Procedure

A sample was prepared for sequencing by assembling the reaction mixture (Table 3.5) in an 1.5 ml eppendorftube. One primer was used for every 500-2000 bp in the sequence and each tube contained only one primer. The tubes were marked with Eurofins Barcodes.

Table 3.5: Components assembled before sending a sample for sequencing.

Component	quantity
Plasmid DNA	400-500 ng
10 µM primer	2.5 µl
DMSO	0.75 µl
Autoclaved RO-water	to 15 µl

3.2.9 Restriction cutting of DNA

Cutting and ligation of DNA-fragments is essential when making recombinant DNA. Restriction endonucleases are enzymes found in many bacterial strains. They are great tools for the construction of recombinant DNA as they provide the possibility of cutting the DNA at specific recognition sequences along a DNA sequence [77]. The first restriction enzyme to be isolated was from *E. coli* strain B [127]. This was shown to be a type I restriction endonuclease which recognizes and bind to specific sequences, but cleave DNA at nonspecific sites up to 1000 bp from the recognition sequence [115]. Later, a restriction enzyme that cleaved DNA only at unique recognition sequences was discovered in *Haemophilus Influenzae* [128]. This was the first type II restriction endonuclease, a

class of enzymes that cleave DNA within the recognition sequence. After this discovery, numerous type II restriction endonucleases have been identified [115].

Because of their properties, type II restriction endonucleases are widely used in cloning work. They normally recognize and cleave palindromic sequences of 4-6 bp and leave either blunt ends with no overhang or sticky ends with a 2-4 nucleotide overhang [115]. This enables isolation of specific DNA fragments from larger elements. Additionally, the restriction enzymes can be used to confirm the presence of certain DNA fragments.

Procedure

The restriction endonuclease reaction was set up with the components given in Table 3.6.

Table 3.6: Components added to a restriction endonuclease reaction.

Component	Quantity
DNA	1-17 µl
Restriction enzyme	0.5µl per enzyme
Buffer	2 µl
Autoclaved RO-water	to 20 µl

Depending on the reaction, one or two restriction enzymes were used. The suitable restriction enzyme(s) was chosen using Benchling or CloneManager. The different enzymes required either 10X NEB Buffer 3.1 or CutSmart to give optimal results. The reaction was incubated at 37°C for 1 hour to ON.

3.2.10 Ligation of DNA fragments

DNA fragments produced by restriction endonuclease reactions, PCR, or from other sources can effectively be joined by ligase enzymes. These enzymes create a phosphodiester linkage between adjacent 3'-hydroxyl and 5'-phosphate termini, using ATP or NADH as energy sources [115]. DNA ligases are essential to all living organisms as they are involved in DNA replication, DNA repair and recombination events [77]. The T4 DNA ligase, originally isolated from bacteriophage T4, can ligate both blunt-end and sticky-end fragments successfully, although blunt-end joining is less efficient [129].

Ligation of restriction fragments requires that the fragments have compatible ends. If they have overhangs, these must be able to base-pair before the ligase can do the job of covalently joining the phosphate backbone of the fragments [77]. If the fragments have incompatible ends, blunting of the ends can be performed before ligation. The Klenow Fragment of *E. coli* Polymerase I has inherent 5'-3'polymerase activity and 3'-5' exonuclease activity and can thus fill in or remove nucleotides at sticky ends to produce blunt ends [130].

Procedure

The vector DNA and insert DNA was mixed together to a total of 17 µl. Typically 3 µl of the vector and 14 µl of the insert was used. 2 µl NEB 10X T4 DNA Ligase Buffer and

1 µl NEB T4 DNA Ligase was added. The reaction was incubated at room temperature for 2 hours, or at 16° ON before transforming the reaction mixture to competent cells or storing it at -20°C.

When blunting of DNA fragments was necessary, 1 µl 10 mM dNTP mix and 0.5 µl Klenow was added after cutting the DNA with restriction enzymes. This was allowed to incubate for 15 minutes at 37°C before putting it on ice for further use or storing at -20°C.

3.2.11 Gel electrophoresis: visualisation and separation of DNA

For the purposes of visualizing DNA and separating DNA of different sizes and conformations, gel electrophoresis is a useful tool [131, 132]. It is based on the movement of DNA molecules through a gel with specific pore size, when electrical voltage is applied. Because the phosphate backbone of DNA carries one negative charge per nucleotide, the ratio of charge to length remains constant. Thus the migration rates of a DNA-molecule through the gel changes with respect to size and conformation [77].

The procedure is set up in a chamber with a positive and negative electrode on opposite sides. The chamber is filled with a buffer capable of carrying electric current [77]. Tris acetate EDTA (TAE) buffer, composed of Tris, acetic acid and EDTA is commonly used [131]. EDTA is a chelating agent and binds divalent cations. This prevents DNA-degrading enzymes dependent upon such cations from functioning [133]. The gel, which often is composed of agarose, is placed in the buffer between the two electrodes. Agarose is a copolymer composed of repeating units of 3,6-anhydro-L-galactose and D-galactose. When heated, followed by cooling, the agarose gelates and a network of fibers form [134]. In this way, the agarose gel act as a filter, separating DNA of different sizes [77]. Wells for loading the sample is formed in the gel. The sample or the gel is normally prepared with a buffer containing ethidium bromide (EtBR), which binds to DNA and fluoresces under UV-light. This enables the visualisation of the DNA [77]. GelGreen and GelRed are designed fluorescent dyes, that is less mutagenic than EtBr since it does not penetrate the skin. They are just as sensitive as EtBr and therefore good options [135]. When loading the sample into the well, a loading dye is commonly applied to monitor the migration rate through the gel [77].

The sizes of DNA fragments can be approximated by running fragments with known sizes together with the samples. Separated DNA-fragments can be cut out from the gel and purified for downstream cloning or other purposes. This is described in the next section.

Procedure

A 0.8% agarose gel was casted in a tray with a comb inserted. This was left to dry for a minimum of 15 minutes before the comb was removed and the tray was inserted into the electrophoresis chamber containing TEA buffer. The gel was completely submerged in the buffer. The samples were prepared by mixing 5 µl PCR product or 100-200 ng plasmid DNA together with 2 µl 6X purple loading dye and water to a minimum of 15 µl. This was loaded into the wells. Lambda DNA cut with either HindIII or PstI restriction enzyme was used as standards for size estimation. These can be found in appendix C. The

gel elecrophoresis was run for 30-90 minutes at 100 V before visualization using BioRad ChemiDoc XRS. When using GelGreen, a blue light conversion screen was used. DNA Fragments were excised with a scalpel while exposed to UV-light.

3.2.12 Extraction of DNA from agarose gel

Fragments separated or visualized by gel electrophoresis can be purified for use in further experiments. There are several methods available for the extraction of DNA from agarose gels, among them electroelution and filtration. Most commercial kits involves denaturation of DNA by chaotropic salts, allowing it to bind to a membrane of silica, glass beads or diatomacheous earth. Contaminants can then be washed away before eluting DNA with water or TE buffers [136]. In this work, the Monarch DNA Gel Extration kit from NEB was used for gel extraction of DNA. This kit includes a gel dissolving buffer containing sodium iodide and guanidine thiocyanate, which are both chaotrophic agents [137, 138]. A silica membrane is used for the binding of DNA while washing [139].

Procedure

The excised gel containing the DNA of interest was dissolved in 600 µl Gel Dissolving Buffer and incubated at 50°C until the gel was completely dissolved (5-15 minutes). The solution was transferred to a Monarch DNA Cleanup Column placed in a collection tube and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded before the column was reinserted to the collection tube. 200 µl DNA wash buffer was added before another centrifugation. The washing was repeated once and the column was then transferred to an eppendorf tube. 15 µl DNA elution buffer was added directly to the membrane. This was incubated for 1-5 minutes before centrifugation for 1 minute at 13 000 rpm to elute the DNA. The DNA was then immediately used or stored at -20°C.

3.3 Transfer of DNA

3.3.1 Transformation

Certain bacterial species are competent, and thus have the ability to take up DNA from their environment by the process known as transformation. The transformation process is an example of mechanisms enabling horizontal gene transfer in bacteria [77]. Most bacteria require specific conditions to reach the physiological state of competency and are only transiently competent. This state is dependent on the expression of specific, conserved genes, termed *com* genes [140]. During natural transformation, DNA uptake is dependent on the binding of DNA to a transformation pilus extruding from the cell wall. One strand of the DNA is degraded, while the other strand is transported into the cell through a transmembrane channel [141]. In *E. coli* however, the uptake of dsDNA has been suggested [142]. When internalized, the DNA can either stay as a separate entity or integrate into the chromosome by transposition or homologous recombination [140].

Transformation provides a means for the delivery of recombinant DNA into bacterial cells. The natural transformation frequency is found to be low, especially in *E. coli*, which is often used in the lab. In order to efficiently use transformation in cloning work,

the frequency may be increased by chemical treatments of the cells. This is further described in section 3.2.5. The chemical treatment is often followed by a short heat-shock. This may increase the transformation efficiency up to 10^8 CFU/µg DNA used [143]. Another method of inducing DNA-uptake is by applying an electric shock to the cells. This procedure is known as electrotransformation [144]. In addition to increasing the transformation efficiency, artificial transformation enables the introduction of circular plasmid DNA [145]. The exact mechanism of artificial transformation is not fully known. It is suggested that the chemical treatment enhances DNA-binding to the cell surface. The heat pulse or electropulse likely creates pores in the outer membrane by lipid-release, and decreases membrane potential of the cytoplasmic membrane. Together, this enhances the frequency of genetic transformation [144].

Procedure

For transformation by heat-shock, chemically competent cells were thawed on ice. Up to 10 µl DNA was added to 100 µl cells and mixed gently by flicking the tube, before 30-60 minutes incubation on ice. The cells were then heat-shocked in a 37°C water-bath for 2 minutes and quickly transferred back to ice, where they were incubated for 2 minutes. 900 ml SOC medium was added and the culture was incubated for 1-2 hours at 37°C with 225 rpm shaking. 200 µl and 800 µl was plated on LA-plates containing the selective antibiotic and incubated ON.

Electrotransformation of *A. chroococcum* was done by the procedures outlined by Korànyi et al. and Sultana et al. [146, 147]. In short, exponentially growing cells were harvested and washed with 10 % glycerol 3 times and the volume was reduced to 40-400 µl by resuspending the cells in less glycerol than the culture volume. The cell suspension and 300-1000 ng DNA was added to a sterile, prechilled electroporation cuvette of suitable size and kept on ice for 5 minutes. A BioRad *E. coli* pulser was used to give the pulse. The apparatus was operated at voltage of 2.50-3.00 kV, a resistance of 200-2000 Ω, and a time constant if 5-9 milliseconds. Immediately after the pulse was given, 1 ml of RM medium was added to the cuvette. This was incubated on ice for 1-5 minutes before the cells were plated on RM agar plates with antibiotics for selection.

3.3.2 Conjugation

Transfer of DNA from one bacterial cell to another bacterial cell through a intercellular channel is another method that enables horizontal gene transfer in some bacterial species. This process is called conjugation and bacteria with specific genes are able to carry out this process. These genes can exist as a single plasmid, a part of a plasmid or integrated in the chromosome [77]. During conjugation, the donor cell produce a conjugative pilus, which brings the cells into physical contact. After an intercellular channel is formed, the DNA is nicked at *OriT*, the origin of transfer. One of the strands is then transferred to the recipient cell through the channel simultaneously with replication [148]. Once transferred to the recipient cell, the transferred DNA is replicated by the recipient cell's replication machinery [77].

Conjugative plasmids are much utilized as DNA vehicles for the purposes of introducing DNA into bacteria. Vectors based on the naturally occuring IncPα group of plasmids

are conjugative plasmids widely used for this purpose. An example of such a plasmid is the RK2 plasmid, which many constructed conjugative plasmids are based on [149, 150, 151]. Some plasmids carry all elements necessary for conjugation, while others are dependent on elements synthesized by the donor. Many plasmids used in conjugation are derived from the same original plasmid, and consequently carry the same elements for conjugations. It is important to be aware of this as plasmids that produce identical pili cannot enter the same cell, a phenomena known as "entry exclusion" [152].

Procedure

Precultures of donor- and recipient strains were made and grown to stationary phase. The donor, *E. coli* S17.1 containing the plasmid to be transferred was grown ON in LB with the apropriate antibiotic. The recipient, *A. vinelandii* or *A. chroococcum* was grown for 1-3 days in BM.

10 ml BM was inocculated with inocculum from the ON culture at 0.1% for *A. chroococcum* and 1% for *A. vinelandii* and grown ON until reaching OD_{600}=0.4-0.6. For *E. coli* S17.1, 10 ml LB was inocculated with inocculum from the pre-culture at 1% and grown for 2-3 hours until reaching OD_{600}=0.4.

4-6 ml of each culture was transferred to a centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The supernatant was carefully removed and the cell pellet was resuspended in 100 µl BM and plated as a drop on LA with m-toluate. The plate was incubated ON at 30 °C.

The conjugation plate was scraped and the cells were resuspended in 1 ml BM. Undiluted and 10^{-1} dilution was plated on BA containing the apropriate antibiotic and m-toluate. The plates were incubated for 3-5 days.

3.3.3 Homologous recombination

Homologous recombination allows organisms to edit DNA by exchanging genetic information between two homologous sequences. The process occurs naturally in bacteria, as well as in higher organisms. Bacteria uses it for different purposes, which includes the exchange of DNA sequences to produce genetic diversity, acquisition of novel genes and for the repair of damaged DNA[153].

The ability of bacteria to carry out homologous recombination offers an easy way for editing of targeted sequences within the chromosome, such as mutagenesis. Mutagenesis of a specific gene may be done by homologous recombination between a plasmid containing a mutated version of the gene and the chromosomal gene [154]. Regions flanking the gene of interest must be homologous to the regions flanking the disrupted gene in the plasmid to enable homologous recombinations at both sides of the gene. The process of homologous recombination begins with introducing a nick in the homologous region of the donor molecule. The single strand is then bound by single-strand binding proteins, and subsequently the protein RecA. This aid in the base-pairing with the homologous region of the recipient molecule. A DNA intermediate with a holiday junction is formed. Resolvase enzymes cuts and rejoins the DNA strands, resulting in two recombinated DNA molecules [77].

The insertion of DNA elements in the chromosome is accomplished by a single- or a double crossover event between homologous regions of the two DNA molecules (Figure 3.2). When undergoing homologous recombination with a pasmid, a single crossover event inserts the complete plasmid, while a double crossover event exchanges a part of the chromosomal DNA with a part of the plasmid DNA [154]. The single crossover event can be screened for by using plasmids that are unable to replicate in the host. The plasmid will then either be integrated into the chromosome, or lost. Due to the selective marker, only cells with the plasmid integrated into the chromosome will survive. If the recombination frequency is low, the chance of obtaining cells with the plasmid integrated into the chromosome is low. A strategy to increase the number of recombination events is to apply conditional suicide plasmids, which allows replication in the host to be controlled. An example is plasmids where replication is controlled by the XylS/Pm system [87]. Omitting the benzoic acid-derivative from the growth medium will in this case only allow cells with the plasmid integrated in the chromosome to survive, as the plasmid cannot replicate without the inducer. Double crossover mutants can be screened for my using a counter-selection marker placed on the vector backbone. The *SacB* gene is a counter-selection marker, whose gene product converts sucrose to levan. Levan is toxic to many cells, including *A. vinelandii*, and sucrose concentrations of 5% in the media is lethal [155]. As it is a possibility that the second crossover events lead to the reversion to wild-type (Figure 3.2), mutants should always be verified by PCR.

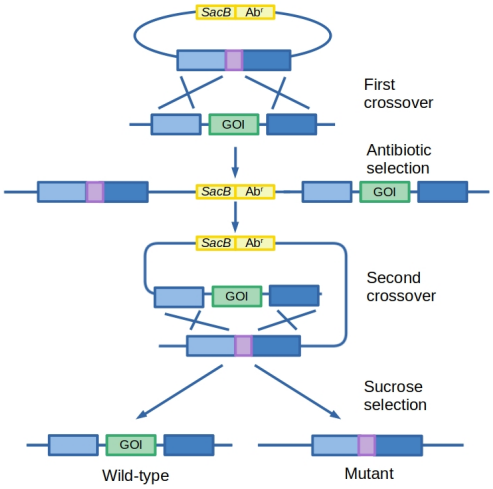


Figure 3.2: Homologous recombination between a plasmid and the chromosome to mutate a gene of interest (GOI). The plasmid contains an antibiotic resistance gene (Ab^r) as a selection marker, *sacB* as a counter-selection marker, and a mutated version of the gene of interest in purple. Blue regions mark homologous regions. Modified from Nakashima and Miyasaki [154]

When doing homologous recombination in *Azotobacter*, care must be taken in order to create pure mutants. As these bacteria often carry several copies of its chromosome, sub-cultivation with selective pressure is often necessary.

Procedure

Four transconjugant colonies were picked and transferred to liquid growth medium (BM) with the antibiotic, as well as to agar plates with the antibiotic. The liquid cultures were subcultured 5 times by transferring 100 µl to 10 ml of fresh media, with the last culture being without the addition of antibiotics. Glycerol stocks of all cultures were prepared to be able to restart the process at any point.

Selection of double cross-over mutants was done by plating dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) on BA plates with sucrose.

3.4 Encystment and analysis of cysts

3.4.1 Induction of cysts and testing of desiccation resistance

As described in section 1.2, *Azotobacter* species are able to differentiate into a dormant cell forms, called cysts. Encystment is induced by *n*-butanol, or related compounds, including β-hydroxybutyrate. In cultures grown in liquid Burk's medium with βHB as the only carbon source, the degree of encystment can reach over 90 % [20]. On Burk's agar plates containing *n*-butanol, percentages of encysted cells ranges around 10 % [75, 22].

The degree of encystment can be measured by counting cells under the microscope, or by plate-counts. When cells undergo encystment, they become optically refractile. A differentiation between vegetative cells and cysts can thus be made based on refractility of the cell [27]. Staining procedures to make the differentiation easier has also been described [156]. These methods does not take into account other physical properties of an observed cyst, and it is therefore common to base the number of cysts on the number of cells surviving desiccation after encystment [23]. The estimation of encystment is then done by streaking the cell samples on agar plates before and after desiccation and counting colonies.

Procedure

Cysts were induced either in liquid BM with βHB as the only carbon source, or on BA with *n*-butanol as the only carbon source. Liquid BM was inocculated with cells maintained on BA plates and grown for 1-2 days. 100 µl of this culture was used as inocculum in 10 ml of new media. This was incubated until the OD_{600} reached 0.8-1.0. The cells were harvested by centrifugation at 6000 rpm for 10 minutes and washed twice in BS by resuspension and subsequent centrifugation. For encystment in liquid encysting medium, the supernatant was removed and the cells were resuspended in 10 ml BM-βHB. The cultures were incubated at 30°C and 225 rpm shaking for five days to complete encystment. For encystment on agar plates, the harvested cells were resuspended in 10 ml BS and 200 µl of this suspension was platet on BA plates with *n*-butanol. The plates were incubated at 30°C for five days to complete encystment.

The desiccation resistance of encysted cells was measured with cell samples from liquid culture or from suspensions of cells from agar plates. To make cell suspensions, cells

encysted on n-butanol agar plates were scraped off the surface of the agar and suspended in BS to yield a suspension with OD_{600}=0.8. 10µl samples of culture or suspension was deposited in open eppendorftubes encased in sterile petri dishes. The samples were incubated for 7 days and estimation of desiccation resistance was done based on cell counts before and after drying. Cell counts were made by making dilution series of the culture-or suspension and plating different dilutions on BA plates. Before drying the 10^{-5} and 10−6 dilutions were plated. After drying undiluted to 10^{-3} dilutions were plated.

3.4.2 Extraction and determination of alkylresorcinol

Phenolic compounds, including alkylresorcinols, react with diazotised reagents to form colored compounds [157, 158]. This gives a possibility of using colorimetric methods for the determination of phenolic compunds in samples. Fast Blue B (4-(4-diazonio-3-methoxyphenyl)-2-methoxybenzenediazonium) is a diazonium compound that forms compounds with yellow to red colors when reacting with phenolic compounds [159]. When it reacts with resorcinol, or its 5-n-alkylderivatives, it forms a violet compound that have an absorbance maximum at 520 nm [159, 160]. Ketoalkenylresorcinols has the same absorbance maximum, while other phenolic compounds has other maxima. This makes it possible to distinguish between resorcinols and other phenolic compounds. Fast Blue B can therefore be used to quantify resorcinol and its 5-n-alkylderivatives in samples, and the method is highly specific (>96%) as well as sensitive (1-10 μg) [158, 160]. While the method is sensitive for alkylresorcinols, it is not possible to differentiate between orcinol and the different alkylresorcinols using this method. Orcinol, resorcinol, and 5-n-pentadecylresorcinol all have the same molar extinction, and can therefore be used as standards when quantifying resorcinols and it's alkylderivatives [158].

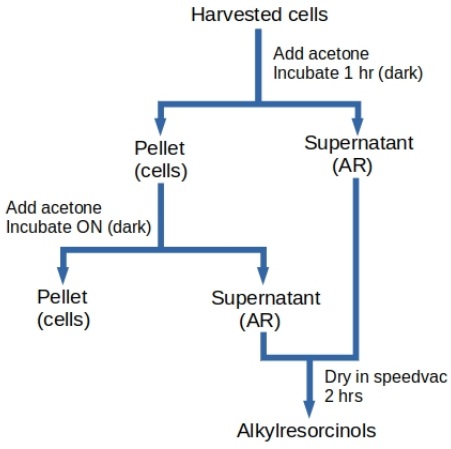


Figure 3.3: Procedure for the isolation of alkylresorcinols from encysted cells of *Azotobacter*. Based on the description of Kozubek and Tyman, and Segura et al. [157, 35]

For precise quantification of total alkylresorcinol in cells, the phenolic lipids must first be extracted. Lipids are soluble in acetone, and can therefore be used to extract alkylresorcinols from cells. This has sucessfully been done in both whole cereal grains, and in cells of *A. vinelandii* [157, 35]. The procedure outline is shown in Figure 3.3. Qualitative analysis of alkylresorcinol on the cell surface does not require extraction of the phenollipids, and can simply be done by observing the color change when adding dissolved Fast Blue B directly to the whole cells [35].

Procedure

Quantification of alkylresorcinols in *A. vinelandii* was done by extracting the phenolic lipids of encysted cells before a colorimetric assay with Fast Blue B as reagent. Cysts were induced in liquid BM-βHB as described in section 3.4.1. The cyst cultures were split into aliquots of 25 ml and harvested by centrifugation at 7000 rpm for 8 minutes. The supernatant was removed and the cells were resuspended in 1 ml 10 mM $MgSO_4$ before a centrifugation at 14000 rpm for to minutes. The supernatant was removed and 1 ml acetone was added. The cells were properly mixed with the acetone with a pipette followed by a short vortexing. The mixture was incubated in the dark for 1 hour at room temperature (no shaking). The mixture was centrifuged for 2 minutes at 14000 rpm. The liquid fraction was pipetted of and stored in the dark at 4°C. The pellet was resuspended in 1 ml acetone and vortexed before incubation in the dark at room temperature ON. The liquid fraction was extracted as described and mixed with the first liquid fraction. Acetone was evaporated in a SpeedVac vacuum concentrator. Orcinol standard for the quantification of alkylresorcinol was made by dissolving the following amounts of orcinol in 1 ml acetone: 1, 5, 10, 25, 50, and 100 µg. These were made from a 1 mg/ml stock solution. 1 ml ethanol Fast Blue B (Appendix B) was added to each of the dried samples. The samples and the standard was incubated at room temperature, protected from light for 1 hour. 50 µl of the samples, including the standard was transferred to a microplate and the absorbance at 520 nm was measured.

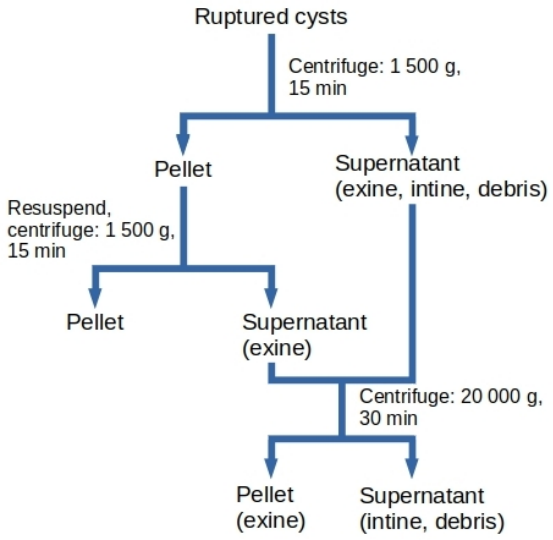
The qualitative analysis of alkylresorcinols on the cell surface of *A. vinelandii* was done by adding 1 ml Fast Blue B acetic acid solution (Appendix B) to agar plates with encysted cells. The encystment of cells on agar plates is described in section 3.4.1.

3.4.3 Fractionation of cysts

The cysts of Azotobacter species are tolerant to a range of treatments that are commonly used to lyse bacterial cells, including sonication [18]. Repaske showed that vegetative cells of *A. vinelandii* were lysed in Tris buffer containing EDTA and lysozyme [161]. It was later showed that cysts could be ruptured in TRIS by the presence of EDTA alone, and also by two other chelating agents, dipicolinic acid and trisodium citrate [162, 163, 19]. The highest degree of cyst lysis (99%) was obtained by suspending mature cysts in a Tris-EDTA solution. To obtain this degree of lysis, it is essential that residual medium and salts are washed off of the cells before suspending the cells in Tris-EDTA as salts hamper the rupturing process [163].

During the lysis of cysts, the exine ruptures in a single line releasing the intine into the solution. The integrity of the central body is maintained, but the viability of these central bodies is $<1\%$ [19]. After lysis, the exine, intine, and central body may be isolated by different centrifugation protocols Figure 3.4[164, 20].

A



B

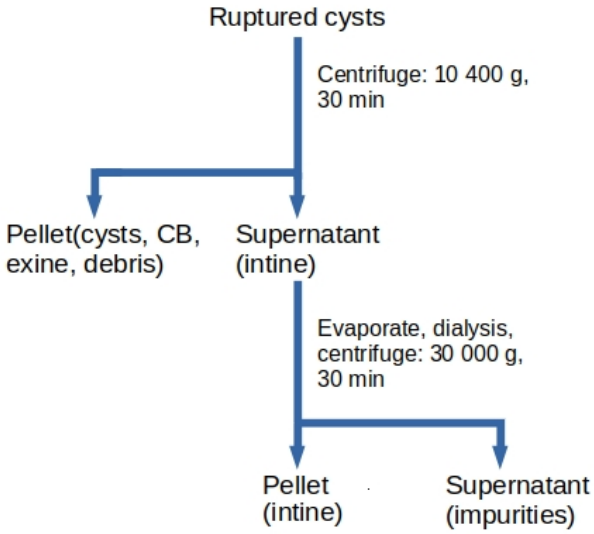


Figure 3.4: Overview of processes for the isolation of exine and intine from a suspension of ruptured cysts. (A) Isolation of exine. Modified from Lin and Sadoff [164]. (B) Isolation of intine. Flow diagram made from method description by Lin and Sadoff [20].

Procedure

Cells of *A. chroococcum* were encysted in liquid media as described in section 3.4.1. The cultures were grown in 7x500 ml shake flasks containing 125 ml medium. After encystment the cultures were harvested by centrifugation at 14 000 rpm for 30 minutes. The supernatant was removed and the harvested cells were washed three times in 0.05 M Tris by resuspension and centrifugation at 14 000 rpm for 30 minutes. After the last centrifugation, the cells were resuspended in 0.05 M Tris with 3 mM EDTA to rupture the cysts. For exine isolation , the suspension was incubated with stirring at room temperature for 15 min. For isolation of intine, the suspension was incubated with stirring at 4°C for 6 hrs.

The procedures for isolating the exine and the intine from the ruptured cysts are outlined in Figure 3.4. For isolation of exine, the suspension of fractured cysts was centrifuged at 1 500 g for 15 minutes and the supernatant was pipetted of and saved. The pellet was resuspended in Tris and centrifuged at 1 500 g for 15 minutes, and subsequantly mixed with the other supernatant fraction. The mixture, with the exine, was centrifuged at 20 000 g for 30. The exine was collected in the pellet. For isolation of the intine, the suspension of ruptured cysts was centrifuged at 10 400 g for 30 minutes. The supernatant, containing the intine, was poured off and the pellet was dicarded. The solution was concentrated from 1 L to 50 ml by evaporation on a rotary evaporator. The remaining 50 ml was dialysed against RO-water for 72 hours, and the water was changed three times a day. The dialysed solution was centrifuged at 30 000 g for 30 minutes to pellet the intine.

3.5 Production and extraction of alginate

The amount, and composition of alginate synthesized by *Azotobacter* species depends on the growth conditions. It has been reported that the concentration of inorganic phosphate has a large effect on the synthesis of alginate, and that phosphate limitation increases the alginate production [165]. In a phosphate limited environment, fructose was found to be a better carbon source with respect to alginate synthesis, than glucose [166]. Alginate production also increase when a nitrogen source is added to the medium [167]. In addition to the specific nutrients, the pH of the medium is particularly important. Clementi et al. found that when pH dropped below 5.5, the alginate production declined to zero [168]. The pH of the medium naturally decreases as alginate is excreted. To oppose this decrease in pH, MOPS was added to the medium, effectively counteracting the acidification of the culture, allowing the continued synthesis and exretion of alginate.

Most of the alginate produced by *Azotobacter* is excreted into the media. Separation of the excreted alginate from the culture can be done in several ways. One very convenient method is to take advantage of the solubility properties of alginate in different solutions. Ethanol, being less polar than water, decreases the solubility of alginate. The addition of inorganic salts further decreases the solubility by neutralizing the negative charges on the alginate molecules, and eventally leads to precipitation of the alginate [169]. Alginate can also be precipitaded by decreasing the pH of the solution sufficiently [55].

Procedure

For production of alginate, 2x500 ml RA1 medium in 3 L shake flasks was inocculated at 0.5 % with *A. chroococcum* grown ON in BM. The cultures were incubated for 48 hrs before alginate was harvested. To harvest the alginate, The cultures were centrifuged at 12 000 rpm for 40 minutes. The supernatant, containing the alginate was filtered through a 0.22 µm filter to remove debris. Deacetylation was done by adding NaOH to 0.1 M and incubating with stirring at room temperature for 1 hr. After incubation, NaOH was added to the pH reached 7.5. The solution was dialyzed agains MQ-water for 48 hrs, changing the water three times a day. Alginate was then precipitated by adding NaCl to 0.2 % and 1 L 96 % ethanol. The alginate was collected by centrifugation at 12 000 rpm for 30 minutes. The supernatant was discarded and the alginate pellet was washed three times with 70 % ethanol, followed by two times with 96 % ethanol. This was done by addition of the ethanol, careful shaking followed by centrifugation at 12 000 rpm for 20 minutes. After removing as much ethanol as possible, the alginate pellet was left to dry in a fume hood for 48 hrs before further analysis.

3.6 Carbohydrate analysis by ^{1}H-NMR and HSQC

Nuclear Magnetic Resonance (NMR) is a versatile and powerful spectroscopic method for the characterization of a wide range of compounds, both organic and inorganic. The method takes advantage of the nuclear spin (I) that certain atoms possess [170]. This is a number that depends on the numbers of protons and neutrons in the atomic nuclei, which both has a spin of $\pm 1/2$. Adding up the spins of protons and neutrons in the nucleus gives the nuclear spin. This is zero for a nuclei with even numbers of both protons and

neutrons, and non-zero for nuclei with odd numbers of neutrons, protons, or both [171]. The spin of the particles makes the atom a magnetic dipole, which is useful for applications such as NMR. The orientation of this dipole is normally random, but when exposed to a magnetic field, it will orient itself according to the magnetic field. The number of possible orientations for a single atom is 2I+1. An atom with spin=$^1/_2$ will thus have two possible orientations, with different energy levels. Exposing the nuclei to electromagnetic radiation will cause the nuclei having the spin-orientation of lowest energy to absorb energy from the radiation and flip to the spin-orientation of highest energy. This is known as resonance. Removing the electromagnetic radiation lets the nuclei revert to the low-energy state. In doing so an oscillating field is generated, generating a signal that can be measured [170]. Many NMR spectrometric analyses employs a method where short pulses of radiowaves are posed on the sample to simultaneously excite the nuclei. To obtain as much synchrony in the excitations as possible, short and strong pulses are applied. The angle of the pulse describes the angle of the pulse relative to the magnetic field. A 90° pulse angle gives the strongest signal, and is therefore often used [172].

The energy it takes to make the nucleus transfer from its low energy state to the high energy state differs for different nuclei, and therefor different frequencies of electromagnetic radiation is necessary to obtain resonance. This is not only dependent on the type of atom, but also on the strength of the magnetic field felt by the atom. When several atoms come together as a molecule, the resonance frequency of a nuclei is affected by its chemical environment. This is a result of nearby electrons, which decreases the magnetic field experienced by the nuclei in a process called shielding. The shielding effect of electrons makes it possible to distinguish identical nuclei in different chemical environments. This is visible in an NMR-specter as a difference in frequency necessary for resonance. The difference is most often given in ppm (parts per million) change in frequency with respect to a standard (Figure 3.5) [170]. The intensity of the signal is most often given as a relative intensity on the y-axis, as compared to the signal of the standard compound. For ^{1}H-NMR, the integrated area under a peak is proportional to the number of H-atoms creating that peak. This makes it possible to calculate relative amounts of different compounds in a sample. This does generally not apply to for example ^{13}C-NMR [172].

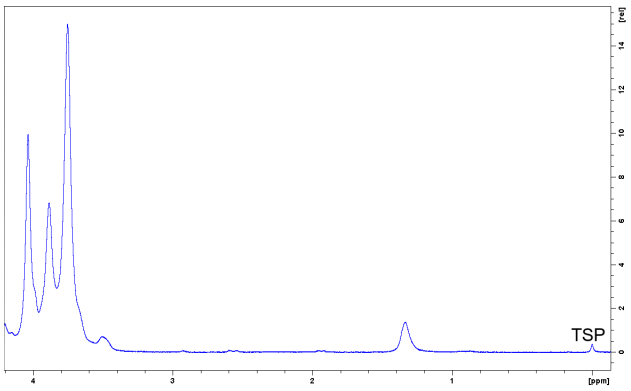


Figure 3.5: A simplified ^{1}H-NMR spectrum, showing the chemical shift of ^{1}H relative to TSP on the x-axis, and the intensity of the signal on the y-axis. The integrative area under the peaks gives the relative amount of protons.

In addition to one-dimensional (1D) ^{1}H-NMR many other NMR spectroscopy techniques exist. Two-dimensional (2D) correlated NMR spectroscopy is a method where the chemical shifts and the measured signal of two NMR "runs" are correlated. In a heteronuclear 2D, the chemical shifts and signal of two different nuclei, for example ^{1}H and ^{13}C, are measured and correlated. Compared to 1D NMR, 2D-NMR is able to obtain higher resolution. The resolution can often be a problem with biomolecules as high molecular weights can cause broad and overlapping signals [170]. Heteronuclear single quantum coherence (HSQC) is an example of 2D correlated NMR spectroscopy with high sensitivity [173].

Procedure

All NMR-experiments in this work was carried out by the Norwegian Biopolymer Laboratory (NOBIPOL) at NTNU. Sample preparation was done by dissolving 10 mg of dry extracted polymer material in 500 µl 99.9% D_2O with 1 µl of 0.5% TSP (3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid) dissolved in D_2O. The dissolved sample was transferred to a 5 mm NMR tube and placed in the spectrometer (Bruker AV-IIIHD 800 MHz). The temperature was set to 25°C and the angle of the pulses was 90°. The length of the pulse-width was calculated for each sample by the "pulsecal" command. The ^{1}H-spectrum was obtained using free induction decay (FID) size of 65536s, spectral width of 12 ppm, 16 scans, and d1 (delay before pulse) of 4s. The ^{1}H-^{13}C HSQC spectrum was obtained using a FID size of 2048s/512 (^{1}H/^{13}C), spectral width of 12/120 ppm, 16 scans, and d1 of 2s. Recording, processing and analyzing of spectra were done in TopSpin 3.5 or 4.0.1.

3.6.1 Carbohydrate monomer analysis by HPAEC-PAD

Chromatography is a method of separation of molecules in a mixture based on their affinity to a material, when passing through this material. The material is known as the "stationary phase", while the analyte mixture is dissolved in what is known as "the mobile phase". Several types of chromatographic methods has been developed by altering the stationary phase, the mobile phase, or the speed of the molecules through the system [115].

In liquid chromatography, the mixture of molecules to be analysed is dissolved in a liquid to make up the mobile phase. During "high performance" liquid chromatography, the liquid mobile phase is exposed to high pressure, forcing it through the column of material making up the stationary phase. Ion chromatography takes advantage of the attraction forces between positively and negatively charges of molecules. The stationary phase, the ion exchanger, is composed of a charged material and the molecules in the analyte are separated based on charge or polarity. High Performance Anion Exchange Chromatography (HPAEC) is performed with a column packed with positively charged material to which the analyte molecules has varying affinities. The molecules will elute at different times, depending on their affinities to the column [115]. When they are eluted, a signal is created. Typically the signal is created by a change in the conductivity of the liquid being eluted. Pulsed amperometric detection (PAD), or pulsed electrochemical detection (PED) is a detection method where the analyte molecules are oxidized on an

electrode, and the current developing is proportional to the concentration of molecule in the solution [174]. Different types of electrodes may be used, and gold is a relatively popular material for this.

HPAEC-PAD is an excellent method for the separation of carbohydrates, including monosaccharides. Due to their acidic nature in high pH solutions, carbohydrates can be separated in an anionic exchange column. The different monosaccharides has different dissociation constants, and consequently they have different affinities to the column at a certain pH, making them separable. PAD offers a very sensitive method of carbohydrate detection [174], and is therefore very suitable for detection of molecules of low quantities.

Procedure

The monomer analysis by HPAEC-PAD was carried out by NOBIPOL at NTNU.

Chapter 4

Results

4.1 Encystment of *Avin05390*, *Avin05750*, and *Avin17310* mutants

It has in previous work been discussed that *Avin05750*, *Avin05750*, and *Avin17310* may have important functions during the encystment process of *A. vinelandii* [89, 90]. These genes are all upregulated during the encystment process (Ertesvåg, unpublished), and hypothetically encodes an exopolysaccharide biosynthesis protein, an alanine dehydrogenase and a regulator of the MarR family, respectively. To investigate the importance of these genes for the cyst formation in *A. vinelandii*, it was necessary to construct mutants where each of the genes were separately inactivated, and to assess the encystment capacity of the mutant strains. Encystment capacities were determined by testing their resistance towards desiccation. Strain ATCC 12518Δ05390 with a mutation in *Avin05390* has previously been made [89], while an *Avin05750* and an *Avin17310* mutant remained to be established.

4.1.1 Cloning of *Avin17310* to construct an *Avin17310* mutant strain

The construction an *A. vinelandi* mutant with inactivated *Avin17310* was planned to be done in three steps. First, a plasmid containing a mutated *Avin17310* gene had to be constructed. This plasmid had to be introduced into *A. vinelandii* by conjugation, and the mutate gene would replace the chromosomal gene by homologous recombination.

Construction of recombination plasmid

The plasmid pAS02, which is shown in Figure 4.1, served as a starting point for the construction of a recombination plasmid that could be used in the inactivation of *Avin17310*. This plasmid, constructed in previous work, carries the downstream element of *Avin17310*, as well as *OriT*, and replication is controlled by the XylS/Pm promoter system. The remaining step in the construction of the final conjugative recombination plasmid, was to

insert the upstream element of *Avin17310* (O17310). This would result in a deletion mutation in the gene. The proposed cloning procedure is outlined in Figure 4.1.

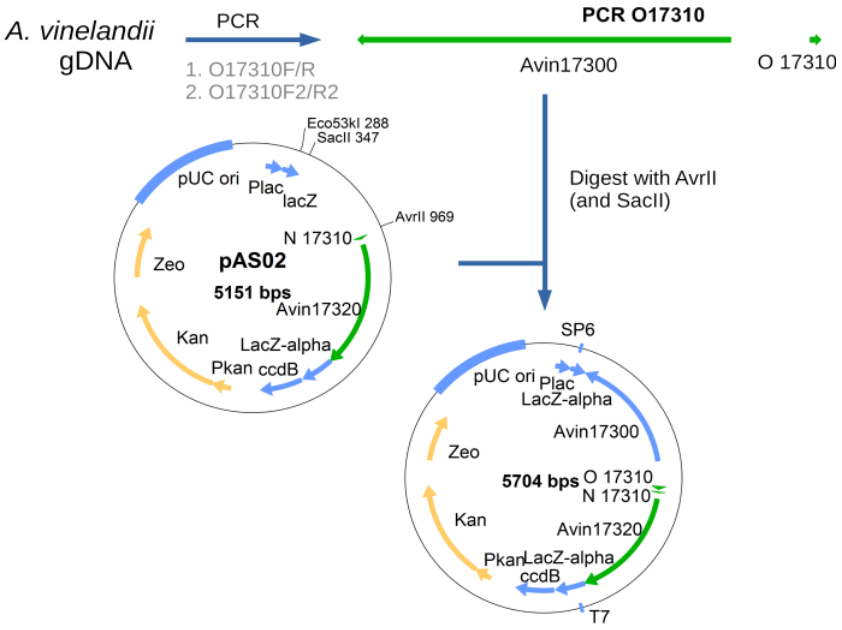


Figure 4.1: Proposed cloning procedure for the cunstruction of a conjugative recombination plasmid for the inactivation of *Avin17310* in *A. vinelandii*. The upsteram element of *Avin17310*, O17310, was amplified by PCR. Two separate primer pairs, marked 1 and 2, were used in separate PCR reactions. The amplified fragment was to be inserted into pAS02 to construct the final plasmid.

A PCR of *A. vinelandii* gDNA with the primers O17310F2 and O17310R2 was performed to amplify the upstream element of *Avin17310*. The product was analyzed by gel electrophoresis, which showed that the desired fragment of 1.1 kb was obtained (Figure 4.2, A). The fragment obtained by PCR was cut out from the gel and purified before it was digested by AvrII-SacII. pAS02 was digested with AvrII-SacII, producing fragments of 0.6 and 4.5 kb (Figure 4.2, B).

Figure 4.2: (A) PCR product from PCR with the primer pair O17310F2/O17310R2, and *A. vinelandii* gDNA as template. The expected fragment of 1.1 kb was obtained. (B) Digestion of the PCR product and pAS02 with AvrII-SacII. Lane 1: pAS02 with the expected fragments of 4.2 and 0.6 kb. Lane 2: Digested PCR-product with the expected size of 1.1 kb.

The digested upstream element of *Avin17310* and the 4.5 kb backbone fragment of pAS02 was cut out from the gel and purified. The fragments were ligated, and the ligation mix was transformed to *E. coli* S17.1. 8 plasmids were isolated from transformants and subsequently digested with AccI-PstI. Successfully inserted PCR product would give fragments of 3.8 and 1.9 kb, while pAS02 would give a single fragment of 5.2 kb. Analysis by gel electrophoresis showed that the PCR product had not been inserted into pAS02 (Figure Figure 4.3).

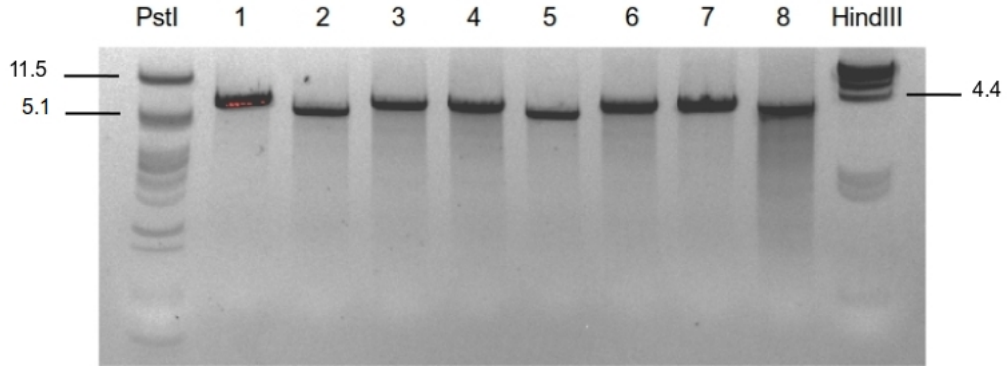


Figure 4.3: Ligation products from the ligation between pAS02 and the *17310* upstream element, digested with AccI-PstI. The expected bands of 3.8 and 1.9 kb were not abtained.

Since SacII is an enzyme that requires binding to two recognition sites for optimal performance [175], SacII was replaced by Eco53kI in another digest of pAS02, together with AvrII. This was done in an attempt to decrease the amount of linearized vector in the ligation reaction. The digest was analyzed by gel electrophoresis, which showed the expected fragments of 0.6 and 4.5 kb. As Eco53kI is a restriction enzyme producing blunt ends, the PCR product was only cut with AvrII before ligation to the Eco53kI-AvrII digested pAS02. Plasmids resulting from this ligation gave fragments of 5.2 kb when digested with AccI-pstI, which indicates that only uncut, or religated pAS02 had been transformed (Figure 4.4).

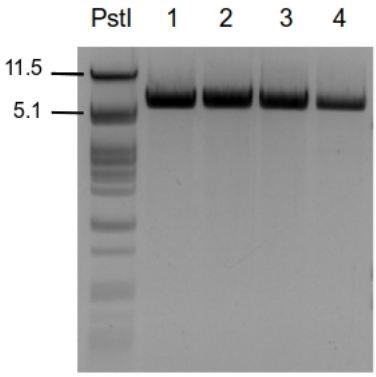


Figure 4.4: Products from ligation between AvrII-Eco53kI digested pAS02 and AvrII digested O17310, digested with AccI-PstI. All plasmids from transformants gave bands of 5.2, which is the size of the vector.

As none of the attempts to insert the PCR fragment directly into pAS02 had been successful, it was decided to try a new approach, which included TOPO cloning of the PCR fragment before cloning it into pAS02. The outline of this cloning strategy is given in Figure 4.5.

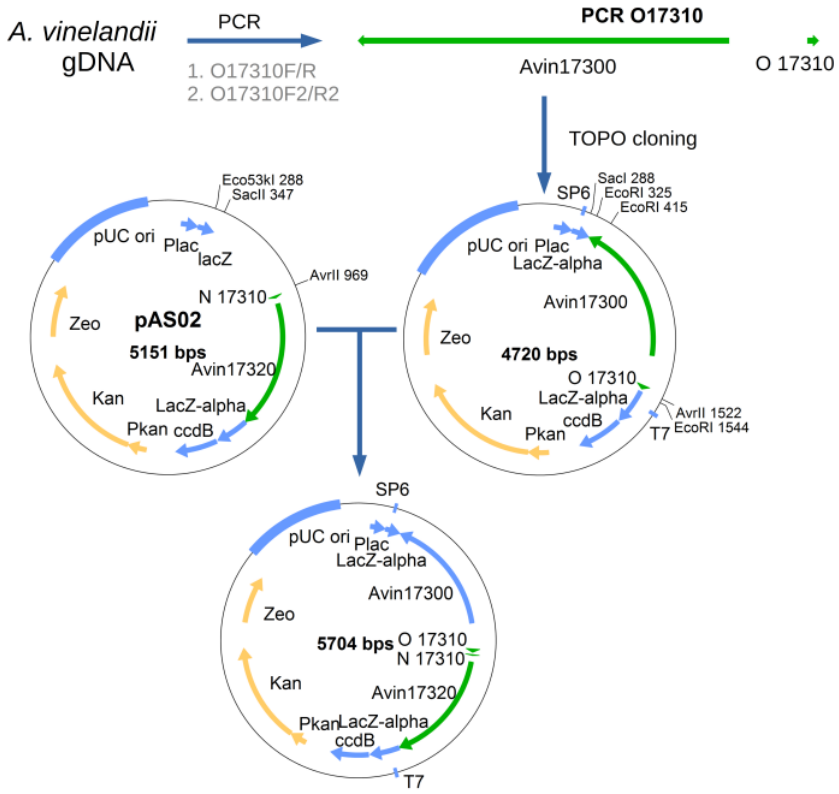


Figure 4.5: Overview of the second cloning strategy implemented to contruct a recombination plasmid for the inactivation of *Avin17310*, which includes TOPO cloning of PCRd17310 fragment obtained by overlap extension PCR, and subsequent cloning of the fragment into the conjugative vector pHE216.

Using TOPO-cloning allowed for the utilization of different restriction enzymes, and possibly also more efficient cutting of the PCR fragment ends. The transformants resulting from the TOPO cloning were checked by isolating plasmids and digesting with EcoRI. Inserted fragment would give bands of 1.1 and 3.5 kb. Only the band of 3.5 kb was visible on the gel for all but one of the transformants. This indicates that the PCR fragment was not inserted and that only the TOPO vector was transformed (Figure 4.6). The transformant carrying a plasmid with an insert, had an insert of the incorrect size. It was also attempted to TOPO clone a 2.2 kb PCR fragment containing both the upstream and downstream elements of *Avin17310*. This resulted in transformants containing only the TOPO vector, without any insert.

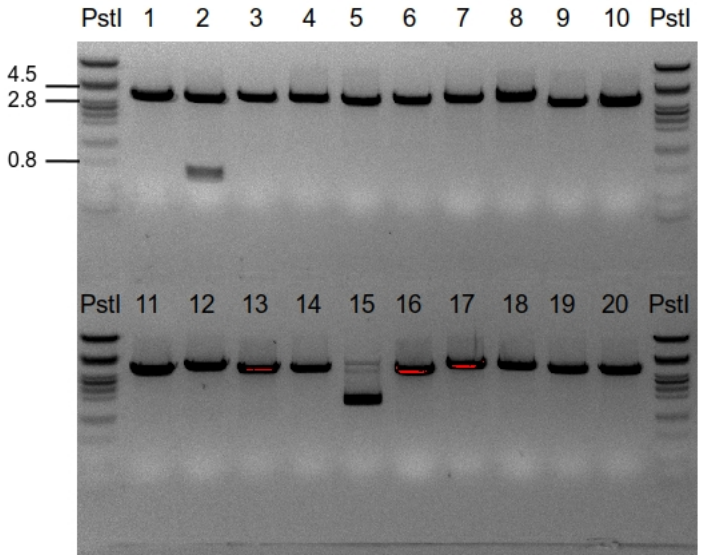


Figure 4.6: Plasmids resulting from TOPO cloning of the upstream element of *17310*. The plasmids were digested with EcoRI, resulting in a 3.5 kb band, instead of the bands of 3.5 and 1.1 kb, expected if the PCR product was inserted. The vector in lane 2 contained an insert, but of the wrong size.

Due to unsuccessful attempts of cloning the upstream part of *Avin17310*, it was decided to run PCR with a new pair of primers, 17310fragmentF and 17310fragmentR. The new primer pair was used in a PCR reaction with *A. vinelandii* gDNA as template, which resulted in a fragment of 2.3 kb (Figure 4.7, A). This fragment was used as a template in a new PCR reaction with O17310F2 and O17310R2 as primers. The correct fragment of 1.1 kb was obtained (Figure 4.7, B).

Figure 4.7: (A) PCR product constructed with the primer pair 17310fragmentF/17310fragmentR, and *A. vinelandii* gDNA as template. The expected band of 2.3 kb was obtained. (B) PCR product constructed with the primer pair O17310F2/17310R2 and the product in A as template. The expected band of 1.1 kb was obtained.

The cloning procedures described in Figure 4.1 and Figure 4.5, including direct cloning into pAS02 and TOPO cloning of the upstream PCR fragment was repeated, but with the same result. As none of the many attempts to clone *Avin17310* had been successful, it was decided to discontinue this work.

Cloning of *Avin17310*

Although the cloning of the 2.2 kb recombination fragment, containing a mutated *Avin17310* gene, and the 1.1 kb upstream fragment of *Avin17310*, failed, two vectors carrying shorter fragments with *Avin17310* were easily constructed by PCR amplification. This was done by a PCR with the primer pairs 17310NdeI/17310ER and 17310NdeI/1731020ER, followed by TOPO cloning. These PCR fragments were 0.4 and 1.7 kb, and appeared to be clonable, as opposed to the 2.2 and 1.1 kb fragment, which appeared to be unclonable (Figure 4.8). An extra verification of the clonability of these fragments was obtained by successful cloning of the fragments into pHE216. Both the 0.4 kb fragment and the 1.7 kb fragments were sequencend and shown to contain the correct sequence. The sequence alignments are given in Appendix G and Appendix E.

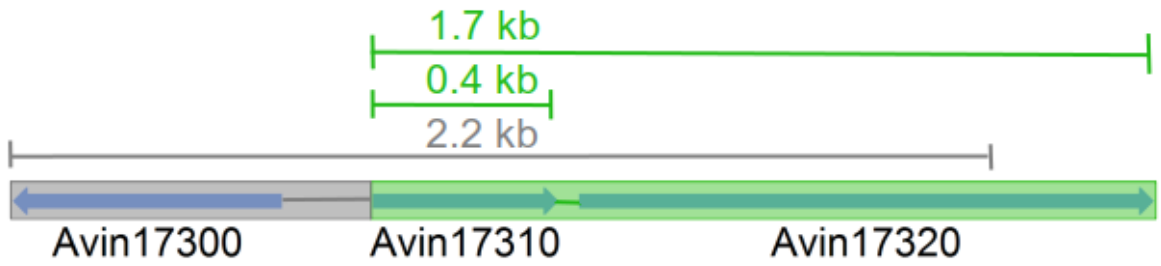


Figure 4.8: Clonable (green) and unclonable (grey) parts of the PCR products containing *Avin17310*.

4.1.2 Construction of a *Avin05750* mutant strain

The inactivation of *Avin05750* in *A. vinelandii* was done by construction of a conjugative recombination plasmid, which was introduced into *A. vinelandii* by conjugation, and subsequent homologous recombination.

Construction of recombination plasmid

A conjugative recombination plasmid carrying *avin05750* has been made previously [90]. This plasmid, designated pAS03, is based on an RK2 derived vector carrying the XylS/Pm system for replication, making it a conditional suicide vector. The final step in the process of making a vector for the purpose of knocking out *Avin05750*, was to insert tetracyclin resistance genes in the center of *avin05750*. This would make the gene nonfunctional, and upon recombination with the chromosome, the chromosomal gene would be exchanged with this nonfunctional gene. The outline of the cloning procedure is given in Figure 4.9.

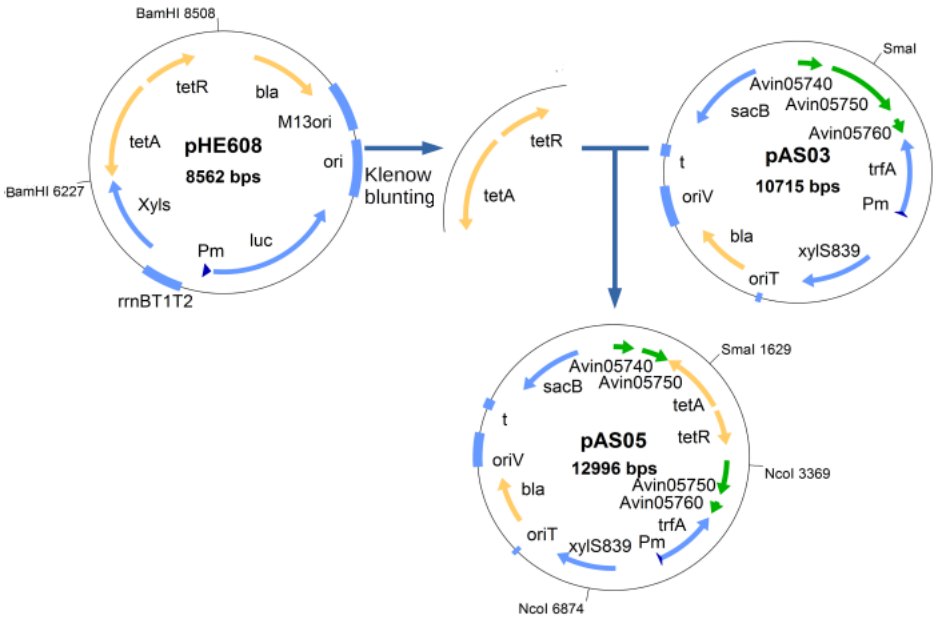


Figure 4.9: Cloning procedure to construct the recombination plasmid pAS05 for deletion of *Avin05750* in *A. vinelandii* Tetracycline resistance genes were cut otu from pHE608 by digestion with BamHI. The fragment was blunted with klenow and ligated to SmaI digested pAS03.

The tetracyclin resistance genes were cut out from pHE608, by digestion with BamHI, and the ends were blunted, using Klenow. This produced fragments of 6.3 and 2.3 kb (Figure 4.10).

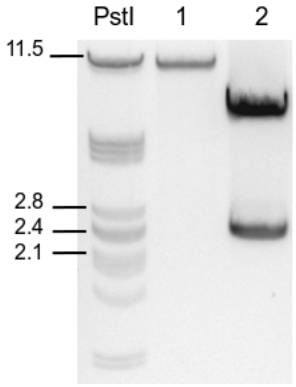


Figure 4.10: Digested pAS03 and pHE608. Lane 1: pAS03 digested with SmaI giving the expected band of 10.7 kb. Lane 2: pHE608 digested with BamHI giving the expected bands of 6.3 and 2.3 kb.

The 2.3 fragment, with blunt ends, was cut from the gel and purified before it was ligated to SmaI digested pAS03, which also has blunt ends (Figure 4.10). The ligation products were transformed to *E. coli* S17.1. Plasmids from transformants growing on selective plates were isolated and digested with NcoI-SmaI to verify the insertion of the tetracyclin resistance genes at the correct site within the plasmid. All four tested transformants carried the correct plasmid, two of each orientation of the insert (Figure 4.11). The plasmid obtained was designated pAS05.

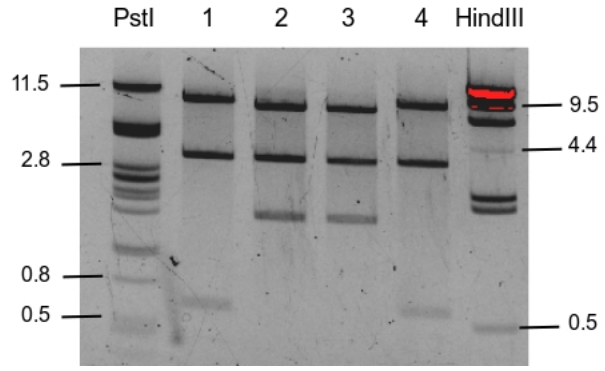


Figure 4.11: Digestion of product formed by ligation between pAS03 backbone and pHE608 fragment. Lane1 and 4: Products digested with NcoI-SmaI, giving the expected band lengths of 8.8, 3.5, and 0.6 kb for the foreward orientation of the fragment. Lane 2 and 3: Products digested with NcoI-SmaI, giving the expected band lengths of 7.8, 3.5, and 1.7 for the reverse orientation of the fragment.

4.1.3 Inactivation of *Avin05750* by conjugation and homologous recombination

To create an *Avin05750* mutant, the chromosomal *Avin05750* had to be replaced with the disrupted version of the gene, carried by pAS05. The constructed recombination plasmid, pAS05, was first transferred to *A. vinelandii* ATCC 12518 by conjugation. BA plates containing apramycin was used to select for transconjugants. Several colonies appeared on the selective plates.

The exchange of the chromosomal *Avin05750* with the disrupted *Avin05750* on pAS05, was carried out by homologous recombination. This was done by first transferring transconjugants to liquid growth media, without m-toluate, to select for cells that had integrated the plasmid into their chromosome. After several sub-cultivations, the cells were plated on plates with sucrose and tetracycline to select for cells that had undergone a second crossover and displaced the plasmid, while integrating the mutated *Avin05750*. A confirmation of the genetic exchange was done by checking for growth on apramycin, while elimination of the plasmid backbone was confirmed by the inability to grow on ampicillin.

To ultimately confirm the inactivation of *Avin05750*, and that no wild-type genes resided on some of the chromosome copies of the mutated strains, PCR of gDNA from cells growing on apramycin, but not on ampicillin, was performed (Figure 4.12, A). The primers used were ADH R2 and ADH NdeI. The mutated *Avin05750* would give a band of 3.5 kb, while the wild-type *Avin05750* would give a band of 1.2 kb. As the first PCR was inconclusive with regards to the mutants being pure, an additional PCR of colony 9 and 10 with the primer pair ADH F/ADH R was performend to confirm that no wild-type genes were present. These primers would produce a 4.4 kb fragment for the mutated gene, and a 2.0 kb fragment for the wild-type gene. Both colonies appeared to contain only mutated gene copies (Figure 4.12, B). The mutated strain was designated ATCC 12518 Δ05750.

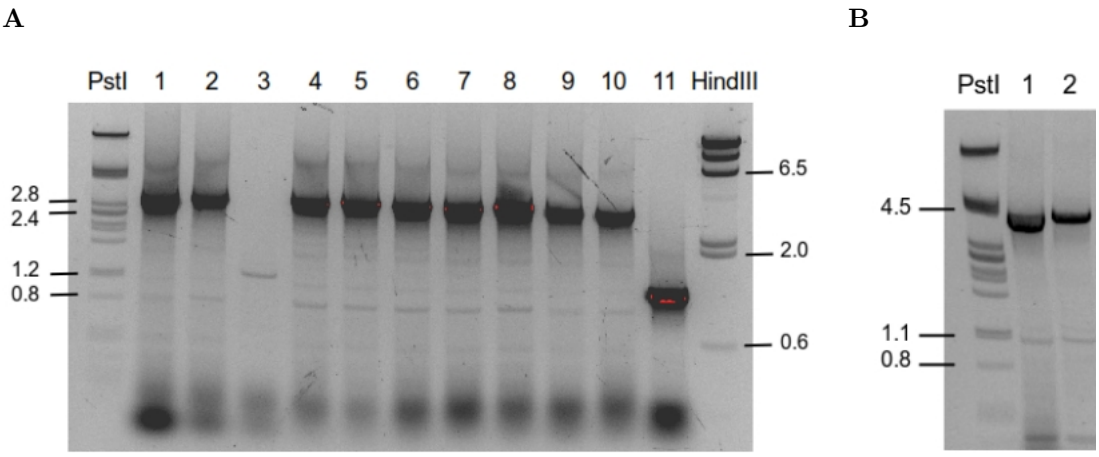


Figure 4.12: Verification of *Avin05750* mutants by pcr. (A) PCR with the primer pair ADH R2/ADH NdeI. Expected band size of mutated gene was 3.5 kb, and expected band size of wild-type gene was 1.1 kb. Lane 1-10: PCR products from colony 1-10 of *Avin05750* mutants. Lane 11: PCR product from *A. vinelandii* WT. (B) PCR with the primer pair ADH F/ADH R. Expected bands were 4.4 kb for the mutated gene, and 2.0 for the wild-type gene.

4.1.4 Construction of a complementation vector for the complementation of *Avin05750* mutant

It is important to verify that changes in phenotype after performing gene deletions is a result of the specific mutation of the gene rather than secondary mutations or other unknown parameters. This is done by complementing the mutant strain with the respective wt gene. The resulting complemented strain should be screened for restoration of the wt phenotype, which would confirm the genetic basis of the change in phenotype seen in mutants. To complement the mutant strain ATCC 12518Δ05750, a complementation vector was first constructed. This vector was based on RK2 and the XylS/Pm system for expression of *Avin05750*. The construction of the vector involved PCR, TOPO cloning followed by restriction digests and ligation. The cloning procedure is outlined in Figure 4.13.

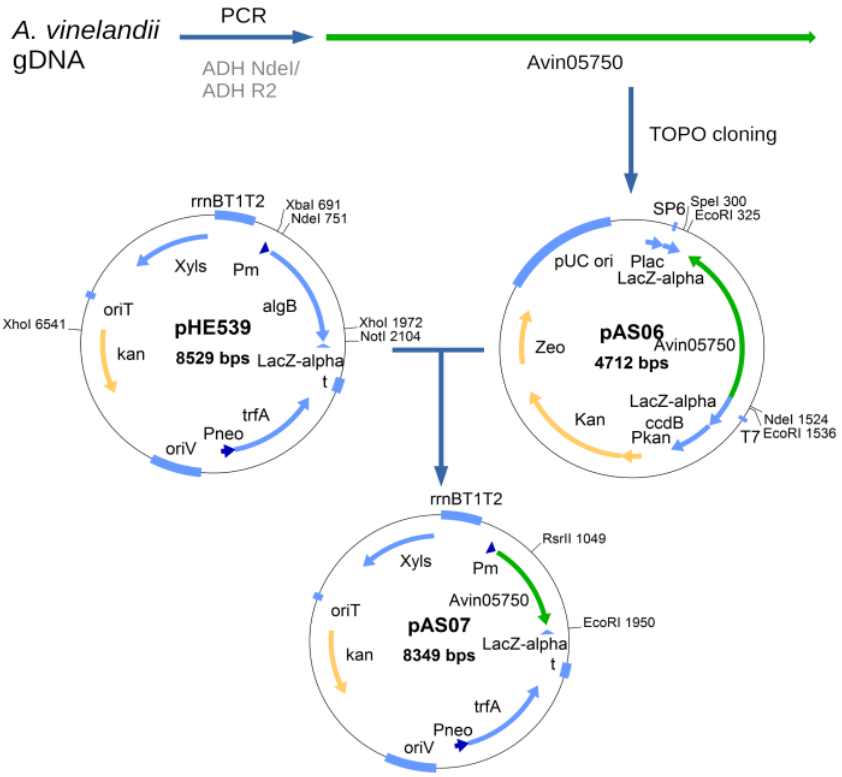


Figure 4.13: Cloninc procedure to obtain a complementation vector containing *Avin05750*, which included PCR with the primer pair ADH NdeI/ADH R2, TOPO cloning of the PCR fragment, and insertion into pHE539.

Avin05750 was first amplified by PCR of *A. vinelandii* gDNA with the primer pair ADH NdeI/ADH R2. This produced a fragment of 1.2 kb (Figure 4.14), which was the expected size.

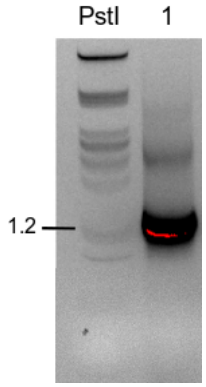


Figure 4.14: PCR products obtained by PCR of *A. vinelandii* gDNA with the primer pair ADH NdeI/ADH R2. The expected band of 1.2 kb was obtained.

The PCR product, containing *Avin05750*, was inserted into the pCR-Blunt II-TOPO vector and cloned in *E. coli* DH5α. Plasmids isolated from TOPO transformants were digested with EcoRI to verify the insertion of *Avin05750*. Two of the four digested plasmids gave the expected bands of 1.2 and 3.5 kb (Figure 4.15). The obtained plasmid was designated pAS06.

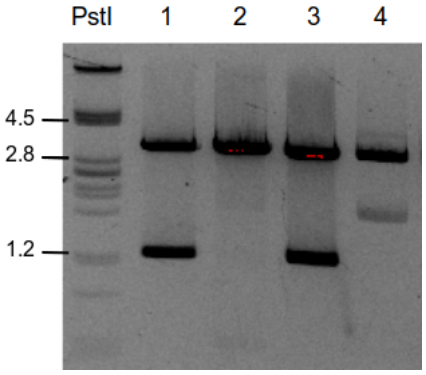


Figure 4.15: Plasmids from TOPO transformants, digested with EcoRI. Expected band sizes were 1.2 and 3.5 kb, which was obtained by the clones in lane 1 and 3.

Since PCR amplification in certain instances can introduce mutations into the amplified sequence, the PCR product was sequenced after insertion into the TOPO vector, using the primers M13F and M13F. The sequence was aligned to the sequence *Avin05750*, obtained from the NIH sequence database GenBank. The alignment can be found in Appendix F. No deviations from the sequence of *Avin05750* was detected.

Avin05750 was cut out from pAS06 using NdeI and SpeI and inserted into NdeI-SpeI digested pHE539. The digests gave the expected fragments of 1.2 and 3.5 kb for pAS06, and 1.4 and 7.1 kb for pHE539 (Figure 4.16).

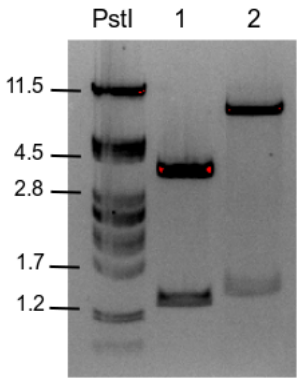


Figure 4.16: Restriction digest of pAS06 and pHE539 using NdeI and SpeI. Lane 1: pAS06 with the expected band sizes of 1.2 and 3.5 kb. Lane 2: pHE539 with the expected band sizes of 1.4 and 7.1 kb.

The 7.1 kb vector backbone of pHE539 and the 1.2 kb fragment of pAS06 was cut out from the gel, purified and ligated. The ligation mixture was transformed into *E. coli* S17.1, which were plated on selective plates. Plasmids were isolated from transformants and digested with EcoRI and RsrII. The correct ligation product would yield fragments of 0.9 and 7.4 kb. Plasmids from all four tested transformants yielded fragments of the expected size (Figure 4.17), confirming the correct ligation product. This plasmid was designated pAS07, and contains *OriT*, enabeling conjugation by *E. coli* S17.1, and the expression of *Avin05750* is under the control of the *Pm* promoter.

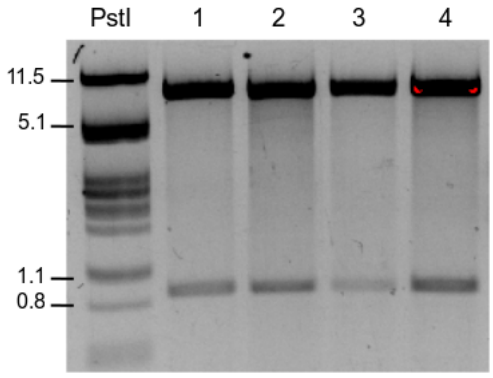


Figure 4.17: Plasmids resulting from ligation of pAS06 fragment into pHE539, digested with EcoRI-RsrII. All tested plasmids gave the expected band sizes of 0.9 and 7.4 kb.

4.2 Desiccation resistance in *Avin05390* and *Avin05750* mutants

Measuring the degree of desiccation resistance in an *Azotobacter* culture is a reliable way of determining the degree of encystment in the culture [23]. This means that only desiccation resistant cysts are counted as true cysts. To investigate whether the genes *Avin05390* and *Avin05750* have an effect on the encystment abilities of *A. vinelandii*, the desiccation resistance of the two mutants ATCC 12518Δ05390 and ATCC 12518Δ05750 was assessed and compared to that of the wild type. This was done by growing cells in liquid- or solid encystment media for five days before drying culture samples for seven days. Plate counts were done before and after drying.

Cultures grown in liquid BM with βHB as the encysting agent did not produce cysts. The experiment was repeated in a total of 9 replicates for ATCC 12518 and ATCC 12518Δ05390, and 3 replicates for ATCC 12518Δ05750. Each replicate was plated and counted three times. No cultures surived desiccation for seven days (Table 4.1).

Table 4.1: Desiccation resistance of strains grown in liquid BM with βHB for encystment. The cultures were dried for seven days.

Strain	Genotype	Desiccation resistance [%]
ATCC 12518	wild type	<0.001
ATCC 12518Δ05390	*5390*::Apr	<0.001
ATCC 12518Δ05750	*05750*::Tet	<0.001

Since *A. vinelandii* 12518 WT did not seem to form cysts when grown in the liquid encysting medium, it was decided to grow the cells on solid *n*-butanol agar for encystment. Five replicates of each stran (ATCC 12518 WT, ATCC 12518Δ05390, and ATCC 12518Δ05750) were included, and each replicate was plated and counted three times. When growing the cells on this solid encystment medium, a small fraction of some of the cultures were able to withstand the desiccation treatment (Table 4.2).

Table 4.2: Desiccation resistance of strains grown on solid *n*-butanol for encystment. The cultures were dried for seven days.

Strain	Genotype	Desiccation resistance [%]
ATCC 12518	wild type	0.008
ATCC 12518Δ05390	*5390*::Apr	<0.001
ATCC 12518Δ05750	*05750*::Tet	0.001

4.3 Desiccation resistance in three wild type srains of *A. vinelandii*

Finding that the encystment of *A. vinelandii* ATCC 12518 WT and both mutants tested (section 4.2) was insignificant, prompted the investigation of the encystment abilities of

A. vinelandii ATCC 12518 as compared to other WT strains of *A. vinelandii*. In the comparison, *A. vinelandii* strain ATCC 12837 and strain E were used as references. Two separate cultures of each strain was tested by the method described in section 3.4.1, and three replicates of each culture were counted. The results, given in Figure 4.18, is the mean of the percentage encystmend obtained in the two cultures.

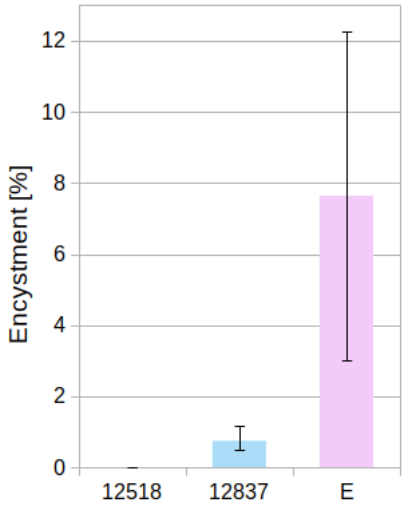


Figure 4.18: Encystment in *A. vinelanii* WT strains, measured by desiccation resistance. The given values are the mean of two separate cultures, and the error bars represents the minimum and maximum values.

A. vinelandii 12518 showed <0.001 % encystment. In contrast both *A. vinelandii* 12837 and *A. vinelandii* E showed encystment of over 0.5%, with strain E showing encystment up to 12.3%. Cells grown on encysting medium for five days were also inspected by phase-contrast microscopy. The cells appeared to be refractile and the intracellular PHB-granules was clearly visible for all strains.

4.4 Alkylresorcinol in *arsF*, *arsG*, and *arsH* mutants

4.4.1 Total alkylresorcinol in mutants defective in alkylresorcinol transport

The genes *arsF*, *arsG, and arsH*, encodes proteins thought to be involved in the transport of alkylresorcinols out of the cell during encystment in *A.vinelandii*. Dalland showed that mutations in either of these genes, inhibits the transport of alkylresorcinols to the cell surface [43]. To investigate whether the mutations of these genes also affects the synthesis of alkylresorcinols itself, it was necessary to do an assay of the total alkylresorcinol contents of the cells. To do this, alkylresorcinols were extracted with acetone and measured by the Fast Blue B method, as described in section 3.4.2.

An equation, giving the relationship between optical density and the concentration of alkylresorcinols, was first obtained by measuring OD_{620} of samples with known alkylre-

sorcinol concentration and fitting a line to the measurements(Figure 4.19). The measurements were done twice, and the given values are the mean of the two measurements.

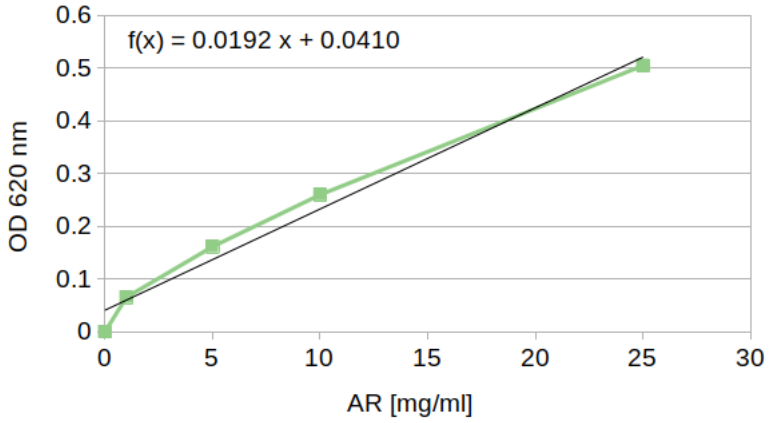


Figure 4.19: OD_{620} measurements of samples with known concentration to construct a standard, giving the relationship between concentration of alkylresorcinols and OD_{620}.

The total alkylresorcinols in *A. vinelandii* ATCC 12518, which was included as wild-type for comparison, and in each of the three mutants are given in Figure 4.20. The cells were grown for five days in liquid BM supplemented with 0.2% βHB for encystment before harvesting the cells and extracting alkylresorcinols. Two separate replicates of each strain were included, and each replicate was measured three times.

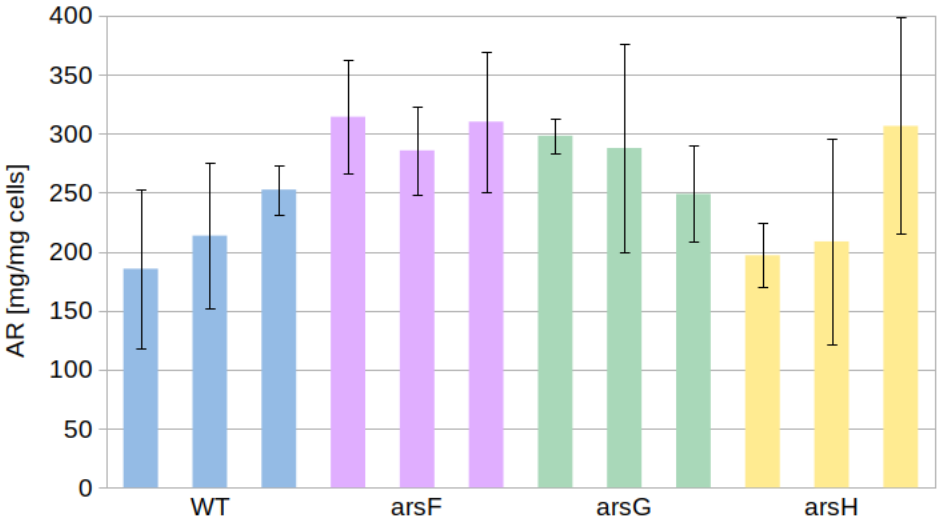


Figure 4.20: Alkylresorcinols extracted from *A. vinelandii* atcc 12518 wt, *arsF*, *arsG* and *arsH* mutants and measured by the Fast Blue B method. Two biological replicates were measured, and each bar is the average of three measurement of each biological replicate. The error bars represents standard deviations of the three measurements.

The alkylresorcinol concentration of the mutated strains do not deviate from the concentration of alkylresorcinols in ATCC 12518 WT. Nor is there any difference between

the three mutated strains. The standard deviation is, however, large due to technical difficulties when performing the measurements.

4.4.2 Complementation of *arsF*, *arsG*, and *arsH* mutants

To verify that the mutations of *arsF*, *arsG*, and *arsH* are the underlying causes of the lacking transport of alkylresorcinols to the cell surface, as described by Dalland [43], all three mutants were complemented with the wt version of the respective genes. This was done by constructing three complementation vectors, each containing the wild-type gene of either *arsF*, *arsG*, or *arsH* and transferring them to their respective mutant strains by conjugation. Assay of alkylresorcinols on the cell surface was subsequently done by adding Fast blue B solution directly to the encysted colonies.

Construction of complementation vectors

The construction of complementation vectors for the complementation of *arsF*, *arsG*, and *arsH* was performed in two steps. The first step involved the insertion of the constitutively active promoter, $P_{constitutive}$, from pHH106 into pHE539, resulting in plasmid pAS10. $P_{constitutive}$ was used since *Pm* requires m-toluate for activation. m-toluate has previously been shown to affect the presence of alkylresorcinols on the cell surface [94], and is therefore not suited in this case. After constriction of pAS10, *arsF*, *arsG*, and *arsH* was inserted into pAS10, and under control of $P_{constitutive}$. This resulted in the three complementation vectors pAS11 for complementation of ATCC 12518Δ*arsF*, pAS12 for complementation of ATCC 12518Δ*arsG*, and pAS13 for complementation of ATCC 12518Δ*arsH*. The outline of the cloning procedure is given in Figure 4.21.

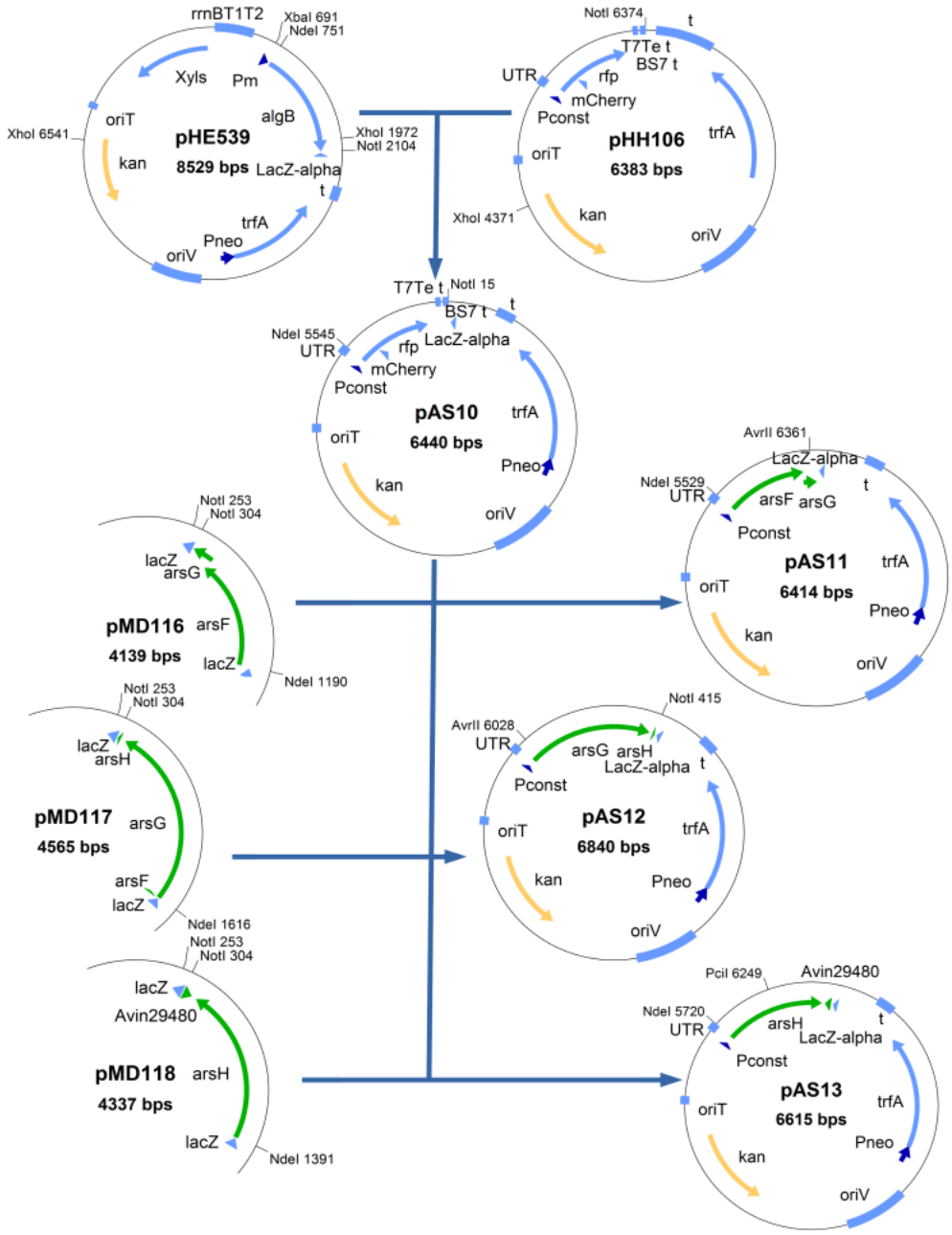


Figure 4.21: Construction of the three complementation vectors pAS11, pAS12, and pAS13. The promoter $P_{constitutive}$ was first cloned into pHE539, resulting in the plasmid pAS10. *arsF*, *arsG*, and *arsH* were separately cloned into pAS10, resulting in the complementation vectors pAS11, pAS12, and pAS13.

$P_{constitutive}$ has previously been shown to function in *A. vinelandii* [176], and was therefore cloned into pHE539, to ultimately be able to express *arsF*, *arsG*, and *arsH*. This was done by digesting pHH106 with XhoI and NotI, and pHE539 with XhoI, XbaI, and NotI (Figure 4.22).

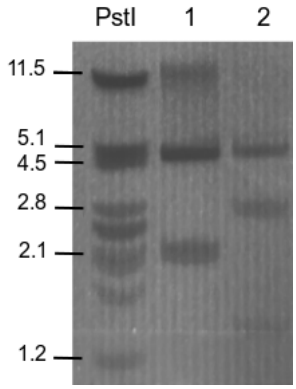


Figure 4.22: pHH106 digested with NotI-XhoI and pHE539 digested with NotI-XbaI-XhoI. Lane 1: Partly digested pHH106 with the expected band sizes 4.4 and 2.0 kb. Lane 2: Digested pHE539 with the expected band sizes 4.4, 2.8, 1.3 and 0.1 kb (0.1 not visible).

The 2.0 kb fragment of pHH106, and the 4.4 kb fragment of pHE139 were cut out from the gel and purified. The fragments were ligated and transformed into *E. coli* S17.1. Plasmids from transformants growing in the presence of kanamycin were digested with DraIII-SpeI for verification of the correct ligation product. The expected fragments were 4.5 and 1.9 kb. All tested plasmids were the desired ligation product (Figure 4.23). The new plasmid was designated pAS10.

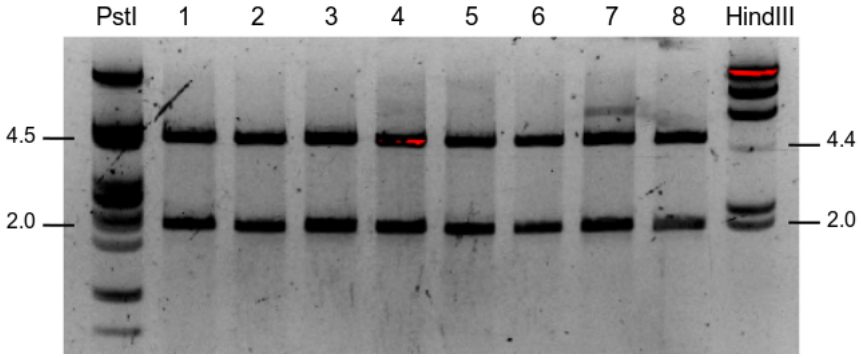


Figure 4.23: Products, formed by ligation of pHH106 and pHE539 fragments, digested with DraIII-SpeI. All digested plasmid gave the expected band sizes of 4.5 and 1.9 kb.

pAS10 has *OriT* for transfer by conjugation, and a promoter, $P_{constitutive}$ for the expression of genes placed under the control of it. To be able to express *arsF*, *arsG*, and *arsH* from $P_{constitutive}$, the genes were cloned into pAS10, in connection with $P_{constitutive}$. The first step of this was to digest pAS10 with NdeI-NotI, giving the vector backbone with a size of 5.5 kb, and a fragment of 0.9 kb. pMD116, pMD117, and pMD118 were digested with NdeI-NotI, giving fragments of 0.9, 1.3, and 1.1 kb, respectively, in addition to the vector backbone of 3.2 kb (Figure 4.24).

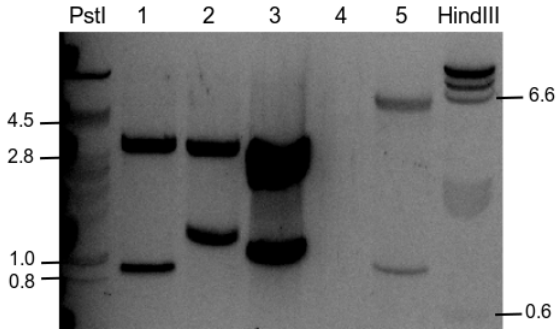


Figure 4.24: Digestions of pAS10, pMD116, pMD117, and pMD118 with NdeI-NotI. Lane 1: Digested pMD116 with the expected band sizes of 3.2 and 0.9 kb. Lane 2: Digested pMD117 with the expected band sizes of 3.2 and 1.3 kb. Lane 3: Digested pMD118 with the expected band sizes of 3.2 and 1.1 kb. Lane 4: Digested pAS10 with the expected band sizes of 5.5 and 0.9 kb.

The fragments of 0.9, 1.3, and 1.1 kb from pMD116, pMD117, and pMD118, respectively, were cut from the gel and purified. The 5.5 kb backbone of pAS10 was also cut from the gel and purified. The fragments were separately ligated into the 5.5 kb backbone of pAS10 and transformed into *E. coli* S17.1 cells. Plasmids were isolated from the colonies growing in the presence of kanamycin. To verify that the correct ligation products were obtained, a set of digestions were set up: the pAS10-pMD116 ligation product was digested with AvrII-NdeI, the pAS10-pMD117 ligation product was digested with AvrII-NotI, and the pAS10-pMD118 ligation product was digested with NdeI-PciI. The expected fragments were 5.6 + 0.8kb, 5.5 + 0.9, and 5.6 + 0.5, respectively. All tested colonies contained the correct ligation product (Figure 4.25). The new plasmids were designated pAS11, pAS12, and pAS13, and carrying *arsF*, *arsG*, and *arsH*, respectively. These plasmids now contained all elements necessary for complementation of the *A. vinelandii* ATCC 12518 Δ*arsF*, Δ*arsG*, and Δ*arsH* strains.

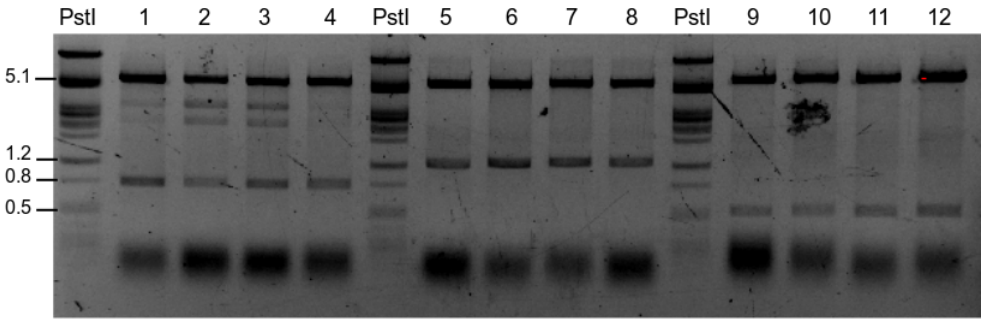


Figure 4.25: Products formed by ligation between pAS10 and pMD116, pMD117, and pMD118, verfied by restriction digestions. Lane 1-4: Ligation product from pAS10 and pMD113, digested with AvrII-NdeiI, resulting in the expected bands sizes of 5.6 and 0.8 kb. Lane 2-8: Ligation product from pAS10 and pMD117, digested with AvrII-NotI, resulting in the expected band sizes of 5.5 and 0.9 kb. Lane 9-12: Ligation product from pAS10 and pMD118, digested with NdeI-PciI, resulting in the expected band sizes of 5.6 and 0.5 kb.

Complementation and testing of alkylresorcinol transport

Alkylresorcinol present on the cell surface can be detected by the addition of Fast blue B, which reacts with the alkylresorcinols to produce a readily visible red color. The mutant strains ATCC 12518Δ*arsF*, ATCC 12518Δ*arsG*, and ATCC 12518Δ*arsH* do not transport alkylresorcinols to the surface of the cell, and hence do not give the red color when Fast blue B is added. If the mutated genes are the cause of the lacking alkylresorcinol transport to the cell surface, complementation with functional genes should restore the transport of alkylresorcinols to the cell surface, and consequently result in a red color when sprayed with Fast blue B solution.

To investigate whether complementation restored the transport of alkylresorcinols to the cell surface, pAS11, pAS12, and pAS13 were first conjugated into ATCC 12518Δ*arsF*, ATCC 12518Δ*arsG*, and ATCC 12518Δ*arsH*, respecitvely. Additionally, the three mutant strains and ATCC 12518 wt were complemented with pAS10 as control. Colonies growing in the presence of kanamycin were regarded as complemented. The complemented strains were grown on *n-butanol* agar plates with 0 µg/ml, 2 µg/ml, and 4 µg/ml kanamycin to induce encystment. After five to seven days of incubation, the plates were sprayed with Fast blue B solution to reveal any alkylresorcinol on the cell surfaces. No alkylresorcinol was detected on the cell surfaces of either of the mutants complemented with the wild-type genes (Figure 4.26, B).

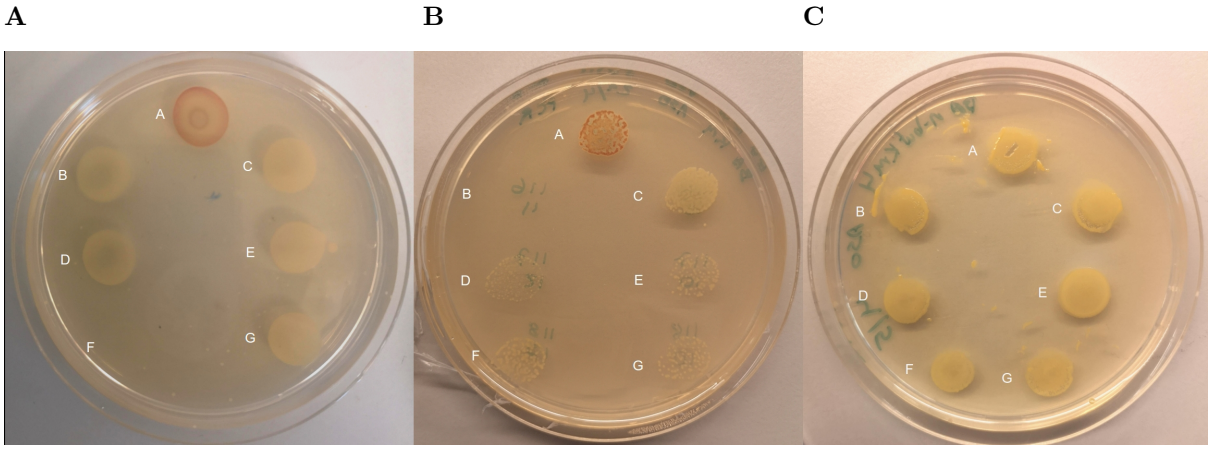


Figure 4.26: *A. vinelandii* cells grown on *n*-butanol agar plates with (A) 0µg/ml, (B) 2µg/ml, and 4µg/ml kanamycin for encystment, followed by the addition of Fast blue B solution. A: ATCC 12518 with pAS10, B: ATCC 12518 ΔarsF with pAS11, C: ATCC 12518 ΔarsF with pAS10, D: ATCC 12518 ΔarsG with pAS12, E: ATCC 12518 ΔarsG with pAS10, F: ATCC 12518 ΔarsH with pAS13, ATCC 12518 ΔarsH with pAS10.

All strains, except ATCC 12518ΔarsH complemented with pAS13 grew well in the presence of 0 µg/ml kanamycin. On *n*-butanol plates with 2 µg/ml the growth was sparse for all strains, while all strains grew well in the presence of 4 µg/ml kanamycin (Figure 4.26). The effect of kanamycin on the growth and the presence of alkylresorcinols on the cell surface was invesitgated by plating *A. vinelandii* ATCC 12518 with the control plasmid pAS10 on *n*-butanol agar plates 0, 2, and 4 *microg*/ml. Wt cells was also plated on plates without kanamycin as reference (Figure 4.27). Wt plated on 0 µg/ml kanamycin gave a strong red color, while the cells containing the plasmid pAS10 gave a weaker red color

on plates with both 0 µg/ml and 2 µg/ml kanamycin. The cells did, however, show less growth on the plates containg kanamycin than the plate with no kanamycin. On plates with 4 µg/ml kanamycin, the cells grew well, but did not react with Fast blue B to give any red color.

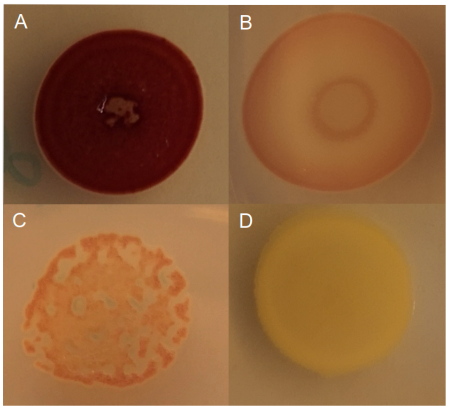


Figure 4.27: *A. vinelandii* ATCC 12518 with and without the plasmid pAS10 grown on *n*-butanol plates with varying consentration of kanamycin, and subsequently sprayed with Fast blue B solution. (A) WT cells on 0 µg/ml kanamycin. (B) Cells with pAS10 grown on 0 µg/ml kanamycin. (C) Cells with pAS10 grown on 2 µg/ml kanamycin. (D) Cells with pAS10 grown on 4 µg/ml kanamycin.

The amount of growth of wt cells on plates with 2 µg/ml kanamycin compared to on plates withot kanamycin was low. When re-streaking transconjugant colonies from the original BM plate onto new plates with 2 µg/ml kanamycin, fewer colonies appeared for each re-streaking. Plasmid loss in cells that lost the ability to grow on 2 µg/ml kanamycin was confirmed by PCR. This showed that the plasmid was unstable in all of strains. The complemented strains did not restore alkylresorcinol transport to the cell surface, and complementation by plasmid carrying complementing genes was therefore unsuccessful.

4.4.3 Complementation of *arsH* mutant by transposon integration

Since the complementation of the mutant strains ATCC 12518ΔarsF, ATCC 12518ΔarsG, and ATCC 12518ΔarsH by insertion of plasmid carrying wt genes was unsuccessful, another strategy, involving transposition, was implemented. A transposon plasmid, pOG115 [94], carrying *arsH*, was transferred to *A. vinelandii* ATCC12518ΔarsH by conjugation. A transconjugant colony was sub-cultured five times to allow for the spread of the gene to as many chromosome copies as possible. The resulting strain, designated ATCC 12518ΔarsHTnOG115, was induced to encyst on *n*-butanol plates and incubated for seven days before the plates were sprayed with Fast blue B solution. The wt control showed a strong red color, while the strain mutant in transport of alkylresorcinols showed no color change (Figure 4.28), as expected. The complemented strain showed a weak red color when sprayed with Fast blue B solution, demonstrating the presence of alkylresorcinols. A PCR was run on the four tested colonies, to verify the insertion of the wt gene by transposition. The bands obtained when separating the PCR products on a gel, was of the expected sizes for both the wt gene and the mutated gene, 1.3 and

3.4 kb, respectively. This verifies the presence of the wt gene, insterted by transposition, and successful complementation.

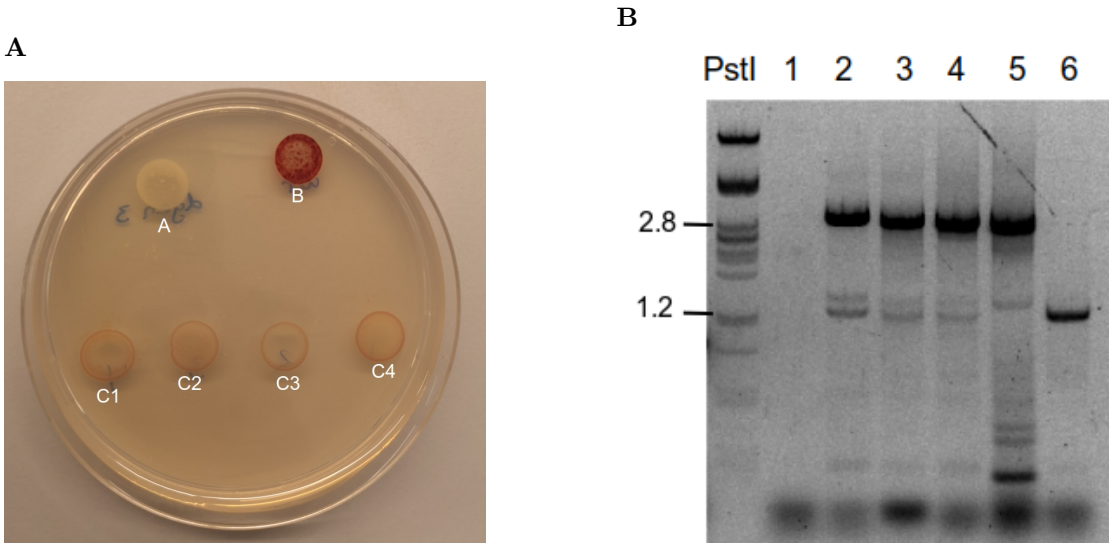


Figure 4.28: (A)Cultures grown on *n*-butanol agar plates for 7 days for encystment, and subsequently sprayed with Fast blue B solution. A: ATCC 12518 ΔarsH , B: ATCC 12518 wt, C1-C4: ATCC 12518 ΔarsH complemented by integration of transposon. (B) PCR of DNA from ATCC 12518ΔarsH complemented strains with the primer pair dgen3FA/dgen3RA. Expected band sizes were 1.3 kb for wt gene, and 3.4 kb for the mutaded gene. Lane 1-4: Complemented strains with the expected band sizes for both wt and mutaded *arsH*. Lane 5: ATCC 12518 ΔarsH with the expected band size (3.4 kb) for the mutated gene. Lane 6: ATCC 12518 wt with the expected band size of the wt *arsH*.

4.5 Characterization of exocellular- and cyst coat polysaccharides of *A. chroococcum*

The analysis of the excreted- and cyst coat polysaccharides of *A. chroococcum* is of interest first and foremost to reveal differences when compared to the cyst coat polysaccharides and exopolysaccharides of *A. vinelandii*. Differences can aid in the understanding of mechanisms underlying phenomena such as encystment and the necessities of certain components for cellular functions. In addition to broadening the knowledge on the encystment process on a more general basis, the determination of the monomer composition of the carbohydrates of the cyst coat could also tell something about how the three epimerases of *A. chroococcum* works *in vivo* during encystment. This work therefore focused on characterising the exopolysaccharides and the carbohydrate components of the cyst coat of *A. chroococcum*.

4.5.1 Characterization of cyst coat polysaccharides of *A. chroococcum*

It was planned to analyse the composition and structure of the carbohydrates in the intine and exine fractions of mature cysts of *A. chroococcum*. Cyst were induced by incubation

in liquid encystment medium, as described in section 3.4.1. The intine and exine were extracted by the procedures outlined in section 3.4.3. The samples were insoluble in both water and DMSO, and could therefore not be analysed by HPAEC-PAD.

4.5.2 Characterization of *A. chroococcum* exopolysaccharides

Exocellular polysaccharides were isolated from stationary phase cultures of *A. chroococcum*, grown for 48 hrs, and subsequently analysed by NMR and HPAEC-PAD.

Analysis of exocellular polysaccharide by ^{1}H-NMR and HSQC

The spectroscopic nalyses of the exopolysaccharides, isolated from *A. chroococcum*, was done by 1D ^{1}H NMR and 2D ^{1}H-^{1}3C HSQC NMR at 25°C and 90° pulse angle and analysed in TopSpin. The main polysaccharide of *A. chroococcum* was found to be an alginate of high molecular weight. The alginate was found to be mainly M-block alginate, with a small fraction of single G-residues (Figure 4.29). The fraction of G-residues, F_G, was found to be 0.06. The degree of acetylation was not assessed in this experiment, as the alginate was deacetylated before analysis. In addition to uronic acids, rhamnose was also detected, and accounted for 5 % of the total extracted exocellular polysaccharides of *A. chroococcum*. The full ^{1}H-NMR spectrum is given in Appendix I.

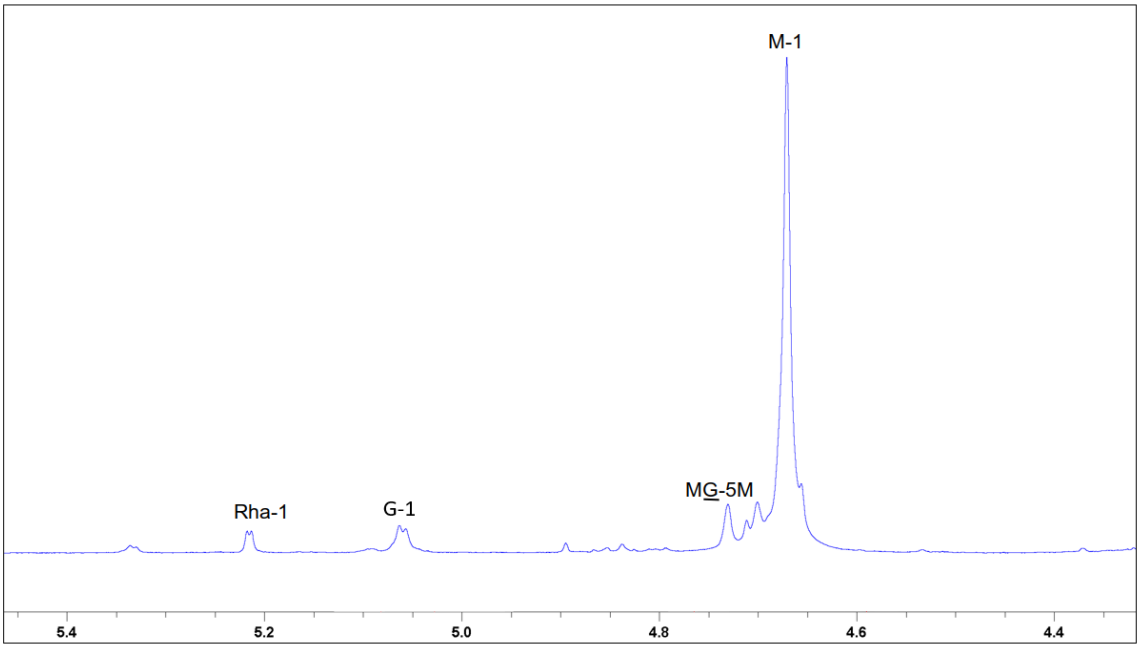


Figure 4.29: ^{1}H-NMR spectrum of exopolysaccharides from *A. chroococcum* grown in RA1 medium for 48 hrs.

The 2D HSQC ^{1}H-^{13}C HSQC NMR has higher resolution than the 1D ^{1}H-NMR, and could potentially give additional information on the composition and structure of the exopolysaccharides of *A. chroococcum*. While it did confirm the findings of the ^{1}H-NMR analysis, it did not reveal any additional information on monomer composition or structure (Figure 4.30). The exocellular polysaccharides consists mainly of an M-block algi-

nate, with a few G-residues interspersed. Additionally rhamnose is present, as seen by a signal from both C-1 and C-5 of the molecule.

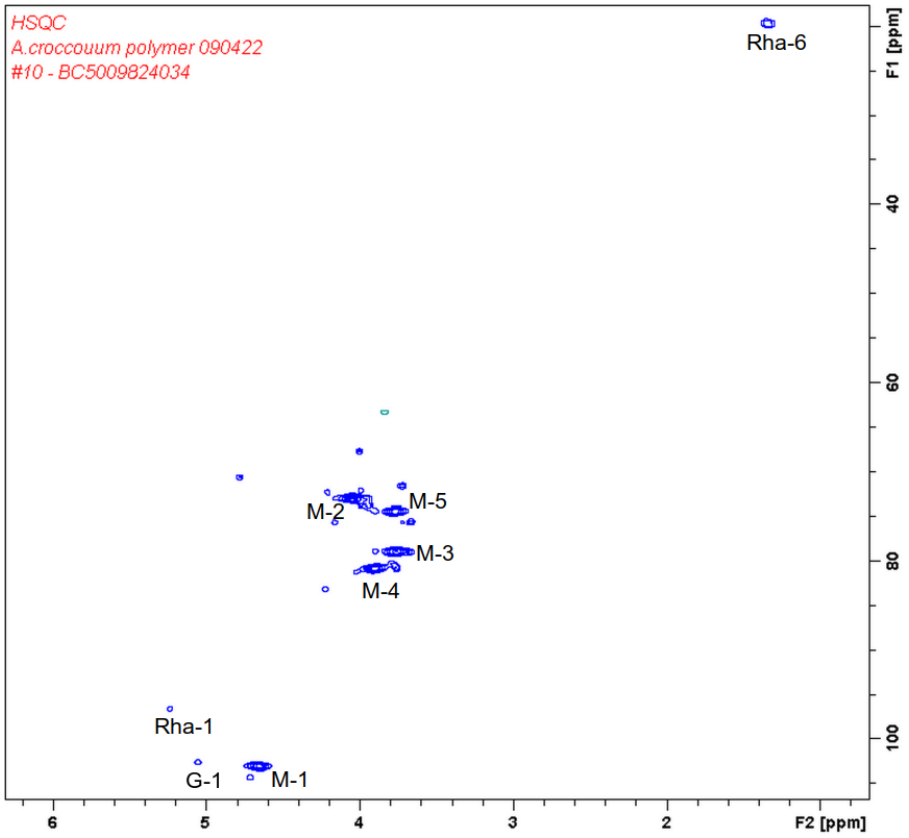


Figure 4.30: Spectrum obtained by HSQC of isolated exocellular polasaccharides from *A. chroococcum* grown in RA1 for 48 hrs.

Component analysis of exocellular polysaccharides by HPAEC-PAD

To be able to discover monomers, not detected by NMR, an analysis by HPAEC-PAD was done. The exocellular polysaccharides of *A. chroococcum* were hydrolysed to obtain the monosaccharides of the polysaccharides before analysis by HPAEC-PAD. Three parallells were run. Eight peaks were obtained (Figure 4.31). Five of the peaks were characterised as arabinose, galactose, rhamnose, glucose, and mannose. Three peaks, marked as X1, X2, and X3 in Figure 4.31 were not identified in this experiment. Although alginate accounts for a large part of the exocellular polysaccharides of *A. chroococcum*, mannuronic acid and guluronic acid is not visible because they are easily degraded by the pre-treatment procedures. The concentrations of the identified carbohydrates were calculated, and are given in Table 4.3. The numbers given are the calculated mean ± standard deviation.

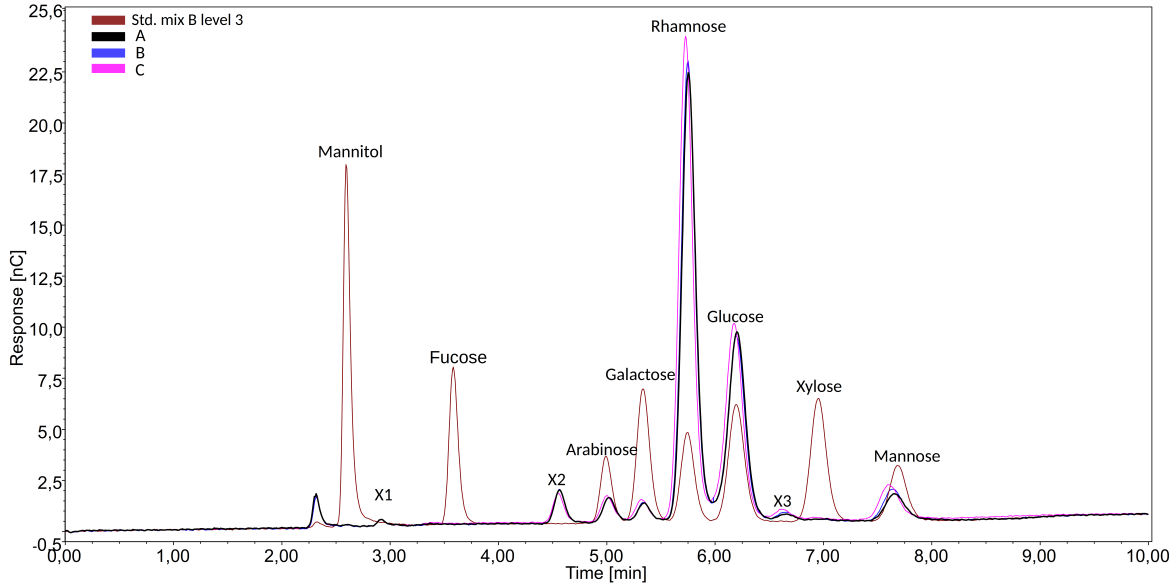


Figure 4.31: Chromatogram of exocellylar polysaccharides obtained from *A. chroococcum* cultures grown in RA1 for 48 hrs.

Table 4.3: Concentrations of monosaccharides in the extracellular polysaccharides of *A. chroococcum*, obtained by HPAEC-PAD. *: values are corrected for mannose in the glucose standard.

Monosaccharide	Concentration [% dry weight]
Rhamnose	2.01±0.03
Glucose*	0.89±0.01
Mannose*	0.19±0.03
Arabinose	0.08±0.00
Galactose	0.07±0.00
Mannitol	n.d
Fucose	n.d
Xylose	n.d

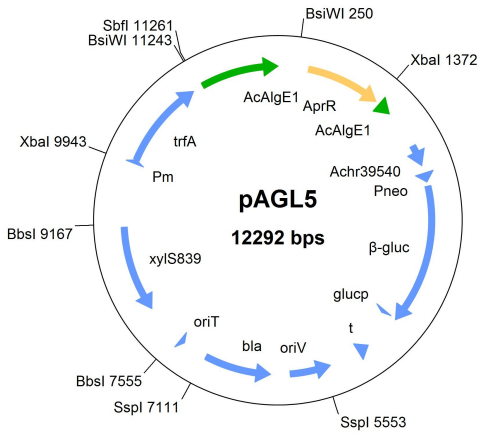
4.6 Inactivation of *AcAlgE1* in *A. chroococcum*

The mannuronan C-5 epimerase AcAlgE1 encoded and expressed by *A. chroococcum* is previously found to be effective in introducing G-residues and forming long G-blocks *in vitro* [76]. To disclose it's function *in vivo*, a mutant carrying a disrupted *AcAlgE1* gene was to be made. It was planned to introduce a previously made plasmid (pAGL5) into *A. chroococcum* by conjugation, followed by homologous recombination.

A conjugation between *E. coli* S17.1 containing pAGL5 as a donor strain, and *A. chroococcum* was set up as described in section 3.3.2. The donor and recipient strain were mixed in a 1:1 ratio and plated on an LA plate. BA plates contaning apramycin was used to select for *A. chroococcum* cells carrying the plasmid. Several conjugation experiments were conducted as described, but gave no transconjugants. Based on a previous report on successful conjugation with *A. chroococcum* as a recipient strain, where RA was used in place of LA on the conjugation plate [177], it was decided to perform the conjugation on RA plates. Conjugation on these plates were tested with different ratios of donor and recipient strains, but without success. As the introduction of pAGL5 into *A. chroococcum* by conjugation was unsuccessfull, it was attempted to introduce the plasmid by it by electrotransformation. Two protocols, one with success in transforming *A. vinelandii* [146], and one with success in transforming *A. chroococcum* strain Azb19 [147], were performed. No positive transformants were obtained by following either of the transformation protocols.

Unsuccessful conjugation- and transformation experiments suggested that there was a problem with either the plasmid itself, or the recipient strain. The plasmid was investigated by setting up several restriction enzyme digests and analyzing the resulting band patterns . The observed band patterns was as expected: 9.3 and 2.5 kb when digested with SbfI-NcoI, 12.3 kb when digested with SbfI-BbsI due to defective BbsI, 8.6, 1.3, 1.3, and 1.2 kb when digested with XbaI-BsiWI, and 8.7, 1.7, and 1.6 kb when digested with SspI (Figure 4.32).

A



B

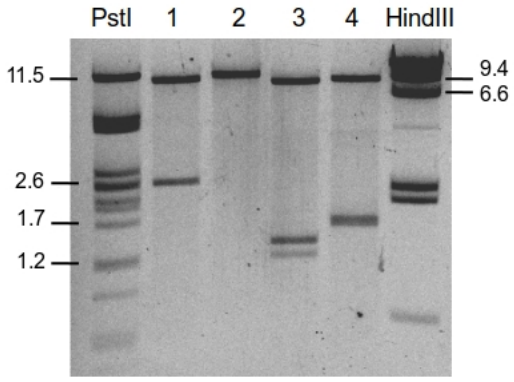


Figure 4.32: Restriction digests of pAGL5. Lane 1: SbfI-NcoI, with the expected bands of 9.3 and 2.5 kb. Lane 2: SbfI-BbsI, expected bands were 8.2, 2.1 and 1.6 kb. Bbs was shown to be unfunctional, hence linearized plasmid. Lane 3: XbaI-BsiWI, with the expected bands of 8.6, 1.3, 1.3 and 1.2 kb. Lane 4: SspI, with the expected bands of 8.7, 1.7 and 1.6 kb.

To assess the ability of pAGL5 to be transferred by conjugation, a conjugation with *A. vinelandii* as the recipient strain was conducted. This resulted in several transconjugant colonies, proving the conjugation and replication functions of the plasmid to be operative.

In the hopes of being able to introduce the plasmid into *A. chroococcum* a strategy to weaken the cell capsule was utilized. It has been shown that growing *A. chroococcum* in iron-limited conditions leads to a change in the cell capsule, causing it to become more loose structured [178]. It is also known that iron-limitation increases the competency in *A. vinelandii* [179]. Knowing this it was suggested that growing *A. chroococcum* in media without iron would allow for the introduction of pAGL5. The growth in liquid media without iron was sparse, and it was not attempted to perform conjugation or transformation of these cultures. While the cells grew well on iron-limited BA plates, the colonies were very mucoid, and separation of cells from the mucoid material was challenging. This made conjugation or transformation procedures difficult. Due to time-limitations, this work was not continued.

Chapter 5

Discussion

The processes of encystment and exopolysaccharide synthesis, specifically alginate synthesis, are two characteristic properties of *Azotobacter* species that makes this bacteria a target of study. To build on the current knowledge, these processes has been studied in two species of *Azotobacter*. The aim was to reveal genes involved in cyst formation in *A. vinelandii*, and to study the composition and structure of exopolysaccharides and cyst coat carbohydrates of *A. chroococcum*, as well as to investigate the *in vivo* function of the secreted mannuronan C-5 epimerase AcAlgE1.

5.1 Encystment of *A. vinelandii* WT and mutant strains

To identify mechanisms underlying the formation of cysts in *A. vinelandii*, a mutational study of genes upregulated during the encystment process was conducted. The three genes *Avin05390*, *Avin05750*, and *Avin17310* putatively encoding an exopolysaccharide biosynthesis protein, an alanine dehydrogenase, and a regulatory protein of the MarR family, respectively, are all upregulated during encystment, and are potentially necessary for the encystment process in *A. vinelandii*. It was therefore decided to separately construct mutants, carrying mutations in each of these three genes, and to assess their encystment capabilities. The *Avin05390* mutant was constructed in a previous work [89], and the *Avin05750* mutant was constructed in this work. The *Avin17310* mutant proved impossible to construct.

5.1.1 Inability to clone the sequence upstream of *Avin17310* might be due to toxic elements

The construction of a mutant carrying a deletorious mutation in *Avin17310* could not be completed due to the failure of cloning *Avin17310*. This was unexpected, and called for an investigation of possible reasons and possible solutions.

The downstream element of *Avin17310* had already been cloned into a TOPO-vector, but every attempt on inserting the upstream element failed. The PCR amplifications of the upstream element alone, and of the whole recombination fragment always produced fragments of the expected size, as analyzed by gel electrophoresis. This suggests that

the problem was the cloning of the PCR products in *E. coli*, and not the amplification by PCR. It is also possible that the insertion into the vector before transformation of *E. coli* cells failed. However, since the downstream element was readily TOPO-cloned, and subsequently cloned into pHE216, this is less likely. Additionally, vectors carrying the complete, undisrupted *Avin17310* gene was easily constructed and cloned in *E.coli*. Based on this, the problem was most likely not the insertion into the vector, but the cloning in *E. coli*. It appeared as if some part of the upstream PCR fragment was unclonable in *E. coli*.

The PCR products that were easily cloned in *E. coli* differed from the unclonable PCR fragments by a 1.2 kb sequence upstream of *Avin17310* (Figure 4.8). Some genes are toxic to *E. coli* when expressed [180]. This sequence does not contain complete protein-encoding sequences, but it is nevertheless a possibility that expression of the partial *Avin17300* gene results in a faulty protein product that is toxic to *E. coli*. It is therefore assumed that this sequence is not clonable in *E. coli*, and that this is the culprit of the troubles in cloning PCR products containing the upstream part of *Avin17310*. Another possible explanation of the inability to clone the part upstream of *Avin17310* is that the 203 bp intergenic region contains sequences that are toxic to *E. coli*. There is a possibility that protein-coding genes are not detected by the annotating programs during sequencing and annotation. Overbeek et al. argues that short protein-coding sequences are frequently overlooked by gene annotation programs [181]. In addition to toxic protein-coding sequences, non-protein coding sequences that are toxic to *E. coli* also exist. Kimelman et al. found that unclonable sequences could encode small regulatory RNA molecules, as well as DNA binding motifs [180].

To solve the problem with the potentially toxic sequence upstream of *Avin17310* PCR, there are several possibilities. First, one can do the cloning in a different host. This could be either a different strain of *E. coli*, or a different species which can tolerate the sequences that are potentially toxic to the *E. coli* strains used in this work. Another possible solution is to exclude the possibly toxic region. This can be done by for example overlap extension PCR. Additionally, the use of CRISPR-Cas has recently been shown to be a potential system for the mutagenesis of genes in *Pseudomonas* species [182, 183], which is closely related to *A. vinelandii*. The use of this system for mutation of *Avin17310* instead of homologous recombination could circumvent the cloning problems experienced in this work, by excluding the potentially toxic gene sequence when cloning in *E. coli*.

5.1.2 Cysts are rarely or never formed by *A. vinelandii* ATCC 12518, and its *Avin05390* and *Avin05750* mutant strains

Encystment of the mutant strains ATCC 12518Δ05390 and ATCCΔ05750 was assessed by measuring resistance towards desiccation. As no mutant of *Avin17310* was obtained, it is excluded from this discussion. Several experiments including both wt and mutant strains were performed, and the results were close to identical in every experiment. Most often, no cells were detected on the plate when plating samples of dried cells. This indicates that less than 0.001% survived desiccation and could be considered real cysts. In a few cases, when encysted on *n*-butanol plates, some cultures of the wt and of the *Avin05750* mutant survived desiccation, but the percentage survival was still very low.

Only two of the six tested cultures showed a percentage of survival just above 0.001 %, of 0.030 and 0.002 %. This is not higher rates of survival than in non-encysting cells of *A. vinelandii* 12837, which survives desiccation for seven days at rates of 0.1% [18]. It is therefore reasonable to assume that the surviving cells of *A. vinelandii* 12518 wt, Δ05390, and Δ05750 strains were not true cysts.

Finding that encystment of *A. vinelandii* 12518 wt both in liquid encystment medium, and on *n*-butanol agar plates was negligible, or even non-occuring, raised the question if this strain is actually able to produce desiccation resistant cysts. Phase-contrast microscopy of cells grown on encysting medium revealed the large intracellular PHB-granules and the rounding of the cells, which is associated with encystment in *Azotobacter*. This gives an indication that encystment was at least initiated, and that some stages of the differentiation process had occured. It is, however, not possible to determine whether the characteristic cyst coat is formed around the central body, when examining the cells by phase-contrast microscopy without staining procedures. This cyst coat appears to be essential for the desiccation resistance of the cyst [35, 33, 184]. To investigate whether *A. vinelandii* 12518 does form true cysts, staining procedures could be applied to cells grown on encysting medium. Another, much simpler and more reliable method was to compare the degree of desiccation survival to that of *A. vinelandii* strains known to form desiccation resistant cysts.

5.1.3 The encystment capacilities of *A. vinelandii* ATCC 12518 is inferior to strains ATCC 12837 and E

Based on the inability to obtain desiccation resistant cysts of *A. vinelandii* ATCC 12518, a comparison to the encystment capabilities of *A. vinelandi* strains ATCC 12837 and E was performed. Both ATCC 12837 and strain E showed encystment to a much greater extent than did ATCC 12518. Strain E showed the highest degree of encystment, with an average of 7.6 %. This number is comparable to what was seen by Gimmestad et al. [75]. On the other hand, the percentage of encystment seen in strain ATCC 12837, which averaged to 0.7 %, was much lower than expected. Previous studies has obtained encystment of up to 100% in cultures of strain ATCC 12837 [21, 184]. It is important to note that these studies measured encystment on a basis of the formation of mature cysts, counted by micropic methods, while in this work encystment was measured on a basis of germination after desiccation treatments. Based on microscopy, the encystment of strain OP, which cannot form cysts due to the lack of alginate synthesis, has previously been reported [42]. This underlines the importance of other strategies than microscopy when quantifying encystment. It also indicates that the percentage of ATCC 12837 cells that formed desiccation resistant cysts in this work may not be lower than one could expect. Additionally, not all encysted cells will germinate, as shown by Gimmestad et al. [75], which were able to markedly increase the germination of encysted cells of strain E, by the addition of an alginate lyase during germination. From this it is reasonable to suggest that a great amount of the encysted cells of strain E simply does not germinate. This could also be the case for strain ATCC 12837 and ATCC 12518. The addition of alginate lyase to encysted cells as described by Gimmestad et. al could verify this.

Encystment is very dependent on the environment the cells are in. The composition

of the encysting medium is of great importance to obtain high degrees of encystment. Both calcium and magnesium are essential for cyst formation, and Page and Sadoff found that a decrease in Ca^{2+} concentration from 0.36 mM to 0.31 mM caused a 75% drop in the encystment of strain ATCC 12837 [21]. Additionally it was found that optimal pH was required for maximal degree of encystment. pH above 7 decreased the extent of encystment in a culture significantly. This findings reveal how only small changes in the composition of the encysting medium affects the extent of encystment. The low degree of encystment found in ATCC 12837 might therefore be a matter of medium composition. The same could apply to strain ATCC 12518, but the very low fraction of cells surviving desiccation in cultures of ATCC 12518 compared to the two other strains points towards impairment in the formation of true cysts, rather than a problem with the encysting medium.

Cagle found, by electron microscopy, that *A. vinelandii* strain O (ATCC 12518) only formed cyst-like cells, as opposed to mature cysts, when incubated on *n*-butanol agar plates [185]. These cells presented as central bodies without the protecting intine and exine coat layers. Strain OP is a derivate of strain ATCC 12518, with a mutation in the *AlgU* gene. When strain OP was complemented with *algU*, it was on the contrary, found to form desiccation resistant cysts at rates of 5 % by Segura et al. [35]. The results of Cagle and Segura et al. are not in agreement, and this may be a result of strain ATCC 12518 not being able to form real cysts. The fact that all literature quantifying encystment employs strain ATCC 12837, or strain E, and that no papers are able to confirm the formation of cysts in *A. vinelandii* ATCC 12518 wt, suggests that strain ATCC 12518 is, at least inferior to strain ATCC 12837 and strain E in forming desiccation resistant cysts. Together with the results in this work, it is most probable that *A. vinelandii* ATCC 12518 is unable to form desiccation resistant cysts, or that cyst formation in this strain is a rarely occuring event.

The observation that *A. vinelandii* strain E encysted to an appreciable extent during the conducted desiccation experiments, opens up a new possibility of assessing the importance of *Avin05390* and *Avin05750* during encystment. Strain E is genetically very similar to strain ATCC 12518 (Ertesvåg, personal communication). Based on this, the vectors constructed for mutation of *Avin05390* and *Avin05750* in ATCC 12518 may be applied for the mutagenesis of these genes in strain E. If transfer of the vectors to strain E and homologous recombination were successful, assessment of the genes importance during encystment would be straightforward.

5.2 Alkylresorcinol transport is restored by the complementation of mutant strains defective in alkylresorcinol transport

Synthesis of alkylresorcinols in *A. vinelandii*, and their transport to the surface of the cells is coupled to the encystment process. Dalland constructed three mutant strains, ATCC 12518ΔarsF, ATCC 12518ΔarsG, and ATCC 12518ΔarsH, which were all unable to transport alkylresorcinol to the cell surface during encystment [43]. It was verified that

all mutated strains were still able to synthesize alkylresorcinols by measurement of the total contents of alkylresorcinol in the cells. The amount of alkylresorcinol synthesizedalso seemed to be unaffected by the mutation of the three genes *arsF*, *arsG*, and *arsH*. Since the mutated strains were still able to synthesize alkylresorcinol, the lack of these lipids on the cell surface of cysts must be a result of the inability to transport the alkylresorcinols to the cell surface. To verify that the inability of alkylresorcinol transport is due to the mutations of *arsF*, *arsG*, and *arsH*, and not secondary mutations, the three mutant strains were complemented with their respective functional genes.

Complementation was carried out by introducing complementation plasmids carrying wt versions of the mutated genes. Reversion to the wt phenotype by complementation would verify that the three genes are indeed responsible for alkylresorcinol transport during encystment in *A. vinelandii*. The complemented strains were induced to encyst on *n*-butanol agar plates containing 2 µg/ml kanamycin for selection, initially for five days. This did often result in the lack of alkylresorcinol on the cell surface of wt cells. It was therefore decided to incubate the cells for two extra days, to increase the chance of completing the encystment process. When incubated for seven days instead of five days, the wt presented with alkylresorcinol on the cell surface in every experiment, as detected by the reaction with Fast blue B. The complemented strains did, however, not show any reaction with Fast blue B even after seven days of incubation, indicating that they were still not able to transfer alkylresorcinols to the cell surface. The chance of having secondary mutations in all three mutant strains, causing inability to transport alkylresorcinols to the cell surface is relatively small. It was therefore suggested that the plasmids were ustable in the *A. vinelandii* strains. This was confirmed, and could explain why the complemented strains were not able to transfer alkylresorcinols to the cell surface. To avoid plasmid loss, the concentration of kanamycin in the agar plates was increased to 4 µg/ml. None of the strains, this time including the wt strain, showed any reaction when Fast blue B was added. This indicates that the cells still were unable to transport alkylresorcinols to the cell surface. The cells grew well on these plates, demonstrating that the plasmid containing the genes necessary for transport of alkylresorcinols to the cell surface was present within the cells. It must therefore be another mechanism causing the lack of alkylresorcinols on the cell surface of these cells.

To find the reason of the inability of the complemented strains to transoprt alkylresorcinols to the cell surface, the possibility of the plasmid having an effect was assessed. Comparing wt cells with and without plasmid grown on the different concentrations of kanamycin (Figure 4.27), reveals that the plasmid most likely had an effect on the ability of *A. vinelandii* to transport alkylresorcinols to the cell surface. Encysted wt cells without plasmid become dark red when Fast blue B was added. On the other hand wt cells carrying the control plasmid obtained a much lighter color. Kanamycin did not seem to have an effect on the presence of alkylresorcinols on the cell surface, as the wt cells carrying the plasmid grown on 0 and 2 µg/ml kanamycin showed the same color intensity when reacting with Fast blue B. Because of the light color obtained by the wt cells with control plasmid, it appears as if only a portion of the cells are able to transport alkylresorcinol to the cell surface. It was observed that higher concentration of kanamycin, with a higher probability of plasmid retainment, diminished the presence of alkylresorcinols on the cell surface. Since the plasmid obviously was present, it is tempting to suggest

that the cells carrying the plasmid were simply not able to form mature cysts, and hence not able to synthesize alkylresorcinols. The presence of these lipids on the cell surfaces of wt cells grown on the lower concentration of kanamycin, or without kanamycin could be a result of the plasmid loss, enabeling the cells to form cysts again. This corresponds well with the weak color obtained in these cultures and the reduced growth on 2 µg/ml kanamycin when compared to 0 µg/ml. Some cells probably still contained the plasmid, while some had lost the plasmid.

As the plasmid itself probably hindered the encystment of *A. vinelandii*, complementation of one of the mutated genes was done by the use of transposon. A transposon with *arsH*, which already existed [94], was successfully introduced into the genome of *A. vinelandii* ATCC 12518ΔarsH. When the resulting strain was allowed to encyst on *n*-butanol plates for seven days, and subsequently sprayed with Fast blue B solution, these strains were colored red. This verifies the presence of alkylresorcinols on the cell surface, showing a successfull complementation of the mutated *arsH* gene. The color of the complemented strains was albeit less intense than the color of the wt strain. Since it was shown that the absence of alkylresorcinols on the cell surface of ATCC 12518ΔarsH is due to the lack of transport, rather than the lack of synthesis. The weak color obtained from the complemented strain is therefore also most likely due to the lack of adequate transport, and accordingly due to subnormal expression of *arsH*. This might be due to fewer copies of the functional gene in the complemented strains than the wt strain. Since *A. vinelandii* can contain several copies of it's chromosome, it is likely that the inserted wt gene is not copied to all chromosomes during the five sub-cultures that were done. It is also a possibility that the strength of the promoter coupled to the wt gene in the transposon, P_{algD}, is lower than the strength of the original promoter during encystment.

Despite the weakness of the color of the complemented cells when reacting with Fast blue B, it is still shown that transport of some amount of alkylresorcinols is restored by inserting a functional *arsH* gene. This is a clear verification that the mutation in *arsH* was the underlying reason of the lack of alkylresorcinols on the cell surface of ATCC 12518ΔarsH. Furthermore it verifies that *arsH* is involved in transport of alkylresorcinols to the cell surface of *A. vinelandii* during encystment. As Dalland described, *arsH* is a part of an operon of three genes that most likely encode an ABC-transporter [43]. This strongly suggests that *arsF* and *arsG* are also involved in the same transport system, and furthermore implicates that the complete ABC-transporter is necessary for transport of alkylresorcinols to the cell surface. However, this needs to be verified by complementation of the *arsF* and *arsG* mutant strains.

5.3 Exocellular and cyst coat polysaccharides of *A. chroococcum*

While much focus has been given to the alginate synthesized by *A. vinelandii*, the literature is limited on information about the extracellular and cyst coat polysaccharides of *A. chroococcum*. Cote and Krull did characterize the exocellular polysaccharides of *A. chroococcum* [71], but no studies characterizing the cyst coat polysaccharides of *A. chroococcum*. Additionally, the knowledge that *A. chroococcum* only harbours one sin-

gle unifunctional mannuronan C-5 epimerase, while *A. vinelandii* harbours five, called for an investigation of the effect this has on the composition of alginate synthesized by these bacteria, and the importance of this protein in the formation of cysts. Extracellular polysaccharides of *A. chroococcum* strain B was therefore isolated and analysed by ^{1}H-NMR, HSQC, and HPAEC-PAD. An analysis of cyst coat monomer composition and structure by NMR was intended, but was not completed due to experimental difficulties.

5.3.1 Cyst coat polysaccharides of *A. chroococcum* demands special methods for solubilization

Cyst coat fractions were isolated and delivered for analysis by NMR. However, they found the isolated fractions to be insoluble in both water and DMSO, causing analysis by NMR to be difficult. Alginates are generally soluble in water, and it is therefore surprising that the cyst coat fractions were not soluble. The cyst coat does, in addition to alginate, contain other constituents, including other carbohydrates, lipids and proteins. These substances might cause the intine and exine to be insoluble. Page and Sadoff applied strong acids to the cyst coat fractions before analysis of carbohydrates and other constituents in *A. vinelandii* [21]. Such treatment could possibly aid in the solvation of the cyst coat fractions and the release of alginate from other constituents in *A. chroococcum* as well. This was not attempted as the alginate monomers are decomposed in strong acid with time [186]. It is also a possibility that the polymers seemed insoluble because they were simply not there. The fractionation procedures could have been disturbed by the presence of large quantities of what appeared to be melanin [187]. Melanin is not synthesized by *A. vinelandii*, and the procedures for fractionation of the cysts were derived for *A. vinelandii*. It might be possible that additional steps should be added to remove the melanin before isolation of the cyst coat fractions, to obtain pure fractions.

5.3.2 Exocellular polysaccharides of *A. chroococcum* probably comprises two polymers

The NMR experiments revealed that the extracellular polysaccharides isolated from *A. chroococcum* grown in RA1 medium for 48 hrs consisted mainly of M-block alginate with a small fraction of G-residues interspersed in the polymer chain. The finding that the main polysaccharide was alginate was as expected, as *Azotobacter* species are known for their alginate synthesis. In addition to alginate residues, a small proportion of rhamnose was detected by NMR. This agrees with the findings of Cote and Krull [71], who found that *A. chroococcum* excreted two types of polysaccharides, which they designated EPS-I and EPS-II. They claimed that EPS-II was an alginate polymer, while EPS-I comprised rhamnose, galactose, mannose and traces of glucose and arabinose. The analysis by HPAEC-PAD, in this work, confirmed the presence of these five monosaccharides. Three unidentified monomers were also detected. These findings supports the results of Cote and Krull, that *A. chrooccum* synthesizes two types of exopolysaccharides. The concentrations of the five monosaccharides identified by HPAEC-PAD were low, compared to the concentration of alginate. Cote and Krull did point out that the ratios of EPS-I to EPS-II, as well as the composition of the exopolysaccharides was highly variable. Other studies has only found a single exopolysaccharide of *A. chroococcum* [188]. This indicates

that the presence of a second polymer is dependent on the culture conditions, and might explain why the concentrations of the monomers corresponding to EPS-I were so low, when compared to the concentration of alginate. The presence of the five monomers, in addition to the M and G residues does, however, suggests that *A. chroococcum* synthesize a second exopolymer under the conditions of this experiment. Altering the growth conditions could increase the synthesis of this second exopolymer, and enable a more detailed study of this polymer. A perhaps more effective method would be to degrade the alginate with a M-specifc alginate lyase to purify the other exopolymer.

Cote and Krull did not analyse the sequence of monomers in the exocellular alginate of *A. chroococcum* [71]. The sequence can say something about the function, as well as the activity of the excreted C-5 epimerases, including AcAlgE1. The analysis of the extracellular alginate of *A. chroococcum*, done by Cote and Krull, was therefore repeated with an analysis of alginate monomer sequence included. The alginate, analysed by ^{1}H-NMR, was of high molecular weight, as indicated by the broad peaks. This is further supported by the lack of any visible peak for the α-or β-anomer of the reducing ends. This factor might make the presence of other constituents difficult to detect by ^{1}H-NMR. Degradation of these high molecular weight alginates with an M-specific lyase could, as already mentioned, potentially ease the analysis of other *A. chroococcum* exopolymer constituents by ^{1}H-NMR. Cote and Krull found the percentage of G-residues to be 10 [71], while in this work the percentage of G-residues was found to be 6. This discrepancy may simply be explained by inaccuracy of the measurements, or by variations in the growth conditions, which is known to affect both the yield and the composition of alginate [91, 165, 33, 71]. There is, however, no doubt that the alginate comprised mainly M residues, with only a very small proportion of G-residues. No consecutive G-residues were detected, meaning that *A. chroococcum* does not synthesize alginates with G-blocks during vegetative growth under the conditions of this experiment. This makes *A. chroococcum* a potential candidate for industrial alginate synthesis, as M-rich alginates are excellent starting points for the synthesis of tailor-made alginates with highly specific properties.

In comparison to the extracellular alginate of *A. vinelandii*, the extracellular alginate of *A. chroococcum* was found to have relatively low amounts of G residues. A G-content of 6 % is remarkably lower than the extracellular alginate of *A. vinelandii*, which has G-contents ranging from 25% up to almost 50 % [33, 91]. The extracellular alginate of *A. chroococcum* seems to resemble the alginate synthesized by an *A. vinelandii* mutant where all the seven secreted epimerases were inactivated, constructed by Steigedal et al. [33]. This mutant synthesized extracellular alginate with 6% G-residues, identical to the proportion of G-residues in the alginate isolated from *A. chroococcum*. This suggests that the G-residues found in alginate excreted by *A. chroococcum* during vegetative growth might be a result of epimerisation by AlgG alone. Gawin et al. found that AcAlgE1 introduces consecutive G-residued [76], which was not detected in the excreted alginate of *A. chroococcum*. According to this the secreted epimerases of *A. chroococcum*, AcAlgE1, may be inactive during vegetative growth.

5.4 Inactivation of *AcAlgE1* in *A. chroococcum* requires special methods

It has been hypothesized that the secreted mannuronan C-5 epimerase of *A. chroococcum*, AcAlgE1 is important for the composition of the alginate synthesized, and the ability of *A. chroococcum* to form desiccation resistant cysts. This hypothesis is strengthened by the findings that the alginate of stationary phase cells of *A. chroococcum* do not contain any consecutive G-residues (section 5.3.2). The goal of this work was therefore to construct a mutant strain where *AcAlgE1* is inactivated, and to assess the importance of this enzyme *in vivo* with focus on excreted alginate structure and the ability to form desiccation resistant cyst. The construction of the mutant was to be done by the introduction of the conjugative plasmid pAGL5, carrying the disrupted *AcAlgE1*, into *A. chroococcum*, and the exchange of the chromosomal gene with the disrupted gene by homologous recombination. The introduction of pAGL5 was attempted by conjugation and by electrotransformation, both with negative results. The focus thus shifted to identify possible reasons for the failure of plasmid introduction. Two hypotheses for the failure of obtaining *A. chroococcum* transconjugants, or transformants was proposed. First, the plasmid could contain mutations in sequences essential for conjugation, or for the XylS/Pm based replication system of the plasmid. Secondly, the problem could be the introduction into the cells itself.

To assess these two possible sources of the problem, a set of experiments including restriction digests and conjugation of the plasmid to *A. vinelandi* confirmed that the plasmid did not contain any mutations affecting its function and that it was functional. It was therefore concluded that the problem was the transfer of the plasmid into *A. chroococcum*, or the maintenance of the plasmid in *A. chroococcum* once transferred, rather than the plasmid itself. Solving this problem needs further work, but possible explanations and suggested solutions are discussed below.

The transfer of a plasmid to *Azotobacter* might, in general, be complicated by both their alginate-containing cell capsule and the exocellular alginate they produce under certain times of incubation. The amount of capsular material and extracellular alginate affects the transformation frequency in *A. vinelandii*, with the capsule having the largest negative effect [16]. A strategy to be able to transform *A. chroococcum* could therefore be to remove, or at least decrease the amount of capsular material around the cells. For *A. vinelandii* this has previously been done by simply altering the media composition[16]. Growth in iron-limited media, to disintegrate the capsular material, was poor and this method was found to be unsuitable for both transformation and conjugation. Another method, which was not tested in this work, is to add alginate lyases to degrade alginate both in the capsule and around the cell. It is shown that removal of the capsular alginate of *A. vinelandii* by washing the cells with glycerol, or by adding an alginate lyase, increases the conjugation frequencies [189]. Although washing the cells with glycerol did not result in any transconjugants in this work, addition of an alginate lyase could possibly be a better solution.

Several genetic and molecular mechanisms may add to the difficulties of introducing plasmid pAGL5 into *A. chroococcum*. *A. chroococcum* strain B harbours six indigenous

plasmids, with sizes up to 311 kb [13]. There has been isolated strains of *A. chroococcum* with variable plasmid numbers, but never more than six [190, 14]. This points towards the incorporation of a seventh plasmid being less likely and the indigenous plasmids might be a contributing factor to the difficulties in introducing plasmids into *A. chroococcum* strain B in the lab. While the number of different plasmids is not necessarily causing the unsuccessful introduction of plasmids, the nature of the plasmids might have a greater effect. The "entry exclusion" phenomena prohibits the entry of a second plasmid containing the same entry exclusion system as a plasmid already existing in the cell [152]. Of the six plasmids *A. chroococcum* carries, one of them might contain an exclusion system that excludes pAGL5, making it nearly impossible to introduce the plasmid by conjugation. Another well known challenge is that of plasmid incompatibilities. The plasmid pAGL5 is derived from RK2, which belongs to incompatibility group IncP, the same incompatibility group as the native plasmid pAcX50c in *A. chroococcum* [14]. It is known that plasmids of the same incompatibility group, having identical origin of replication or partitioning systems are not able to coexist over time without selective pressures. However, in this case, the plasmid was selected for by plating the cells on agar with antibiotics, making plasmid loss due to plasmid incompatibility less likely.

Although two plasmids of the same incompatibility group should be able to coexist in the presence of a selective pressure, the number of surviving cells might be reduced as a result of the incompatibility [84]. Additionally, several other factors can add to the problem of plasmid incompatibility, making the number of generations before plasmid loss occurs few. This includes the fitness cost carrying a plasmid brings. Newly acquired plasmids pose a greater cost on the cells than already existing plasmids, until the cell is adapted to the new plasmid [191]. This can explain why pAGL5, which is a small plasmid, is cured from the cell, rather than the already existing large plasmids, even though large plasmids tend to pose a greater burden on the cell. The indigenous plasmids might also carry genes making their propagation advantageous for the growth of the cells. Additionally, the indigenous plasmid pAcX50c encodes partitioning systems [13], making it more likely that this plasmid will be propagated when competing with another plasmid of the same incompatibility group, such as pAGL5, that does not encode any partitioning systems.

pAGL5 is a low-copy number plasmid, and gave few transconjugants upon conjugation to *A. vinelandii*. Low-copy plasmids have a greater chance of segregational loss, unless apropriate partitioning systems control the distribution of plasmids during cell division. The plasmid pAGL5 is based on the RK2 replicon, which requires the expression of the gene *trfA* for replication. It has previously been shown that mutations in *trfA* can alter the copy number of the plasmid [87, 192]. pAGL5 was constructed to be able to replicate in *A. vinelandii* by introducing a mutated *trfA*, *Ts247Ccop171W* (Ertesvåg, personal communication). While this *trfA* variant replicates both in *A. vinelandii* and in *E. coli*, other *trfA* mutations renders the plasmid unable to replicate in *E. coli* [193], while it replicates very well in *A. vinelandii* (Ertesvåg, personal communication). This demonstrates the species-dependency on replication of plasmids carrying a specific *trfA* variant. Thus it is possible that *A. chroococcum* requires a different *trfA* variant than the one pAGL5 holds. Changing the concentration of m-toluate in the media could also possibly affect the ability of pAGL5 to be maintained in the cell, and consequently survival of the cells [87].

Although the introduction of pAGL5 into *A. chroococcum* was found to be challenging, it is certainly not impossible to do so. Most genetic modifications performed on *A. chroocoocum*, has been carried out on the strain MCD-1, which is cured of four of its six indigenous plasmids. It is additionally a non-mucoid strain. These two alterations together probably increases the conjugation and transformation frequencies enough to be able to introduce new plasmids into the cell. Mutation of *AcAlgE1* in this strain would, however, be of no value when investigating function, as this strain does not synthesize alginate. On the other hand, if possible without using a non-mucoid strain, the curation of the indigenious plasmids could potentially increase conjugation or transformation frequencies. It was also noted that during conjugation to *A. vinelandii*, the pre-conjugation incubation time was very important. If the cultures were grown for an extended period of time, by diluting the culture to keep it in the exponential phase, production of exocellular alginate increased rapidly and complicated the pelleting of the cells. This decreased the conjugation efficiency drastically. These two observations during conjugations to *A. vinelandii* illustrates how small changes in the procedure can lead to large differences in the result. The same is often experienced for electrotransformation procedures. Developing new protocols for conjugation and electrotransformation of *A. chroococcum* might just be what is needed for the successful untroduction of pAGL5 into *A. chroococcum*.

Even though a mutant with *AcAlgE1* was not constructed, the composition and sequence of alginate isolated from stationary-phase cultures of *A. chroococcum* indicates that AcAlgE1 is inactive during vegetative growth. Then the question if the activity of these epimerases are coupled to specific events, such as encystment, arises. The activity the epimerases is out of the scope of this work, but it would be interesting to do an assessment of the expression of these secreted epimerases both during vegetative growth, and during encystment. This can be done by quantitative real-time PCR of the wild-type, meaning that the problems with introduction and maintenance of a recombination plasmid is easily bypassed.

Chapter 6

Conlclusion

The encystment and the structure of polysaccharides, including alginate, of *Azotobacter* species were studied by mutational and analytical methods. The capacity of *A. vinelandii* to form desiccation resistant cysts appeared to be strain specific, and *A. vinelandii* ATCC 12518 did rarely, or never form desiccation resistat cysts when measured by the standard assay. Mutational studies on genes involved in encystment should therefore be performed in other strains of *A. vinelandii*, as for example strain E. Strains with separate mutations in *arsF*, *arsG*, and *arsH* that are unable to transport alkylresorcinols to the cell surface during encystment did synthesize alkylresorcinols, confirming that these genes do not affect this process. The mutant with an inactivated *arsH* gene was successfully complemented with *arsH*, confirming that this gene is involved in the transport of alkylresorcinols to the cell surface. This also strongly suggests that *arsF* and *arsG* are involved in the transort of alkylresorcinols to the cell surface. The composition of cyst coat polysaccharides of *A. chroococcum* were not determined due to insolubility of the extracted intine and exine fractions. The exopolysaccharides of *A. chroococcum* comprised mostly M-block alginate (94% M), with no consecutive G-residues. A second polymer composed of five identified, and three unidentified different monomers also seemed to be present, but the full composition and structure of this polymer was not elucidated. Inactivation of the mannuronan C-5 epimerase AcAlgE1 of *A. chroococcum*, to disclose its function *in vivo*, was not completed due to difficulties in introducing the plasmid into the cells. However, as the exocellular alginate isolated from cells in the stationary phase did not contain consecutive G-residues, it is believed that this enzyme is inactive during vegetative growth, and that it is possibly more active and important during encystment.

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Appendix A

Growth media

Growth media used in this work is presented here. When solid media was prepared, 15 g/l Bacteriological agar was added to the medium before autoclaving. Autoclaving was done at 121°C for 20 minutes for all media. Agar plates were stored at 4°C.

Burk's Medium (BM)

A 5X Burk's salt solution was prepared by mixing the components listed below in RO-water.

$MgSO_4 \cdot 7H_2O$	1.0 g/l
NaCl	1.0 g/l
K_2HPO_4	3.2 g/l
KH_2PO_4	0.8 g/l

Separate 10000X mineral solutions was prepared as listed below and sterile filtrated before storage at -20°.

$FeSO_4$	300 g/l
$CaCl_2$	50 g/l
Na_2MoO_4	21 g/l

Glucose at a final concentration of 20 g/l was dissolved in 50 ml of the total water added to the media and autoclaved separately.

The final media was prepared by mixing the 5X burk's salt solution, mineral stock solutions and glucose to a final concentration given in the following table. The media was stored at room temperature.

Burk's salt	1X
$FeSO_4$	30 mg/ml
$CaCl_2$	5 mg/ml
Na_2MoO_4	2.1 mg/ml
Glucose	20 g/l

For encystment, glucose was replaced by β-hydroxybutyrate at a final concentration of 2 g/l. A stock solution of 200 g/l β-hydroxybutyrate in RO-water was prepared and sterile filtrated. The solution was stored at 4°.

Luria Broth (LB)

The components listed below was mixed in RO-water.

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	5 g/l

The media was autoclaved at 121°C and stored at room temperature.

Psi medium

The component listed below were mixed.

Tryptone	20 g/l
Yeast extract	5 g/l
MgSO4·7H2O 10.24 g/l	

pH was adjusted to 7.6 with 1 M KOH before autoclaving. The media was stored at room temperature.

RA1 medium

The components listed below were mixed to make the base solution.

$MgSO_4 \cdot 7H_2O$	2.0 g/l
NH_4NO_3	1.5 g/l
Peptone	2.0 g/l
Mops	10.5 g/l

The pH was adjusted to 7.0 with 10 M NaOH and autoclaved at 121 °C.

Separate solutions of dipotassium hydrogenphosphate and calcium chloride were prepared. The pH was adjusted to 7.0 before the solutions were autoclaved at 121°C for 20 minutes. The solutions were storat at RT.

K_2HPO_4	50 g/l

$CaCl \cdot 2H_2O$	147.02 g/l

Fructose at a final concentration of 20 g/l was dissolved in 50 ml water and autoclaved separately. To prepare the final medium, the base soluion and the fructose solution were mixed. 10 ml/l of the dipotassium hydrogenphophate solution, and 2 ml/l of the calcium solution was added. Mineral stock solutions were added to the following concentrations:

$FeSO_4$	30 mg/ml
Na_2MoO_4	2.1 mg/ml

RM medium

To make the base of the RM growth medium, the components listed below were mixed and autoclaved at 121° for 20 minutes.

Nutrient broth (Difco)	2 g/l
Yeast extract	0.1 g/l
NaCl	0.2 g/l
$MgSO_4 \cdot 7H_20$	0.2 g/l
$CaSO_4 \cdot 2H_2O$	0.05 g/l

To prepare the final growth medium, sucrose was added to a final concentration of 2%, and minerals were added as described for BM.

SOC medium

The components listed below were mixed.

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
KCl	0.186 g/l
Glucose	3.6 g/l
MgCl2	5.08 g/l

The medium was sterile filtrated before aliquoting 1 ml to 1.5 ml eppendorftubes. The tubes were stored at -20°C.

TFB1

The components listed below were mixed.

KAc	2.94 g/l
RbCl	12.1 g/l
CaCl2 ·2H2O	1.47 g/l
MnCl2 ·4H2O	10.0 g/l
Glycerol	150 ml/l

pH was adjusted to 5.8 with dilute acetic acid and sterile filtrated. The solution was stored at 4°C.

TFB2

The components listed below were mixed.

MOPS	2.1 g/l
RbCl	1.21 g/l
CaCl2 ·2H2O 11 g/l	
Glycerol 150 ml/l	

pH was adjusted with dilute NaOH and sterile filtrated. The solution was stored at 4°C.

Appendix B

Solutions

Stock solutions and other solutions used in this work are presented here.

Antibiotic stock solutions

10000X stock solutions of antiobiotics was prepared by dissolving each antibiotic in RO-water to the concentration below.

Ampicillin	200 g/l
Apramycin	50 g/l
Kanamycin	50 g/l
Tetracycline	5 g/l

The solutions were sterile filtrated and stored in 1.5 ml aliquots at -20°C.

The working concentrations for *E. coli* was 200 µg/l for ampicillin, 50 µg/l for apramycin, 50 µg/l for kanamycin and 5 µg/l for tetracycline. The working concentrations of apramycin and ampicillin for both *A. vinelandii* and *A. chroococcum* were 25 µg/l and 10 µg/l, respectively.

Fast blue B solution

The components below were mixed to the given concentrations. RO-water was used as a solvent. The solution was prepared the same day as it was used, as it quickly deteriorates.

Acetic acid	5%
Fast blue B salt	0.5%

Ethanol Fast blue B solution

The components below were mixed in RO-water. The solution was prepared the same day as it was used, as it quickly deteriorates. Short term storage was done at 4°C. protected from light.

Acetic acid	1 %
Fast blue B salt	0.08 %
Ethanol	80 %

m-Toluate

A stock-solution of 0.5M m-Toluate was prepared by dissolving 0.6870 g of m-toluic acid in 100% ethanol to a total volume of 10 ml. The working concentration was 0.5mM.

Appendix C

DNA standards

The DNA standards used in this work were *Lambda* DNA digested with PstI or HindIII (Figure C.1).

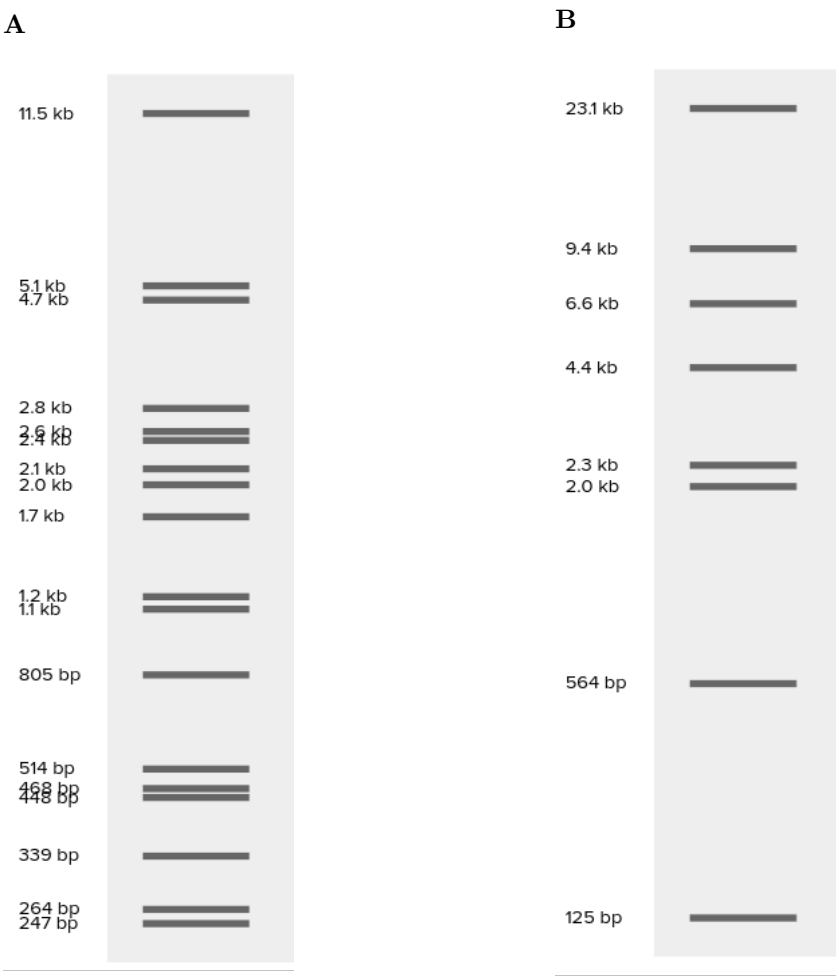
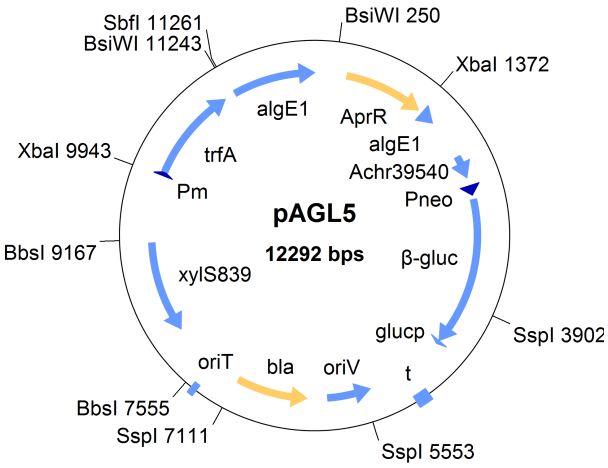
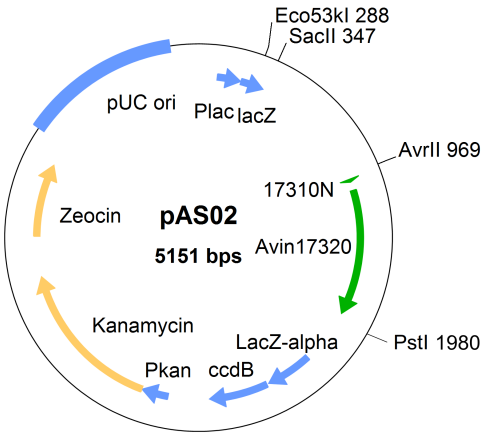


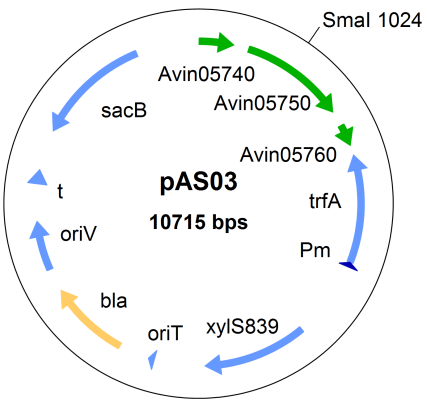
Figure C.1: DNA standards used in this work. *Lamda* DNA digested with (A) PstI and (B) HindiII.

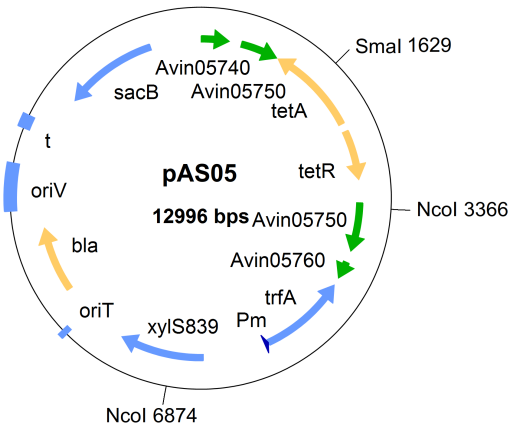
Appendix D

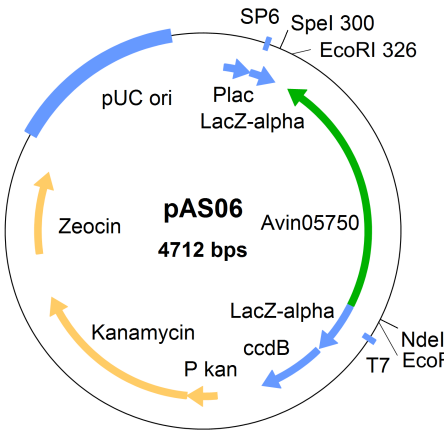
Plasmid maps







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Plac
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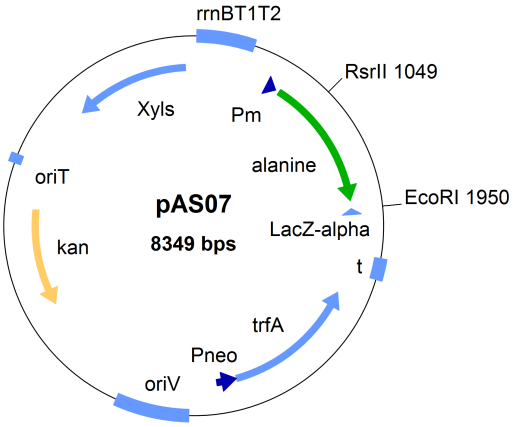
Avin05750
LacZ-alpha
Kanamycin
ccdB
P kan
T7
NdeI
EcoR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Pm
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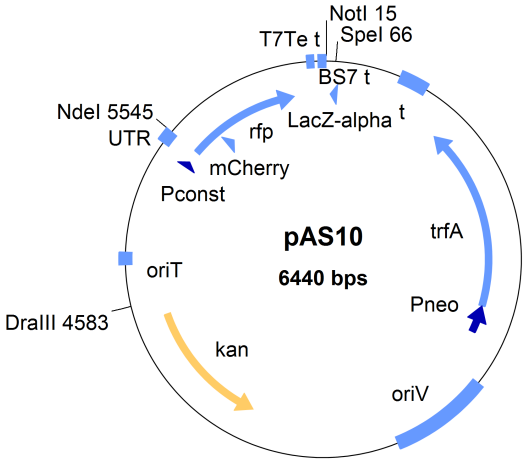
LacZ-alpha
kan
t
trfA
Pneo
oriV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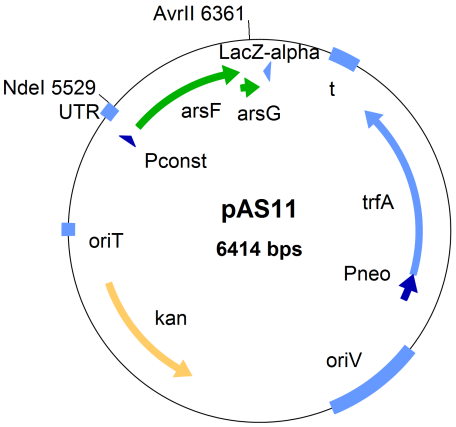
trfA
oriT
Pneo
DraIII 4583
kan
oriV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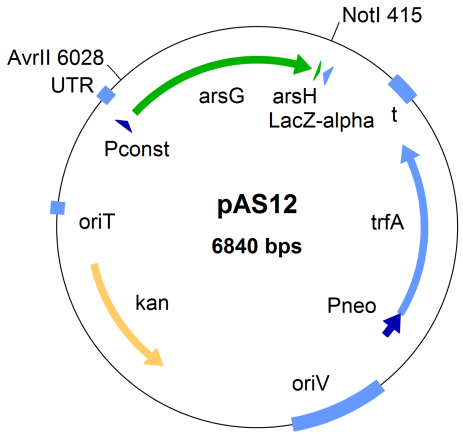
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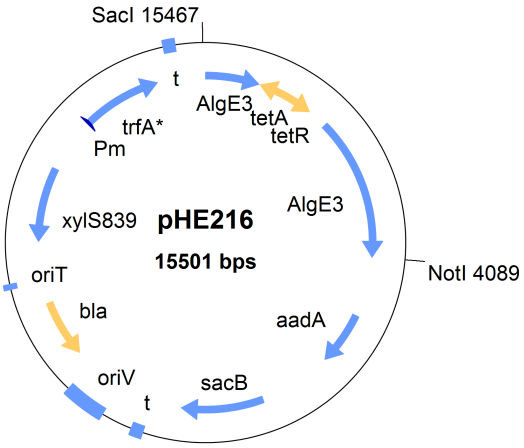
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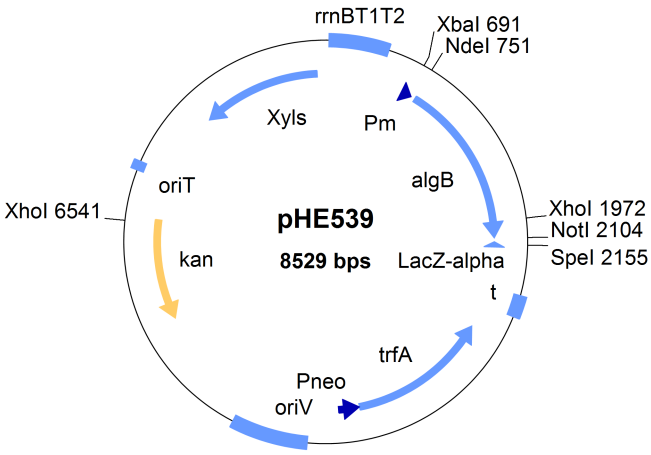
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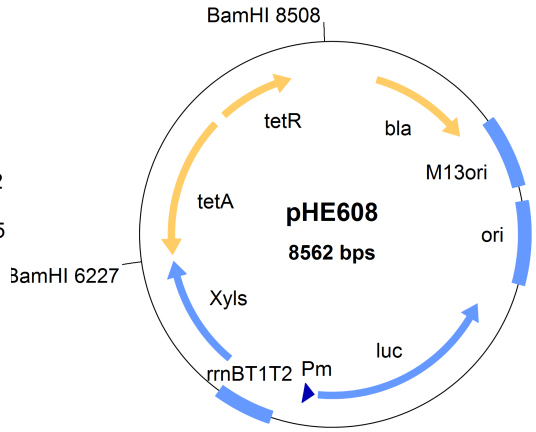
oriV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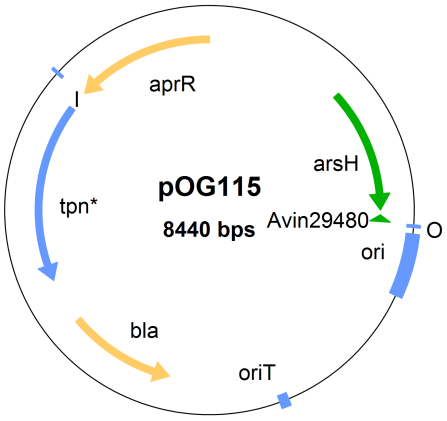
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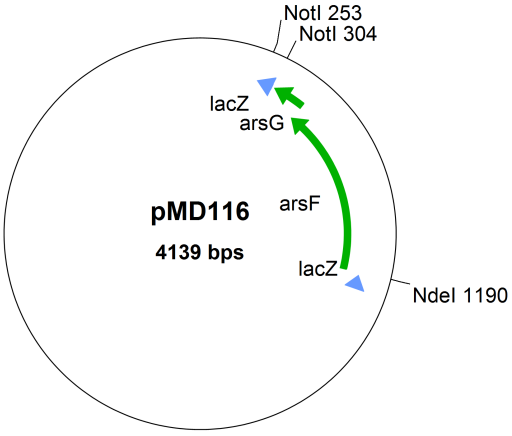
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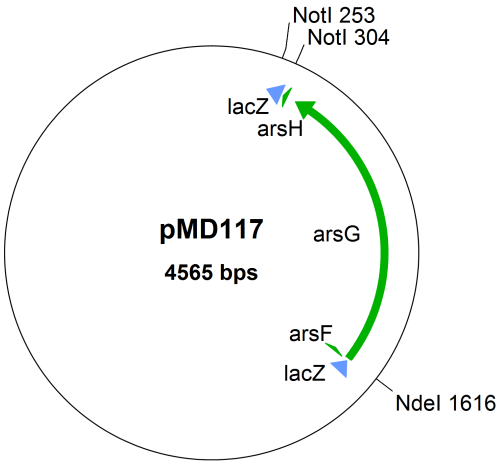
NdeI 1190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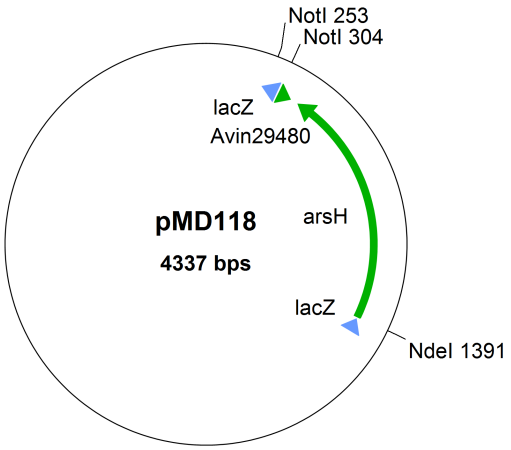
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arsH
lacZ
NdeI 1391

Appendix E

PCR Primers

Primers used in PCR amplifications, together with the sequence and the melting temperature, are given in Table E.1

Table E.1: Primer sequences and melting temperatures of primers used in PCR amplifications.

Primer	**Sequence**	**Tm**[°C]
1731020ER	5' TTGATCGAGTAGCGGATCAC 3'	61
17310ER	5' CCTGCATTTCGAGGAGAG 3'	59
17310NdeI	5'CATATGAAGTCGCTGAGACAGTTG 3'	61
M13F	5' GTAAAACGACGGCCAG 3'	-
M13R	5' CAGGAAACAGCTATGAC 3'	-
N17310R	5' CGTTTATGCCGACGAACCC 3'	63
O17310F2	5' GCCGCCTATCGTGAAGAATC 3'	62
O17310R2	5' ATTCCTAGG-CTGTCGGAACAGGTGAATCG 3'	62
O17310FragmentF	5' GTGCCAGTCGTCCATGAGTGTG 3'	67
O17310FragmentR	5' TCCGGCCCATATGCGAGCAAG 3'	69
Gen3FA	5' TCCGGCCCATATGCGAGCAAG 3'	-
Gen3RA	5' TATGAGATCTGCCCAGAACCTCTACGACAC 3'	-

Appendix F

Avin05750 Sequencing and alignment

Sequence of PCR product obtained from *A. vinelandii* gDNA with the primer pair ADH NdeI/ADH R2 was aligned to the sequence of *Avin05750* from NIH sequence database GenBank. Primers used in sequencing were M13F and M13R. The alignment is given here.

```
Page 1 ADH_compl_align

          1760                                                                1841
Avin05750 ATATGCGTATCGGGATACCCAGGGAACTCAAGAACCACGAATACCGGGTCGGTCTGACGCCGTCCTCGGTGGCCGAGCTGAG
M13R      CCTTCAATGCGTTTGGGATCCCAGGGACTCAAGACCCCGA--ATCCGGGTCGTTTGACG-CGTCCTCGGT-GCCGAGCTGAG
M13F      ATATGCGTATCGGGATACCCAGGGAACTCAAGAACCACGAATACCGGGTCGGTCTGACGCCGTCCTCGGTGGCCGAGCTGAG

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          1842                                                                1923
Avin05750 CGCTCTCGGCCACGAGGTCTGGGTCGAAAGCCGGGCCGGCGCCGCCATCGGCTTCGCCGACCCGGACTACCGGGCCGCCGGG
M13R      C-CTTTTGGCCACGAGGTCT-GGTCGAAAGCCGGGCCGGCGCCGCCATCGGCTTCGCCGACCCGGACTACCGGGCCGCCGGG
M13F      CGCTCTCGGCCACGAGGTCTGGGTCGAAAGCCGGGCCGGCGCCGCCATCGGCTTCGCCGACCCGGACTACCGGGCCGCCGGG

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          1924                                                                2005
Avin05750 GCGCACATCGCCACGGACGGCGCGGCGCTCTACGAGGCCGCCGAACTGATCGTCAAGGTCAAGGAGCCGCTGGCCGAGGAGC
M13R      GCGCACATCGCCACGGACGGCGCGGCGCTCTACGAGGCCGCCGAACTGATCGTCAAGGTCAAGGAGCCGCTGGCCGAGGAGC
M13F      GCGCACATCGCCACGGACGGCGCGGCGCTCTACGAGGCCGCCGAACTGATCGTCAAGGTCAAGGAGCCGCTGGCCGAGGAGC

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          2006                                                                2087
Avin05750 GCGCCCGCCTGCGCCCGCACCACACCCTGTTCACCTACTTGCACCTGGCCCCGGACCGGGCGCAGACCGACGATCTGCTGGC
M13R      GCGCCCGCCTGCGCCCGCACCACACCCTGTTCACCTACTTGCACCTGGCCCCGGACCGGGCGCAGACCGACGATCTGCTGGC
M13F      GCGCCCGCCTGCGCCCGCACCACACCCTGTTCACCTACTTGCACCTGGCCCCGGACCGGGCGCAGACCGACGATCTGCTGGC

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          2088                                                                2169
Avin05750 CAGCGGCGCCACCTGCATCGCCTACGAGACGGTCAGCGACGCCCAGGGCCGCCTGCCGTTGCTGGCGCCGATGTCCGAGGTC
M13R      CAGCGGCGCCACCTGCATCGCCTACGAGACGGTCAGCGACGCCCAGGGCCGCCTGCCGTTGCTGGCGCCGATGTCCGAGGTC
M13F      CAGCGGCGCCACCTGCATCGCCTACGAGACGGTCAGCGACGCCCAGGGCCGCCTGCCGTTGCTGGCGCCGATGTCCGAGGTC

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          2170                                                                2251
Avin05750 GCCGGACGGATGGCGATCCAGGCCGGCGCCCACTGCTTGGAAAAGGCCCAGGGCGGGCGCGGCGTGCTGCTCGGCGGAGTGC
M13R      GCCGGACGGATGGCGATCCAGGCCGGCGCCCACTGCTTGGAAAAGGCCCAGGGCGGGCGCGGCGTGCTGCTCGGCGGAGTGC
M13F      GCCGGACGGATGGCGATCCAGGCCGGCGCCCACTGCTTGGAAAAGGCCCAGGGCGGGCGCGGCGTGCTGCTCGGCGGAGTGC

..................................................................................................

          2252                                                                2333
Avin05750 CCGGGGTGCCGGCCGGCAAGGTCGCGATCCTCGGCGGCGGGGGTGGTGGGCAGCCAGGCGCTGGCCATGGCCGTCGGCCTGGG
M13R      CCGGGGTGCCGGCCGGCAAGGTCGCGATCCTCGGCGGCGGGGGTGGTGGGCAGCCAGGCGCTGGCCATGGCCGTCGGCCTGGG
M13F      CCGGGGTGCCGGCCGGCAAGGTCGCGATCCTCGGCGGCGGGGGTGGTGGGCAGCCAGGCGCTGGCCATGGCCGTCGGCCTGGG

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          2334                                                                2415
Avin05750 CGCGGAGGTCACCGTGCTCGACCGCGATCTGGACGTCCTGCGCCGGCTGGACGCCCGCTACGGCAACCGCATCGCCACCCTG
M13R      CGCGGAGGTCACCGTGCTCGACCGCGATCTGGACGTCCTGCGCCGGCTGGACGCCCGCTACGGCAACCGCATCGCCACCCTG
M13F      CGCGGAGGTCACCGTGCTCGACCGCGATCTGGACGTCCTGCGCCGGCTGGACGCCCGCTACGGCAACCGCATCGCCACCCTG

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          2416                                                                2497
Avin05750 CACGCCACGGGGGCGGCGGTGCGCGAGCAGGTGCTGGCGGCGGATCTGGTGATCGGTGCCGTGCTGATCCCCGGCGCGGCGG
M13R      CACGCCACGGGGGCGGCGGTGCGCGAGCAGGTGCTGGCGGCGGATCTGGTGATCGGTGCCGTGCTGATCCCCGGCGCGGCGG
M13F      CACGCCACGGGGGCGGCGGTGCGCGAGCAGGTGCTGGCGGCGGATCTGGTGATCGGTGCCGTGCTGATCCCCGGCGCGGCGG

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          2498                                                                  2579
Avin05750 CGCCCAAGCTGATCGGCGCGGAAATGGTCGCGCGGATGAAGCCCGGCGCGGTGCTGGTGGACGTGGCCATCGACCAGGGCGG
M13R      CGCCCAAGCTGATCGGCGCGGAAATGGTCGCGCGGATGAAGCCCGGCGCGGTGCTGGTGGACGTGGCCATCGACCAGGGCGG
M13F      CGCCCAAGCTGATCGGCGCGGAAATGGTCGCGCGGATGAAGCCCGGCGCGGTGCTGGTGGACGTGGCCATCGACCAGGGCGG

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          2580                                                                  2661
Avin05750 CTGCGCCGAGACCTCGCATCCGACCACCCACGCCGAGCCGACCTATGTGGTCGATGGCGTGGTGCACTACTGCGTGGCCAAC
M13R      CTGCGCCGAGACCTCGCATCCGACCACCCACGCCGAGCCGACCTATGTGGTCGATGGCGTGGTGCACTACTGCGTGGCCAAC
M13F      CTGCGCCGAGACCTCGCATCCGACCACCCACGCCGAGCCGACCTATGTGGTCGATGGCGTGGTGCACTACTGCGTGGCCAAC

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          2662                                                                  2743
Avin05750 ATGCCCGGCGCGGTGGCCCGCACCTCGACCCTGGCGCTGAACAACGCGACCCTGCCGTTCGTCGTCGCCCTGGCGGAG-AAG
M13R      ATGCCCGGCGCGGTGGCCCGCACCTCGACCCTGGCGCTGAACAACGCGACCCTGCCGTTCGTCGTCGCCCTGGCGGAG-AAG
M13F      ATGCCCGGCGCGGTGGCCCGCACCTCGACCCTGGCGCTGAACAACGCGACCCTGCCGTTCGTCGTCGCCCTGGCGGAGAAAG

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          2744                                                                  2825
Avin05750 GGCGTGCGCCGCGCCCTGGAGGAAGATCCGCACCTGCGCGACGGCCTCAACCTGGCGCGCGGCCAGCTCGCCTGCGCCAGCG
M13R      GGCGTGCGCCGCGCCCTGGAGGAAGATCCGCACCTGCGCGACGGCCTCAACCTGGCGCGCGGCCAGCTCGCCTGCGCCAGCG
M13F      GGCGTGCGCCGCGCCCTGGAGGAAGATCCGCACCTGCGCGACGGCTCAACTGGGGCG---GGGCAGTTCG-CTGGGCCAGCG

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          2826                                                                  2907
Avin05750 TGGCCGAAGCCCACGGCCTGCCGTACCGCAACGCCGCCGAGTTGCTGGCGCGGTTGTGAGCGGCGGGGAATCGCTCCGGCCA
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M13F      GGGC--GAAGCCACGGGCTGC--GTACGCAACGCCGCC-AATTGCTGGC-CGGTTG-GAACGGC-GGGAATCCCTCCGGCCC

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          2908                                  2950
Avin05750 CGCTGACGGCGAGACGCCCGCGCGCGAGTGCATTCATCCATCC
M13R      CGCTGACGGCGAGACGCCCGCGCGCGAGTGCATTCATCCATCC
M13F      GCTGACGGGAGAACCCCGGCGCGAGGGTTTCTCCTCCCAAGGG

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```

Appendix G

Avin17310 Sequencing and alignments

Sequence of PCR product obtained by PCR of *A. vinelandii* gDNA with primer pair 17310NdeI/17310ER was aligned to the sequence of *Avin17310* from NIH sequence database GenBank. Primers used for sequencing were M13F and M13R. The alignment is given here.

```
Page 1 17310_align

          1018                                                                1099
Avin17310 CATATGAAGTCGCTGAGACAGTTGCTGCGCGAACGCCACGAATGGATGGAAGCGCGGATATTCGAGGGGGCCGAGCGAAAAG
M13R      CATATGAAGTCGCTGAGACAGTTGCTGCGCGAACGCCACGAATGGATGGAAGCGCGGATATTCGAGGGGGCCGAGCGAAAAG
M13F      CATATGAAGTCGCTGAGACAGTTGCTGCGCGAACGCCACGAATGGATGGAAGCGCGGATATTCGAGGGGGCCGAGCGAAAAG

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          1100                                                                1181
Avin17310 GCTATGGAGGAATCACGCCGGCCATGAGCCGTCTGTATGGCCAGATAAGTCGCGAGCCGATCGGTCTGTCCGAATTGGCTCG
M13R      GCTATGGAGGAATCACGCCGGCCATGAGCCGTCTGTATGGCCAGATAAGTCGCGAGCCGATCGGTCTGTCCGAATTGGCTCG
M13F      GCTATGGAGGAATCACGCCGGCCATGAGCCGTCTGTATGGCCAGATAAGTCGCGAGCCGATCGGTCTGTCCGAATTGGCTCG

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          1182                                                                1263
Avin17310 GCAAATGGGGATATCTCGCCAGGCCATTCACAAGATGGTGGGCGAGGGGGTGCAAGCGGGATTTCTGGAGTTGGTGGACAGC
M13R      GCAAATGGGGATATCTCGCCAGGCCATTCACAAGATGGTGGGCGAGGGGGTGCAAGCGGGATTTCTGGAGTTGGTGGACAGC
M13F      GCAAATGGGGATATCTCGCCAGGCCATTCACAAGATGGTGGGCGAGGGGGTGCAAGCGGGATTTCTGGAGTTGGTGGACAGC

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          1264                                                                1345
Avin17310 GAGGAAGACCGGCGCATCAAACTCGTTCGTTTCAGTCTCGAAGGATTGCGCATGGCCGATGCCGCCAGACAGGAAATGGAAT
M13R      GAGGAAGACCGGCGCATCAAACTCGTTCGTTTCAGTCTCGAAGGATTGCGCATGGCCGATGCCGCCAGACAGGAAATGGAAT
M13F      GAGGAAGACCGGCGCATCAAACTCGTTCGTTTCAGTCTCGAAGGATTGCGCATGGCCGATGCCGCCAGACAGGAAATGGAAT

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          1346                                                                1427
Avin17310 CGATCGAACAGGAACTTGCGGAAAGAATCGGTGGAGAGGATCTGGAGGAGTTGCGCCGTATTCTGGCCAAAGCCTGGTATTG
M13R      CGATCGAACAGGAACTTGCGGAAAGAATCGGTGGAGAGGATCTGGAGGAGTTGCGCCGTATTCTGGCCAAAGCCTGGTATTG
M13F      CGATCGAACAGGAACTTGCGGAAAGAATCGGTGGAGAGGATCTGGAGGAGTTGCGCCGTATTCTGGCCAAAGCCTGGTATTG

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          1428                1452
Avin17310 ATTGCTGAAAAGTTTCCGGTACAAG
M13R      ATTGCTGAAAAGTTTCCGGTACAAG
M13F      ATTGCTGAAAAGTTTCCGGTACAAG

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```

Sequence of PCR product obtained by PCR of *A. vinelandii* gDNA with primer pair 17310NdeI/1731020ER was aligned to the sequence of *Avin17310* and *Avin17320* from NIH sequence database GenBank. Primers used for sequencing were M13F and M13R. The alignment is given here.

```
Page 1 17310_17320

            337                                                                                 418
Avin17310... TTGATCGAGTAGCGGATCACCAGATGGCGATGGTGTAGTCCATGTTTATCCGCAACTCGTTCACATCGCGGGAATAACTACT
M13F         TTGATCGAGTAGCGGATCACCAGATGGCGATGGTGTAGTCCATGTTTATCCGCAACTCGTTCACATCGCGGGAATAACTACT
M13R         -------------------------------------------------------------------------------

.....................................................................................................

            419                                                                                 500
Avin17310... CTGGTAATCGGCCTGGCGAACGCGAAACGCCATATCCTTCAGCGGCCCCGACTGGATCACATAGGACAGCTCCAGATATCGC
M13F         CTGGTAATCGGCCTGGCGAACGCGAAACGCCATATCCTTCAGCGGCCCCGACTGGATCACATAGGACAGCTCCAGATATCGC
M13R         -------------------------------------------------------------------------------

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            501                                                                                 582
Avin17310... TCGCTTTCGTGCGCCCTGCTGGCACTGAAGGCGGGCATGTTTATGTGACTGCCATCGGCATAGCGAAACGTAGCCAGCAGGC
M13F         TCGCTTTCGTGCGCCCTGCTGGCACTGAAGGCGGGCATGTTTATGTGACTGCCATCGGCATAGCGAAACGTAGCCAGCAGGC
M13R         --------------------------------TCCCCAAATAGTGTTCTTGAAGAGTAAAGGGCGGGCGCGGTTTATCAGGA

.....................................................................................................

            583                                                                                 664
Avin17310... CGGGCAACCCGCTTGCCGCAAAGTCGTAATCGTAACGCACCTGCCATACCCGCTCGTCCGGGTTGCCCATCTCGCCGTCGAG
M13F         CGGGCAACCCGCTTGCCGCAAAGTCGTAATCGTAACGCACCTGCCATACCCGCTCGTCCGGGTTGCCCATCTCGCCGTCGAG
M13R         TTTTCTGGTTTTTGTTGGGGCCACCCATCCCCCCACAAAACCACACTTTTCTCTTCCGCAAGGGGGGGTATTTTGCACAAAA

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            665                                                                                 746
Avin17310... TAGTCCCTGGCTGTGCGTGTGCGTGAAGGTCGCGTTTATGCCGACGAACCCGTGTTCGCCGAAAATCCGGTAGTAACCGGCG
M13F         TAGTCCCTGGCTGTGCGTGTGCGTGAAGGTCGCGTTTATGCCGACGAACCCGTGTTCGCCGAAAATCCGGTAGTAACCGGCG
M13R         AAAACCCCGGACCCCCAATAGTTTATCACCCCCCCCTCTTTTGGTTCTTTCCGGAGTTCTGGTGGGGGGGAGGGGTGTTTAC

.....................................................................................................

            747                                                                                 828
Avin17310... CCGAACGCATGCCCCTTGTACTTGTAGGTCGAGAAAAATCCCGCATAGCGATTGTCGATATCCCCGGCCCGCGCCTCGCCCT
M13F         CCGAACGCATGCCCCTTGTACTTGTAGGTCGAGAAAAATCCCGCATAGCGATTGTCGATATCCCCGGCCCGCGCCTCGCCCT
M13R         GAAAACTTTTTCCAAATCGTTTAACCGGCGCGAAACCTCCCCTTTTTTTTGGTGAAAAATCCCCAAAGGTTTTGAATTCCCG

.....................................................................................................

            829                                                                                 910
Avin17310... GCTCGCTGCTGGAGAAATAGCGAAAATCATGCTGCAACTTGCCGGGCCCCAGGGCATGGCTGAACTGCACGCCCAGGTAATG
M13F         GCTCGCTGCTGGAGAAATAGCGAAAATCATGCTGCAACTTGCCGGGCCCCAGGGCATGGCTGAACTGCACGCCCAGGTAATG
M13R         CCCCGCCCTCCCCTTCCGTTGGGGAAAAAAGGGAAATCATGTGAAATTTCCGGGCCCCGGGCAGGGTGAACTGCCGCCCAGG

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            911                                                                                 992
Avin17310... CTGCTTGTACACATCGTCGAGATAGCCGAAGTAATGGTTCGTGACCAGCCCGGGAGTCCAACGGTAATCGGCGCCGACGAAA
M13F         CTGCTTGTACACATCGTCGAGATAGCCGAAGTAATGGTTCGTGACCAGCCCGGGAGTCCAACGGTAATCGGCGCCGACGAAA
M13R         TAATCTGCTGTACCCTTGTCGAGATACCGAAGTAATGTTTCGGACCAGCCCGGGAGTCCAACGGTAATCGGC-CCGACGAAA

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            993                                                                                1074
Avin17310... GCGAAGTGACTGCCCTCGGCGCTGGACTCGAAACGCCCCCAGGGGCTGGCTATCCGCAATGCCTCGAAGTTGGTGGAGTTGC
M13F         GCGAAGTGACTGCCCTCGGCGCTGGACTCGAAACGCCCCCAGGGGCTGGCTATCCGCAATGCCTCGAAGTTGGTGGAGTTGC
M13R         GCGAAGTGACTGCCCTCGGCGCTGGACTCGAAACGCCCCCAGGGGCTGGCTATCCGCAATGCCTCGAAGTT-GTGGAGTTGC

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            1075                                                                1156
Avin17310... GCTCCTCGATGCGATCGAAATAGCCCGCGTGCAGGGTGAGATCGCGAAATTCCCCCGACTTCATCTGCGCGCCGCGAAACAC
M13F         GCTCCTCGATGCGATCGAAATAGCCCGCGTGCAGGGTGAGATCGCGAAATTCCCCCGACTTCATCTGCGCGCCGCGAAACAC
M13R         GCTCCTCGATGCGATCGAAATAGCCCGCGTGCAGGGTGAGATCGCGAAATTCCCCCGACTTCATCTGCGCGCCGCGAAACAC

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            1157                                                                1238
Avin17310... CGGCGGAAACAGGCGCACCGTCGGCGAAACCAGTATCGGCACCATGGGCGTCAGACCGCCGACGGCCAGTTCGGTCTGCGCG
M13F         CGGCGGAAACAGGCGCACCGTCGGCGAAACCAGTATCGGCACCATGGGCGTCAGACCGCCGACGGCCAGTTCGGTCTGCGCG
M13R         CGGCGGAAACAGGCGCACCGTCGGCGAAACCAGTATCGGCACCATGGGCGTCAGACCGCCGACGGCCAGTTCGGTCTGCGCG

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            1239                                                                1320
Avin17310... GCGCGCGCCTTGAGGACGAAATCGAACTGGCTGTAACTGCTCTCCGCCTTGCCATCCGGCCCATATGCGAGCAAGCCGGTGC
M13F         GCGCGCGCCTTGAGGACGAAATCGAACTGGCTGTAACTGCTCTCCGCCTTGCCATCCGGCCCATATGCGAGCAAGCCGGTGC
M13R         GCGCGCGCCTTGAGGACGAAATCGAACTGGCTGTAACTGCTCTCCGCCTTGCCATCCGGCCCATATGCGAGCAAGCCGGTGC

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            1321                                                                1402
Avin17310... CCACGCGATCGGGGGAGGAATCGAGCTTGACGCCGGTCTTGCTCGTCAGGCCGACGGCGAAGCCGACAGGTCCTTCGGTATA
M13F         CCACGCGATCGGGGGAGGAATCGAGCTTGACGCCGGTCTTGCTCGTCAGGCCGACGGCGAAACCGACAGGTCCTTCGGT-TA
M13R         CCACGCGATCGGGGGAGGAATCGAGCTTGACGCCGGTCTTGCTCGTCAGGCCGACGGCGAAGCCGACAGGTCCTTCGGTATA

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            1403                                                                1484
Avin17310... ACCGGATTGAACATTCAATATGAAACCCTGTGCCCATTCCGCCTGACGCGACTGCGACTGAAAACGCGTGGCGTCGCCCTTG
M13F         ACCGGATTGAACATTC-ATATGAAA-CCTGTGCCATTCCCCTGACCGACTGCGAATGAAAACGCGTGGCGTCCCCTTGAATC
M13R         ACCGGATTGAACATTCAATATGAAACCCTGTGCCCATTCCGCCTGACGCGACTGCGACTGAAAACGCGTGGCGTCGCCCTTG

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            1485                                                                1566
Avin17310... TAGTCGCGATCCATATAGAAATTCCGCAATGTCAGGGTAGCCTTGCTATCGGCCAGGAAGTCCCCGCTGGCGGGCATGGACA
M13F         CCGATCCAAAAGAAATTCCGCAAGTCAGGGAACCTTGCTACGCCAGAAATCCCGCTGGGGGCTGGAAAAATGGCAGATGCCG
M13R         TAGTCGCGATCCATATAGAAATTCCGCAATGTCAGGGTAGCCTTGCTATCGGCCAGGAAGTCCCCGCTGGCGGGCATGGACA

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            1567                                                                1648
Avin17310... ATACTGCACAGATGCCGGAAGCAAAAACCGCCCCTGCATTTCGAGGAGAGAACATTTTTATTGCGACTCCGAATTATTATTC
M13F         AAACAAAACGCCCCGGTTTCGGGAGAAAATTTTTTTGGCCCCCATTTTTTTTTTTTCGGAAAATCTCCAAAAACACGGTTGGG
M13R         ATACTGCACAGATGCCGGAAGCAAAAACCGCCCCTGCATTTCGAGGAGAGAACATTTTTATTGCGACTCCGAATTATTATTC

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            1649                                                                1730
Avin17310... TTGTACCGGAAACTTTTCAGCAATCAATACCAGGCTTTGGCCAGAATACGGCGCAACTCCTCCAGATCCTCTCCACCGATTC
M13F         AAAAGGGCAACCCCCAACCCCCCCATTTCCAGTTGTTTTAAATTTTTTTGGGGGGGGGGAACCAAAAAAAGAAAAAGGGGCT
M13R         TTGTACCGGAAACTTTTCAGCAATCAATACCAGGCTTTGGCCAGAATACGGCGCAACTCCTCCAGATCCTCTCCACCGATTC

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            1731                                                                1812
Avin17310... TTTCCGCAAGTTCCTGTTCGATCGATTCCATTTCCTGTCTGGCGGCATCGGCCATGCGCAATCCTTCGAGACTGAAACGAAC
M13F         CCCCCCAAAAAGCCGCCCCCCAGGGGAGGAAAAAAAAAAGAAAGAAAAAAGATGGGAGGGTTTTTTTTCTGCCTCCCTGGTAT
M13R         TTTCCGCAAGTTCCTGTTCGATCGATTCCATTTCCTGTCTGGCGGCATCGGCCATGCGCAATCCTTCGAGACTGAAACGAAC

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             1813                                                                1894
Avin17310... GAGTTTGATGCGCCGGTCTTCCTCGCTGTCCACCAACTCCAGAAATCCCGCTTGCACCCCCTCGCCCACCATCTTGTGAATG
M13F         GTTGAGGGAAAATAATATTATCACTCCCATAGGT-----------------------------------------------
M13R         GAGTTTGATGCGCCGGTCTTCCTCGCTGTCCACCAACTCCAGAAATCCCGCTTGCACCCCCTCGCCCACCATCTTGTGAATG

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             1895                                                                1976
Avin17310... GCCTGGCGAGATATCCCCATTTGCCGAGCCAATTCGGACAGACCGATCGGCTCGCGACTTATCTGGCCATACAGACGGCTCA
M13F         -------------------------------------------------------------------------------
M13R         GCCTGGCGAGATATCCCCATTTGCCGAGCCAATTCGGACAGACCGATCGGCTCGCGACTTATCTGGCCATACAGACGGCTCA

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             1977                                                                2058
Avin17310... TGGCCGGCGTGATTCCTCCATAGCCTTTTCGCTCGGCCCCCTCGAATATCCGCGCTTCCATCCATTCGTGGCGTTCGCGCAG
M13F         -------------------------------------------------------------------------------
M13R         TGGCCGGCGTGATTCCTCCATAGCCTTTTCGCTCGGCCCCCTCGAATATCCGCGCTTCCATCCATTCGTGGCGTTCGCGCAG

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             2059               2082
Avin17310... CAACTGTCTCAGCGACTTCATATG
M13F         ------------------------
M13R         CAACTGTCTCAGCGACTTCATATG

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Appendix H

Cyst cell counts

CFU counted before and after drying of culture samples for estimations of encystment are given in Table H.1 for experiments with ATCC 12518 wt, ATCC 12518Δ05390, and ATCC 12518Δ05750 grown in liquid BM supplemented with βHB for encystment. Table H.2 presents cell counts before and after drying for ATCC 12518 wt, ATCC 12518Δ05390, and ATCC 12518Δ05750 grown on n-butanol for encystment. Cell counts before and after drying of the three wt strains ATCC 12518, ATCC 12837, and E are given in Table H.3. Letter designates cultures, while numbers designates replicate platings of the same culture. 10 µl culture samples were dried if not otherwise stated. For plating after dessication, the dried cells were dilutet in 100µl BS and 50 µl of this was plated.

Table H.1: Cell counts and calculated CFU before and after drying of cultures encysted in liquid BM supplemented with βHB. Letter designates culture, and numbers designates replicate platings of the same culture. *: 1 ml samples of culture dried. n.a.: not assesse, or overgrown.

		Before drying			**After drying**		
Strain	parallell	10^{-5}	10^{-6}	CFU [ml^{-1}]	10^{0}	10^{-1}	CFU [ml^{-1}]
ATCC 12518	A1	114	8	$1.14{\cdot}10^{8}$	0	0	0
	A2	95	15	$9.50{\cdot}10^{7}$	0	0	0
	A3	102	12	$1.02{\cdot}10^{8}$	0	0	0
	B1	121	21	$1.21{\cdot}10^{8}$	0	0	0
	B2	103	6	$1.03{\cdot}10^{8}$	0	0	0
	B3	125	10	$1.25{\cdot}10^{8}$	0	0	0
	C1	177	18	$1.77{\cdot}10^{8}$	0	0	0
	C2	120	12	$1.20{\cdot}10^{8}$	0	0	0
	C3	171	14	$1.71{\cdot}10^{8}$	0	0	0
	D1	59	6	$5.90{\cdot}10^{7}$	0	0	0
	D2	54	3	$5.40{\cdot}10^{7}$	0	0	0
	D3	26	1	$2.60{\cdot}10^{7}$	0	0	0
	E1	171	18	$1.71{\cdot}10^{8}$	0	0	0
	E2	182	21	$1.82{\cdot}10^{8}$	0	0	0
	E3	167	17	$1.67{\cdot}10^{8}$	0	0	0
	F1	170	18	$1.70{\cdot}10^{8}$	0	0	0

	F2	163	7	$1.63 \cdot 10^8$	0	0	0
	F3	201	27	$2.01 \cdot 10^8$	0	0	0
	G1*	197	12	$1.97 \cdot 10^8$	n.a.	0	0
	G2*	166	18	$1.66 \cdot 10^8$	n.a.	0	0
	G3*	179	22	$1.79 \cdot 10^8$	n.a.	0	0
	H1*	86	13	$8.6 \cdot 10^7$	n.a.	0	0
	H2*	93	6	$9.3 \cdot 10^7$	n.a.	0	0
	H3*	144	12	$1.44 \cdot 10^8$	n.a.	0	0
	I1*	111	10	$1.11 \cdot 10^8$	n.a.	0	0
	I2*	134	12	$1.34 \cdot 10^8$	n.a.	0	0
	I3*	121	15	$1.21 \cdot 10^8$	n.a.	0	0
Δ05390	A1	233	19	$2.33 \cdot 10^8$	0	0	0
	A2	209	21	$2.09 \cdot 10^8$	0	0	0
	A3	202	26	$2.02 \cdot 10^8$	0	0	0
	B1	128	10	$1.28 \cdot 10^8$	0	0	0
	B2	99	12	$9.9 \cdot 10^7$	0	0	0
	B3	132	15	$1.32 \cdot 10^8$	0	0	0
	C1	197	20	$1.97 \cdot 10^8$	0	0	0
	C2	239	29	$2.39 \cdot 10^8$	0	0	0
	C3	215	18	$2.15 \cdot 10^8$	0	0	0
	D1	20	2	$2.0 \cdot 10^7$	0	0	0
	D2	26	1	$2.6 \cdot 10^7$	0	0	0
	D3	24	0	$2.4 \cdot 10^7$	0	0	0
	E1	88	13	$8.8 \cdot 10^7$	0	0	0
	E2	81	5	$8.1 \cdot 10^7$	0	0	0
	E3	90	3	$9.0 \cdot 10^7$	0	0	0
	F1	126	12	$1.26 \cdot 10^8$	0	0	0
	F2	128	14	$1.28 \cdot 10^8$	0	0	0
	F3	135	13	$1.35 \cdot 10^8$	0	0	0
	G1*	157	18	$1.57 \cdot 10^8$	n.a.	0	0
	G2*	172	12	$1.72 \cdot 10^8$	n.a.	0	0
	G3*	156	17	$1.56 \cdot 10^8$	n.a.	0	0
	H1*	101	17	$1.01 \cdot 10^8$	n.a.	0	0
	H2*	192	20	$1.92 \cdot 10^8$	n.a.	0	0
	H3*	169	21	$1.69 \cdot 10^8$	n.a.	0	0
	I1*	184	21	$1.84 \cdot 10^8$	n.a.	0	0
	I2*	n.a.	n.a.	n.a.	n.a.	0	0
	I3*	176	19	$1.76 \cdot 10^8$	n.a.	0	0
Δ05750	A1*	242	30	$2.42 \cdot 10^8$	0	0	0
	A2*	174	21	$1.74 \cdot 10^8$	n.a.	1	100
	A3*	220	29	$2.20 \cdot 10^8$	n.a.	2	200
	B1*	158	13	$1.58 \cdot 10^8$	n.a.	2	200
	B2*	136	28	$1.36 \cdot 10^8$	n.a.	1	100
	B3*	211	25	$2.11 \cdot 10^8$	n.a.	0	0
	C1*	201	17	$2.01 \cdot 10^8$	n.a.	0	0
	C2*	200	23	$2.00 \cdot 10^8$	n.a.	1	100

	C3*	211	22	$2.11{\cdot}10^8$	n.a.	1	100

Table H.2: Cell counts and calculated CFU before and after drying of cultures encysted in liquid BM supplemented with βHB. Letter designates culture, and numbers designates replicate platings of the same culture. n.a.: not assessed.

		Before drying			**After drying**		
Strain	parallell	10^{-5}	10^{-6}	CFU [ml^{-1}]	10^0	10^{-1}	CFU [ml^{-1}]
ATCC 12518	A1	280	21	$2.80{\cdot}10^8$	8	0	$1.6{\cdot}10^4$
	A2	266	31	$2.66{\cdot}10^8$	1	0	$2.0{\cdot}10^3$
	A3	250	31	$2.50{\cdot}10^8$	114	16	$2.28{\cdot}10^5$
	B1	187	20	$1.87{\cdot}10^8$	0	0	0
	B2	156	28	$1.56{\cdot}10^8$	0	0	0
	B3	183	14	$1.83{\cdot}10^8$	0	0	0
	C1	163	17	$1.63{\cdot}10^8$	12	0	$2.4{\cdot}10^4$
	C2	165	36	$1.65{\cdot}10^8$	2	0	$4.0{\cdot}10^4$
	C3	140	28	$1.40{\cdot}10^8$	0	0	0
	D1	n.a.	459	$4.59{\cdot}10^9$	0	0	0
	D2	n.a.	471	$4.71{\cdot}10^9$	0	0	0
	D3	n.a.	199	$1.99{\cdot}10^9$	0	0	0
	E1	419	41	$4.19{\cdot}10^8$	0	0	0
	E2	462	49	$4.62{\cdot}10^8$	0	0	0
	E3	428	51	$4.28{\cdot}10^8$	0	0	0
Δ05390	A1	269	31	$2.69{\cdot}10^8$	0	0	0
	A2	284	22	$2.84{\cdot}10^8$	0	0	0
	A3	272	30	$2.72{\cdot}10^8$	0	0	0
	B1	231	26	$2.31{\cdot}10^8$	0	0	0
	B2	249	30	$2.49{\cdot}10^8$	0	0	0
	B3	252	26	$2.52{\cdot}10^8$	0	0	0
	C1	328	30	$3.28{\cdot}10^8$	0	0	0
	C2	329	29	$3.29{\cdot}10^8$	0	0	0
	C3	338	32	$3.38{\cdot}10^8$	0	0	0
	D1	188	20	$1.88{\cdot}10^8$	0	0	0
	D2	164	24	$1.64{\cdot}10^8$	0	0	0
	D3	159	14	$1.59{\cdot}10^8$	0	0	0
	E1	318	38	$3.18{\cdot}10^8$	0	0	0
	E2	313	35	$3.13{\cdot}10^8$	0	0	0
	E3	310	25	$3.10{\cdot}10^8$	0	0	0
Δ05750	A1	259	20	$2.59{\cdot}10^8$	0	0	0
	A2	239	24	$2.39{\cdot}10^8$	0	0	0
	A3	242	29	$2.42{\cdot}10^8$	1	0	$2.0{\cdot}10^3$
	B1	365	44	$3.65{\cdot}10^8$	2	0	$4{\cdot}10^3$
	B2	360	33	$3.60{\cdot}10^8$	7	0	$1.4{\cdot}10^4$
	B3	368	41	$3.68{\cdot}10^8$	0	0	0
	C1	327	26	$3.27{\cdot}10^8$	0	0	0

C2	285	29	$2.85{\cdot}10^8$	0	0	0
C3	266	29	$2.66{\cdot}10^8$	0	0	0
D1	125	12	$1.25{\cdot}10^8$	1	0	$2.0{\cdot}10^3$
D2	115	10	$1.15{\cdot}10^8$	4	0	$8{\cdot}10^3$
D3	132	16	$1.32{\cdot}10^8$	0	0	0
E1	246	22	$2.46{\cdot}10^8$	0	0	0
E2	249	20	$2.49{\cdot}10^8$	0	0	0
E3	276	18	$2.76{\cdot}10^8$	0	0	0

Table H.3: Cell counts and calculated CFU of wt strains before and after drying of cultures encysted in liquid BM supplemented with βHB. Letter designates culture, and numbers designates replicate platings of the same culture.n.a.: not assessed, or overgrown.

		Before drying			**After drying**			
Strain	parallell	10^{-5}	10^{-6}	CFU [ml^{-1}]	10^0	10^{-1}	10^{-2}	CFU [ml^{-1}]
ATCC 12518	A1	227	30	$2.27{\cdot}10^8$	1	0	0	$2{\cdot}10^3$
	A2	254	27	$2.54{\cdot}10^8$	0	0	0	0
	A3	226	21	$2.26{\cdot}10^8$	0	0	0	0
	B1	183	21	$1.83{\cdot}10^8$	3	0	0	$6{\cdot}10^3$
	B2	203	20	$2.03{\cdot}10^8$	2	0	0	$4{\cdot}10^3$
	B3	192	18	$1.92{\cdot}10^8$	0	0	0	0
ATCC 12837	A1	86	9	$8.6{\cdot}10^7$	182	12	2	$3.64{\cdot}10^5$
	A2	102	12	$1.02{\cdot}10^8$	426	49	5	$8.52{\cdot}10^5$
	A3	165	6	$1.65{\cdot}10^8$	107	10	0	$2.14{\cdot}10^5$
	B1	73	6	$7.3{\cdot}10^7$	1312	208	5	$2.62{\cdot}10^6$
	B2	89	6	$8.9{\cdot}10^7$	57	6	1	$1.1{\cdot}10^5$
	B3	98	11	$9.8{\cdot}10^7$	39	6	0	$7.8{\cdot}10^4$
E	A1	214	30	$2.14{\cdot}10^8$	n.a.	222	16	$4.44{\cdot}10^6$
	A2	205	32	$2.05{\cdot}10^8$	n.a.	160	10	$3.20{\cdot}10^6$
	A3	190	33	$1.90{\cdot}10^8$	n.a.	535	49	$1.07{\cdot}10^7$
	B1	393	51	$3.93{\cdot}10^8$	n.a.	940	106	$2.12{\cdot}10^7$
	B2	329	45	$3.29{\cdot}10^8$	n.a.	n.a.	554	$1.11{\cdot}10^8$
	B3	356	23	$3.56{\cdot}10^8$	40	16	0	$3.2{\cdot}10^5$

Appendix I

^{1}H-NMR spectrum of *A. chroococcum exopolysaccharides*

The full NMR-spectrum obtained by ^{1}H-NMR of extracted exopolysaccharides from *A. chroococcum* grown in RA1 medium for 48 hrs is given in Figure I.1.

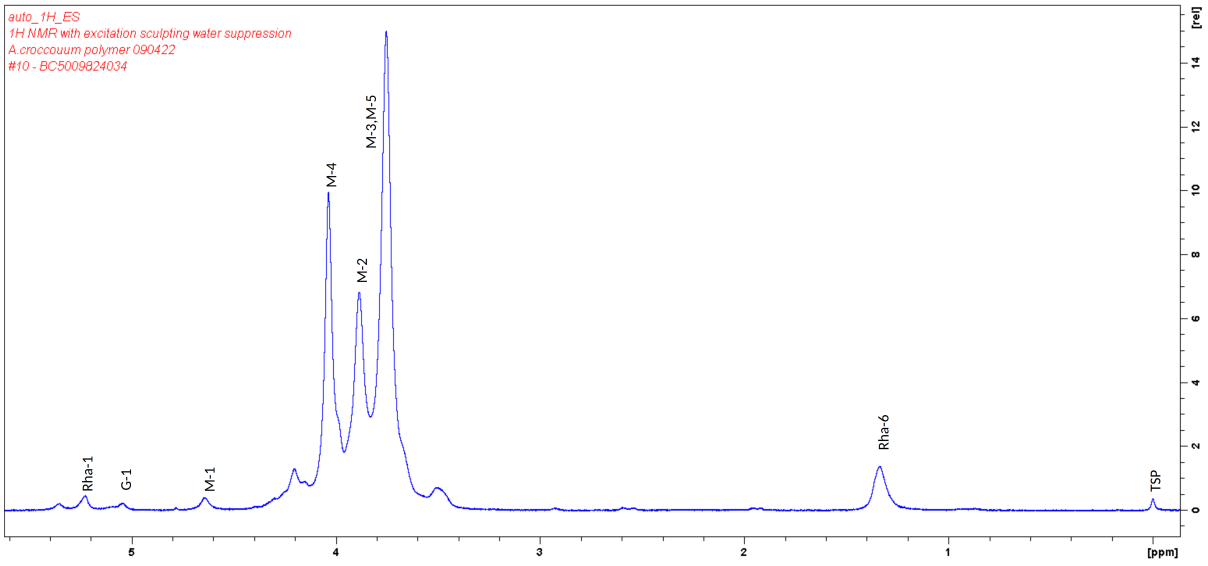

Figure I.1: NMR-Spectrum of exopolysaccharides from *A. chroococcum* grown in RA1 medium for 48 hrs. Peaks are marked with their respecitve molecule and the number of carbon the protons belongs to.

Anita Solem

Investigation of Encystment and Characterization of Exopolysaccharides in *Azotobacter* Species

NTNU
Norwegian University of
Science and Technology