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Regulation of alginate biosynthesis in Azotobacter vinelandii

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Regulation of Alginate Biosynthesis in *Azotobacter Vinelandii*

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Abstract

Alginate is a linear exopolysaccharide composed of various amount of (1-4) linked β-D mannuronic acid and its C5 epimer α-L-guluronic acid. Alginate is synthesized by two bacterial genera *Azotobacter* and *Pseudomonas. Azotobacter vinelandii* is a gram negative bacterium that can fix atmospheric nitrogen and form desiccation resistant cyst.

Transcriptional activation of *algD* gene is a main point in regulation of alginate biosynthesis pathway in *A.vinelandii.* In *A.vinelandii* alginate biosynthesis gene cluster is organized in transcriptional units of algD promoter. *AlgR*, *AlgB* and *AmrZ* are the major regulators required by *algD* promoter for its expression. Alternative sigma factor σ^{22} encoded by *algU* directly controls the *algD* promoter and its expression.

In this study, a vector expressing *AlgW* in *A.vinelandii* was constructed. Also, the plasmids for measuring *algD* expression and *algC* expression using mcherry as a reporter gene were constructed. Alginate production and *algD* gene expression was compared in strains lacking *algW* or *algB* with wildtype strain overproducing these proteins. Also, alginate was assayed enzymatically.

Based on results it was found that *algB* controls *algD* not only in *Pseudomonas* species but also in *Azotobacter*.

The study of effect of succinate on growth and alginate revealed that wildtype *A.vinelandii* has maximum growth in the medium supplemented with succinate

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CHAPTER 1

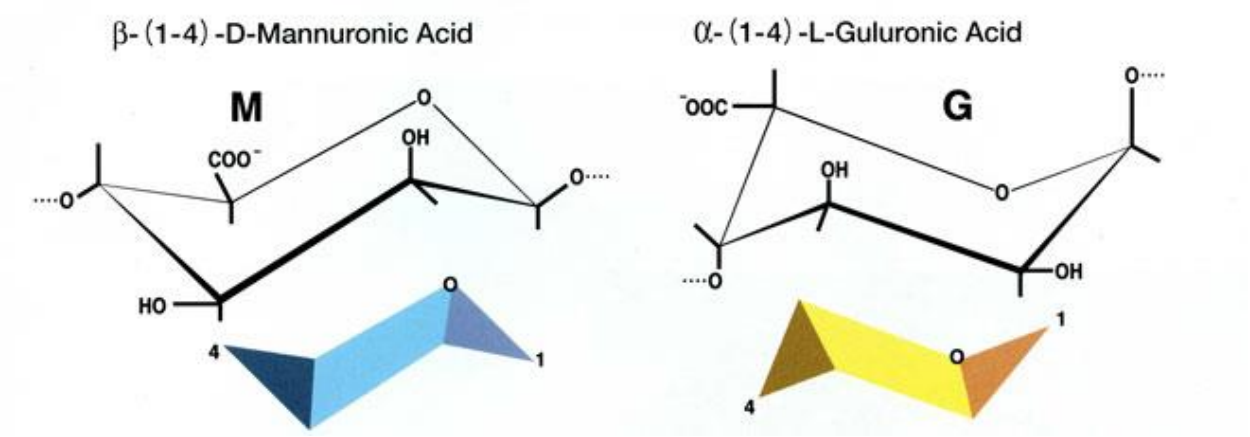
1 INTRODUCTION

Azotobacter vinelandii is the best studied model organism for biological and genetic studies of biological nitrogen fixation having one of the highest respiratory rates of any known bacterium. *A.vinelandii* is an aerobic bacterium capable of fixing atmospheric nitrogen. *A.vinelandii* is a free living gammaproteobacteria which is capable to adapt its mechanism to diverse source of nutrients. Under favourable environmental conditions *A. vinelandii* forms desiccation resistant cyst, which are differentiated cells surrounded by a capsule composed of a high proportion of the exopolysaccharide alginate (3) (4).

A.vinelandii contains molybdenum containing nitrogenase encoded by *nif* and two other oxygen sensitive nitrogenase one iron nitrogenase encoded by *anf* and vanadium nitrogenase encoded by *vnf* (4).

Bacterial alginate is a linear exopolysaccharide composed of various amount of (1-4)-linked B-D-mannuronic acid and its C5 epimer α-L-guluronic acid (5). Bacterial alginate is partially O-acetylated at 2nd and 3rd carbon positions of mannuronic acid residues and monomer distribution is variable throughout the polymer due to epimerization of some mannuronic acid residues (6). Alginate is essential for encystment process, since non mucoid strain fail to encyst(3).

A



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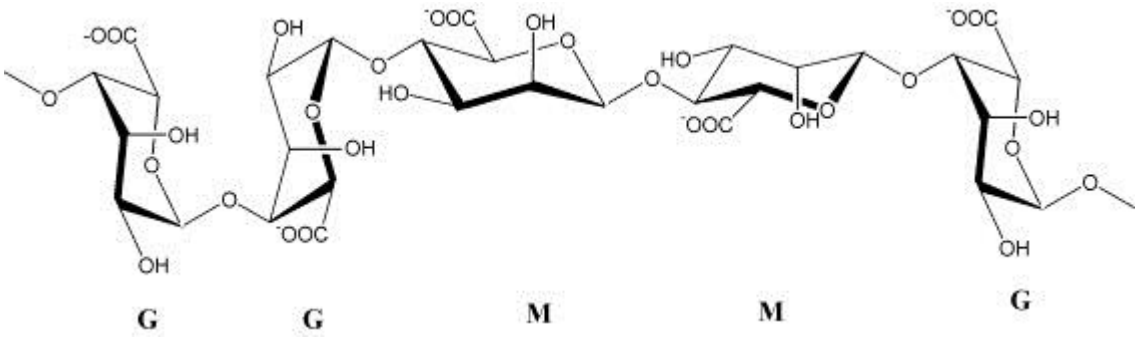


Figure 1-1 **Alginate building blocks and its structure(7)**.

A) The two building blocks of alginate. B) The chemical structure of alginate.

1.1 Distribution and Organization of Alginate genes

Alginate genes are distributed throughout the pseudomonas rRNA homology group I-Azotobacter-Azomonas lineage, while in pseudomonas groupV (Xanthomonas) and enteric lineage alg genes have been retained but bacteria could not produce alginate. Azotobacter species are closely related to pseudomonas group I. *A. vinelandii* possesses sequences homologous to all *P. aeruginosa* alg genes.

In *A. vinelandii* as in *P. aeruginosa* , all of the biosynthetic genes, except *algC*, are clustered. This cluster is organized in a polycistronic operon transcribed from a promoter located upstream of *algD* in *P. aeruginosa* (8).

Organization of alginate gene is found identical on both *Azotobacter Vinelandii* and *Pseudomonas Aeruginosa*. All biosynthetic genes except *algC* including *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX(alg60)*, *algL*, *algI*, *algJ*, *algF* and *algA* are clustered. These 12 structural genes are organized and transcribed as an operon from *algD* promoter. *AlgC* is transcribed from its own promoter. *AlgZ*. *algR*, *algQ*, *algP* and *algB* controls the regulation of alginate production (5).

A.vinelandii possesses an alginate biosynthetic gene cluster, organized in operons, one of which contains *algD* encoding a GDP mannose dehydrogenase that converts GDP mannose to GDP mannuronic acid which is the substrate for alginate polymerization. The *mucA* and *mucB* genes code for negative regulators of *algU* activity (9).

In *P. aeruginosa*, transcription from *algD* promoter is affected by the products of several regulatory genes. *AlgU* encodes an alternative sigma factor which is required for *algD* transcription. The *mucA* and *mucB* products counteract *AlgU* by suppressing its function (10) The *algR* and *algB* genes encode response regulators where *algR* bind to three sites within the *algD* promoter and to activate transcription from the *algU*-dependent promoter, therefore inactivation of *algR* abrogates alginate production. Another gene *fimS* (also called *algZ*) which is located upstream of *algR* is involved in control of alginate biosynthesis under certain conditions (11).

In *A. vinelandii*, at least two promoters transcribe *algD* and these are recognized by different sigma factors: P_1, recognized by σ^{70}, and P_2 recognized by the alternate σ^E factor (*algU*). The *algUmucABCD* operon seems functionally equivalent to those of *P. aeruginosa*, since inactivation of *algU* abrogated alginate production(10)& (3) *AlgU* activity has been shown to be essential for encystment in addition to its role in alginate synthesis(8,12). When algD was transcribed from two promoters, one putative *algU* dependent promoter P2, and a promoter P1 showing consensus sequences for the negative sigma factor, in a highly mucoid strain of *A.vinelandii*, algu and *mucABCD* genes of *A.vinelandii* were sequenced and identified which showed that they control alginate biosynthesis in a manner similar to that of *P.aeruginosa*. *A.vinelandii* strain UW136 containing a mutation in *algU* gene which encodes AlgU sigma factor doesnot produce any alginate and was also unable to encyst (13,14).

The *mucA* and *mucB* genes code for negative regulators of *algU* activity(9).

Transcription of the other two operons of the alginate biosynthetic cluster is *algU* independent (13,14).

1.2 Bacterial biosynthesis of alginate

Alginate is produced as a capsule when synthesized in active vegetative cell or as the cyst(15). The bacterial alginate biosynthesis occurs in similar manner in both *Azotobacter* and *Pseudomonas* species. Pinder and Brucke were the first to propose alginate biosynthetic pathway in *A. vinelandii* (16). The first polymeric compound formed during bacterial alginate biosynthesis is polymannuronic acid which is later epimerized to guluronic acid residues. The early step in alginate biosynthesis pathway is the conversion of Glucose-6-phosphate to fructose-6-phosphate (fig 1-3) through Enter-Doudoroff pathway. Fructose-6-phosphate is then converted to GDP mannuronate in multiple steps catalyzed by enzyme phosphomannuomutase, GDP mannose pyrophosphorylase and GDP mannose dehydrogenase. GDP mannuronate is then polymerized, modified and exported by polymerase encoded by *Alg8* and *Alg44* to produce polymannuronate and finally to produce alginate (see fig 1-2).

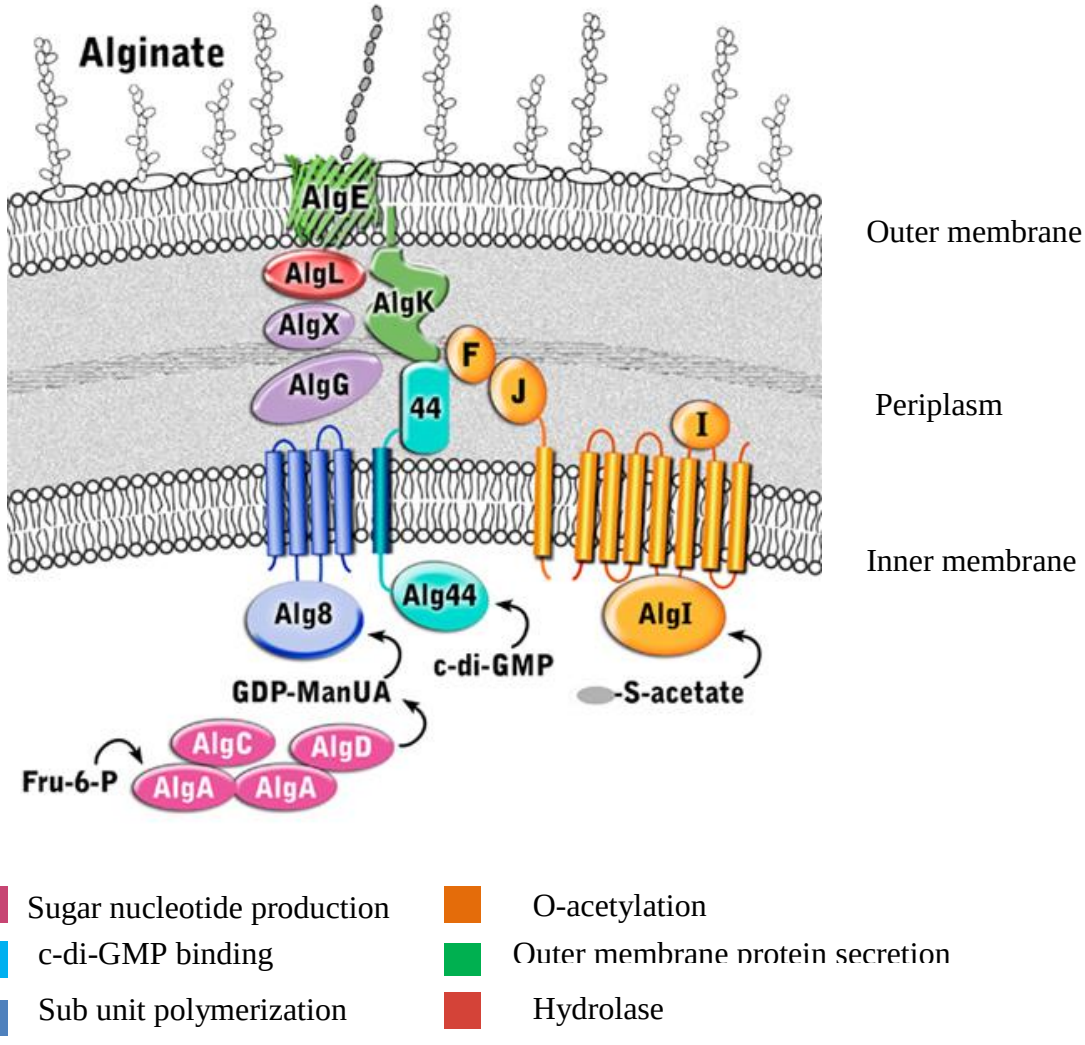


Figure 1-2 The proposed model of alginate biosynthetic complex (2).

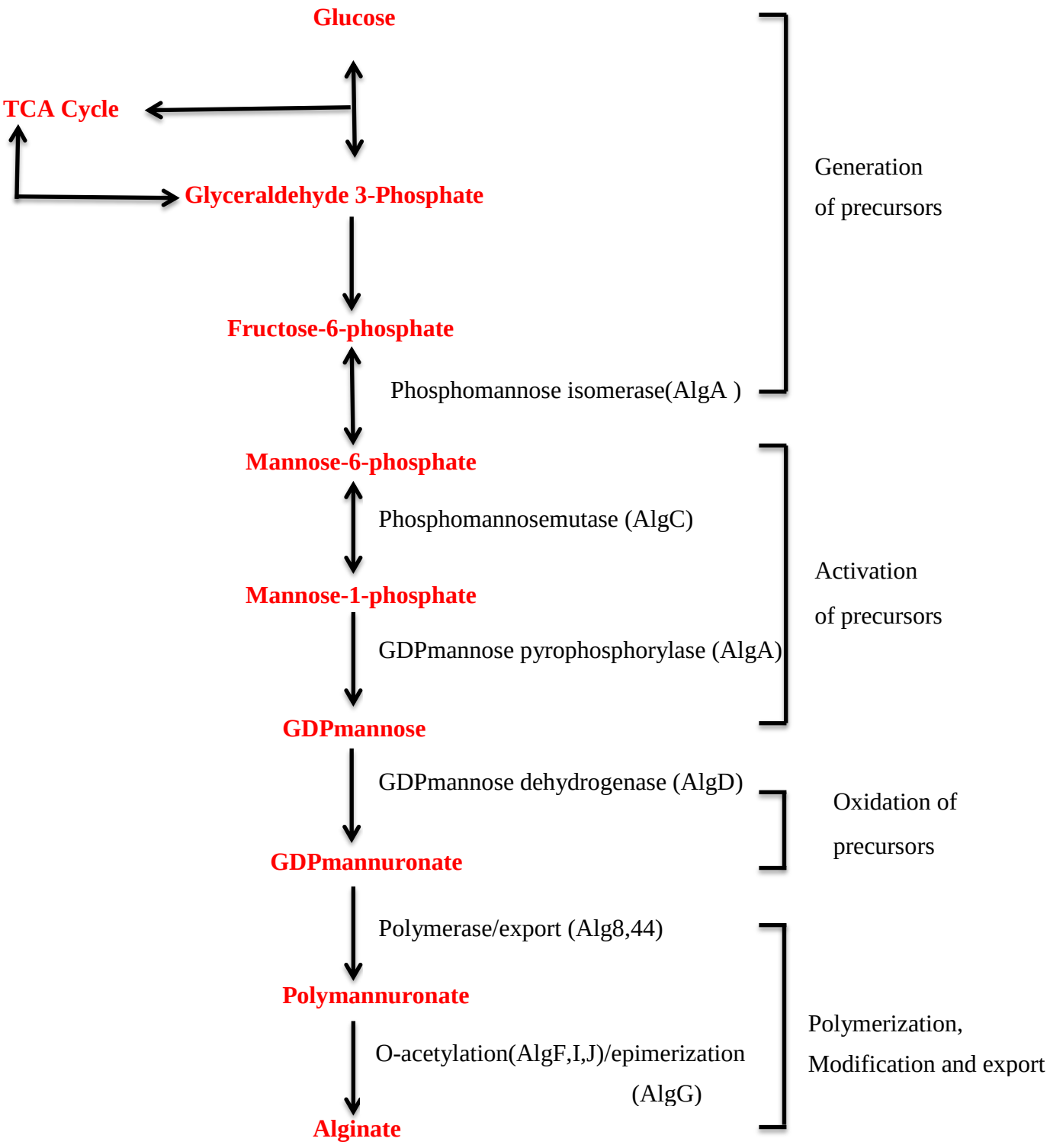


Figure 1-3 Biosynthetic Pathway of alginate in *A. vinelandii* (1)

Table 1 Alginate genes of *A. vinelandii* (5)

Genes	**Products and Functions**
algD	GDP-mannose dehydrogenase
alg8	Subunit of alginate polymerase
alg44	Subunit of alginate polymerase
algK	Translocation/Polymerization
algJ	Porin-like OM protein
algE$_{1\text{-}7}$	Extracellular mannuronan C-$_5$ epimerases
algG	C5 epimerases
algL	Alginate lyase
algV	Acetylation
algI	Acetylation
algF	Acetylation
algA	PMI-GMP
algC	PMM
algR	Response regulator protein of TCSTS
algU	Sigma factor α^{22}
mucA	Anti- α^{22} factor
mucB	Anti- α^{22} factor
mucC	Homologue of phoORF4 product
mucD	Homologue ofserine protease(HtrA)

1.3 Alginate genetics in *A. vinelandii*

A.vinelandii produces alginate both as vegetative state capsule and as an integrated part of a cyst of this organism, which makes alginate genetics in *A.vinelandii* differ than that of *P. aeruginosa*. As *P. aeruginosa* alginate biosynthetic gene cluster is organized in transcriptional units *PalgD, Palg8-alg44-algK* and *palgG-X-L-V-I-F-A* (17). *AlgD* is transcribed from three promoters in which one is indirectly algU dependent and other two *algU* independent. These two *algU* independent promoter has a major role in alginate production. *AlgA* transcription is regulated by its own promoter. Transcription of structural gene *algC* is from two promoters, among which one is directly dependent on the alternative sigma factor. *Azotobacter vinelandii* consist alginate biosynthetic genes of a family of seven extracellular mannuronan C-5 epimerases which catalyze the Ca^{++} dependent polymer level epimerization of β-D manuronic acid to α-L-guluronic acid in alginate (18).

1.4 Alginate regulatory genes

As mentioned earlier genes for alginate biosynthesis in *P.aeruginosa* are clustered in *algD* operon. *AlgR, algB* and *algZ* are major regulators required by *algD* promoter for its expression. Alternative σ^{22} encoded by algU directly controls the *algD* promoter and expression of *algD* promoter. AlgU is negatively regulated by the gene products of *mucA, mucB, mucC* and *mucD* (19).

AlgR is a two component regulator encoding 27.6kDa protein of the two component regulator family. *AlgR* contains a conserved N-terminal domain which is typical of response regulators of two component signal transduction system and C-terminal domain has a *LytTR* domain. *AlgR* interacts with sensory components encoded by *algZ* located upstream of algR and under σ^{22} positive control *algZ-algR* forms an operon. However, *algZ* is not required for alginate production which makes algR both phosphorylation dependent and phosphorylation independent mechanism of gene activation.

AlgB belongs to an Ntrc-family encoding two component regulator. *AlgB* binds within a region relative to the start of *algD* promoter that can activate the promoter utilizing sigma factor other than σ^{54} in that the *algD* promoter utilizes $\sigma^{22.}$ *AlgB* has an N-terminal response regulator domain with conserved Asp-16, Asp-59 and Lys-109 residues that are typically

involved in phosphorylation. *AlgB* has a σ^{54} interaction domain and two walker boxes. The C-terminus of *algB* contains helix-turn-helix for DNA binding that belongs to the fis family of regulatory proteins.

AmrZ gene is an arc like DNA binding protein originally called as "*algZ*" which is an alginate regulator gene. It was renamed as *amrZ* for "alginate and motility regulator". The *amrZ* gene is positively controlled by σ^{22} and encodes a 12kDa protein that is a DNA binding protein of the ribbon –helix-helix family. *AmrZ* can be either a gene activator or gene repressor making it as a multifunctional regulator of alginate and motility (20).

AlgQ (*AlgR1*) is a positive regulator that is needed for the formation of precursor GDP mannose for alginate biosynthesis. *AlgP* (*algR3*) is a highly basic histone like protein required for normal *algD* expression (20). AlgW is a htr like protease. It has been shown to be required for activation of the alginate biosynthetic operon by D-cycloserine and AlgW mediates regulated proteolysis of *mucA* during overexpression of *mucE* (21).

1.5 The XylS/Pm regulator/promoter system

E.coli is one of the widely used host species for the expression and production of recombinant protein since its genetics are better characterized than other microorganisms (22). The modulation of physiological conditions such as pH, temperature and ligands can be used for promoter regulation. Also the transcription regulators that may be activators or repressor, regulates the activity of promoter. *Pm-Xyls* is a widely used promoter system that can be applied in broad host range expression vectors for high level expression of heterologous protein (23).

Pm promoter system is one of the highly used promoter system for recombinant protein expression originated from *Pseudomonas putida* TOL-plasmid pWW0 and regulated by *Xyls* regulator (24). *Xyls* is an archetype transcription regulator belonging to Arac/Xyls family derived from TOL plasmid pWW0 of *Pseudomonas putida*. Pm promoter regulated by *Xyls* is σ^{54} dependent in *Pseudomonas putida* and σ^{70} dependent in *E.coli* and acts as master regulator for genetic switches of meta operon on TOL plamid pWW0. *Xyls* is a positive regulator activated by 3-methylbenzoate. The oxidative catabolism of toluates and benzoate is specified by meta cleavage pathway of TOL plasmid pWW0 which are encoded by a gene that are grouped in a operon which are under control of single promoter pm. Expression of this Pm

promoter for alkyl benzoate degradation is under control of TFSXylR and *Xyls* regulator respectively. The *Xyls* gene can be transcribed from two promoters Ps1 and Ps2 whereas *XylR* can be activated from *Ps1* while *Ps2* transcription is constitutively low. Thus Pm promoter can be activated by the controlled and balanced expression of *Xyls*. Depending upon growth phase, transcription is processed from Pm promoter mediated by RNA polymerase, when effector molecules activates *Xyls* regulator.

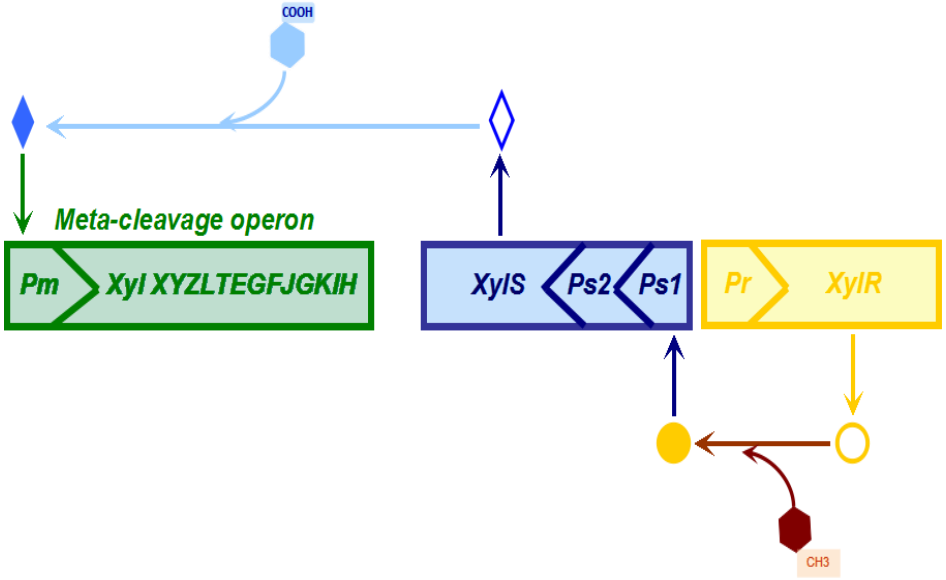


Figure 1-4 **Regulatory circuits controlled by XylS and XylR on the TOL plasmid pWW0** (http://parts.igem.org/Part:BBa_K1031911) downloaded in 04/05/2014.

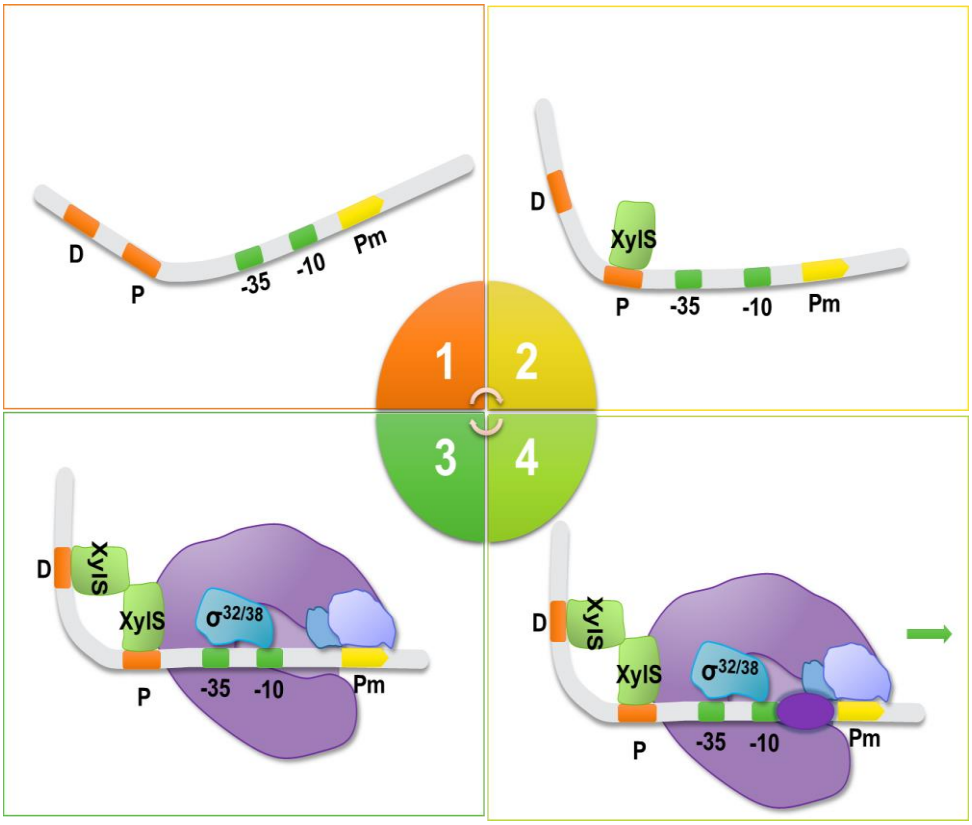


Figure 1-5 **Mechanism of XylS binding to Pm promoter** (http://parts.igem.org/Part:BBa_K1031911) downloaded in 04/05/2014.

1.6 Industrial and pharmaceuticals applications of alginate

Alginate has been widely used in industrial and pharmaceutical field due to its stabilizing, gelling and viscous property. Alginic acid can be used as disintegrating agents in tablets due to its insoluble nature in water and swelling nature when placed in water. Alginate can be used as a coating of drugs for a controlled released system of drugs. Alginate molecules are capable of undergoing immediate hydration to create a hydrocollodial layer of high viscocity making up a diffusion barrier decreasing the movement of small molecules. Alginate is used in modulation of gastrointestinal transit time. Absortion of drugs is influenced by variation in gastric emptying time, mainly in the lower region of digestive tract. For this a buoyant capsule is made with powder consisting of the drug substance in combination with alginate and a pH-independent hydrocolloid gelling agent (25). Alginate is also widely been used as an impression making material in dentistry. Calcium alginate can be used in burn dressing to promote healing which is less painful than conventional dressing (26).

Similarly, alginate is also used in bioseparation of proteins. Alginate consists of blocks units of guluronic and mannuronic acid which shows inherent biological affinity for a variety of enzymes such as pectinase, lipase, phospholipase D, a and ss amylases and glucoamylase. These enzymes can be purified by affinity precipitation, aqueous two phase separation, macroaffinity ligand facilitated three phase partioning, immobilized metal affinity chromatography and expanded bed affinity chromatography utilizing precipitating ability of alginate with Ca^{++} (27).

Alginate is nowadays used as a safest food additives and is applied to various food, such as icecream, jelly, lactic drinks, dressings,instant noodle, beer etc. alginate is widely used in textile printing as a substrate of color paste while applying to print fabrics, scarf, towel etc. alginate is used for welding rods as a flux binder (http://www.kimica-alginate.com/alginate/application.html) downloaded in 05/05/2014.

1.7 Aim of study

The main aim of study was to study some part of alginate gene regulation in A.vinelandii. So to study this, first aim was to make or construct tools for studying. Also the study was focused to know whether the alginate biosynthesis regulated the same way in azotobacter as in pseudomonas.

The aim of the study was to create control vector for mcherry promoter probe for this pTA92 was inserted with apramycin resistant gene since *A.vinelandii* works well with apramycin as a selectable marker. The study was also focused to show that mcherry can be used as a reporter gene to measure the gene expression.

The aim of the work was to measure *algD* expression in the algB strains, A.vinelandii ATTC12518Tc and 12518 to see the effect of the algB inactivation on *algD* expression. Also the algD expression was measured in two mutant strains *algW* and *amrZ*.

The aim of the study was also focused on to know the effect of overexpression of *algW* in *A.vinelandii*. For this, complementation study was carried out to see what happens if *algW* is overproduced. To examine this *algW* was to be cloned into a vector that can be transferred to *A.vinelandii*.

The other aim of the study was to analyse the effect of succinate on alginate and growth. For this wildtype *A.vinelandii* was grown in RAI medium containing fructose only, fructose+sodium, and fructose+succinate.

Luciferase assay is one of the widely used genetic reporter assay which is very expensive and ATP consuming. In this study the aim was to show that mcherry can also be used as a reporter gene for measuring gene expression, which is very less expensive than that of luciferase assay and also does not need ATP at all.

CHAPTER 2

2 Materials and Methods

2.1 Bioinformatics tools

2.1.1 Clone Manager Suite

Clone manager suite is a data program that can be used to design and analyze primer and to plan and simulate cloning. This program can also be used to identify open reading frames, potential start codon, compare sequences, create map of sequences of cleavage sites etc. Clone manager suite is an important tool for restriction enzyme analysis and for drawing graphic maps. (www.scied.com)

2.2 Media and Solutions

50 × TAE- buffer

242g/l Tris base

57.1ml/l acetic acid (100%)

100ml/l EDTA

1M Tris-HCl (pH-8.0)

121g/l Tris base

pH adjusted to 8.0 with concentrated HCl

Autoclave

0.05M Tris HCl, 1.5% NaCl (pH7.5)

15g/l NaCl

6.072g/l Tris -base

pH adjusted to 7.5 with concentrated HCl

Autoclave

0.5M EDTA (pH 8.0)

186g/l EDTA

pH adjusted to 8.0 with 5-10 M NaOH

Autoclave

TE (pH 8.0)

10ml/l 1M Tris-HCl

2ml/l 0.5M EDTA, autoclave (pH8.0)

LB

10 g/l tryptone
5 g/l yeast extract
5 g/l NaCl Autoclave

LA: LB where15 g/l agar are added before autoclaving.

SOC:

20 g/l tryptone
5 g/l yeast extract
0.5 g/l NaCl
2.5 mM KCl
3.6 g/l glucose
5.08 g/l$MgCl_2$
Sterilize by filtration. Store in 1.5 ml tubes at -20 ^{o}C.

Psi-medium

5g/l Yeast extract
20g/l tryptone
5g/l $MgSO_4$ (magnesium sulfate)
pH adjusted to 7.6 using KOH.
Autoclave.

TFB1

0.58g/l 30mM KAc (kaliumacetate)
2.42g/l 100mM RbCl (rubidiumchloride)
0.294g/l 10mM $CaCl_2 \times 2H_2O$ (calciumchloride)
2.0g/l 50mM $MnCl_2 \times 4H_2O$ (mangan(II)chloride)
30ml 15 % v/v Glycerol

pH adjusted to 5.8 using dilute acetic acid
SIV to 200 ml.
sterilized by filtration.

TFB2

0.21g/l 10mM MOPS
1.1g/l 75mM $CaCl_2 \times 2H_2O$ (calcium chloride)
0.121g/l 10mM RbCl (rubidiumchloride)
15ml 15 % v/v Glycerol

pH adjusted to 6.5 with dilute NaOH.
sterilized by filtration

Azotobacter medium

RAI

Salts
2g/l $MgSO_4 * 7H_2O$
1.5g/l NH_4NO_3
2g/l peptone
10.5g/l Mops
900ml H_2O

Adjust pH to 7.0

Autoclave

Phosphate: 2.5 g K_2HPO_4 in 50 ml H_2O. Adjust pH to 7.0

1 M $CaCl_2$: Autoclave

Fructose: 20 g Fructose in 100 g H_2O, autoclave.

$FeSO_4$ (15 mg/ml stock): 2 ml per l medium

Cool down the autoclaved solutions. Mix the salt solution and the fructose. Add 1ml phosphate and 0.2 ml 1 M $CaCl_2$ per 100 ml final medium.

Trace Mineral Solutions

5g/l $FeSO_4 \times 7\ H_2O$

0.39g/l $CuSO_4 \times 5\ H_2O$

0.44g/l $ZnSO_4 \times 7\ H_2O$

0.15g/l $MnSO_4$

0.010g/l $Na_2Mo\ O_4 \times 2\ H_2O$

0.020g/l $CoCl_2 \times 6\ H_2O$

50ml concentrated HCl

The solution was sterile filtered.

Phosphate Solution

50g/l K_2HPO_4

Adjusted pH to 7.0 with HCl.

Autoclave

Burks medium (BM)

0.2g/l $MgSO_4 \times 7\ H_2O$

0.2g/l NaCl

0.64g/l K_2HPO_4

0.16g/l KH_2PO_4

900ml H_2O

Autoclave

Glucose: 20 g glucose in 100 g H_2O, autoclave.

Cool down, add glucose to salt solution. Add (from frozen, sterile-filtered stock solutions. 10000X works nicely) $FeSO_4$ (15 mg/ml medium), $CaCl_2$ (50 mg/liter medium), Na_2MoO_4 (2.1 mg/l medium).

Add NH_4Acetate to 0.15 mM before autoclaving if needs fixed nitrogen.

5× Burks Buffer

1g/l $MgSO_4$× 7 H_2O

3.2g/l K_2HPO_4

0.8g/l KH_2PO_4

1g/l NaCl

This could not be autoclaved, as autoclaving leading to precipitates stored at room temperature

Lysis Mix for luciferase assay

1× CCLR

1.25mg/ml lysozyme

2.5mg/ml BSA

Added water to desired volume (prepared freshly for each tube).

Antibiotics

All antibiotics solutions were sterile filtered and stored at -20°C. Antibiotics were added at temperature below 50°C to avoid loss of function.

Tetracyclin hydrochloride

15mg/ml tetracyclin dissolved in 50% ethanol and the solution was filter sterilized and stored at -20°C and was used in medium in the concentration of 15 μg/ml.

Ampicillin

200mg/ml ampicillin dissolved in distilled water and the solution was filter sterilized and stored at -20°C and was used in the medium in the concentration of 200 μg/ml.

Apramycin

50mg/ml apramycin dissolved in diatilled water and filter sterilized and was used in the medium as 25 μl/ml.

2.3 Bacterial Strains and plasmids

Bacterial Strains	**Description**	**Source**
A.vinelandii ATCC 12518Tc	Derivative mutant of A.vinelandii ATTC12518,where tc^r gene has been inserted.	ATTC
A.vinelandii ATCC 12518	wildtype	ATTC
A.vinelandii AlgB strain	*A.vinelandii*ATTC12518Tc algB::TnCAM140	Maerk unpublished
A.vinelandii Algw strain	*A.vinelandii* ATTC12518Tc algW::TnCAM140	Maerk unpublished
A.vinelandii AmrZ strain	*A.vinelandii*ATTC12518Tc amrZ:TnCAM140	
E.Coli S17.1	E.coli 294 with RP4-2 Tc integrated plasmid:Mu-Km :: Tn7 containing tra and trfA genes	Simon et al.,1993
E.Coli S17.1. λpir	R6K λ phage, lysogenic strain of E.coli S17.1	de Lorenzo et al., 1993
DH5α	E.Coli DH1 derivative with deoR, nupG, Φ80dlaczΔM15 and Δ(lacZYA-argF)U169	
Plasmids		
pUC128	Col E1 based cloning vector , ampr	Keen et al., 1988
pOA3	RK2 based vector encoding plasmid with rrnBT1T2 site,AP^r , trfA gene.Luciferase controlled by the Pm promoter	Omar Abbas,unpublished
pTA92	RK2-based vector encoding m-cherry controlled by the T7 promoter. Km^r	Trine Aakuik strand, unpublished

pHE327	Derivative of RK2 encoding a luciferase gene controlled by *A.vinelandii* algD promoter. Apramycin resistant	Helga Ertesvag
pOA2	Derivative of pLitmus 28 contain Pm,xyls, Ap^r	Omar Abbas
pHE364	Derivative of pUC128 encoding algW	Helga ertesvag
pHE365	Derivative of pUC128 encoding rrnB from *E.coli*	Helga Ertesvag
pJS1	Plasmid obtained by replacing kan^r gene with apr^r gene of pOA2 after digesting with PstI.	This work
pJS2	Same plasmid as pJS1 but obtained in other orientation.	This work
pJS3	Derivative of pJS2 in which mcherry is made controlled by pm promoter by replacing T7RNA polymerase with promoter of pOA3 by digesting with NdeI and AvrII.	This work
pJS4	Derivative of pHE365 in which transcription terminator rrnB is inserted by ligating pHE365 with pJS2 after digesting with PspoMI and AgeI.	This work
pJS5	Derivative of pHE364 in which algW is cloned after digesting with ACC65I and ligating with BglII linker.	This Work

2.4 Cultivation and cryopreservation of bacterial strains

Glycerol (60%) was added to live bacterial strains and the final concentration made to 15% glycerol. This was mixed well and stored upto -80°C. when strains would be grown up, they were streaked on agar plates (RAI or Burks for *A.vinelandii* and LA for *E. coli*) to the form visible colonies. colonies were then inoculated into liquid medium or on discs.

2.5 Plasmid isolation

The Wizard® Plus SV Minipreps DNA purification system from Promega was used to isolate plasmids. During this process at first the cells were grown overnight in incubator at 37°C. The next day six eppendrof tubes were filled with culture, centrifuged at 1300 rpm 2 minutes and the supernantant was removed. 250µl of resuspension buffer is then added to two tubes for each plamids and resuspended. The the resuspension was transferred to the pellet in another tube and again resuspended and finally end up with two tubes for each culture, each with about 250µl resuspension of cells. Then the same volume of cell lysis buffer was added to the each tube and the timer was started. Mixed the solution by inverting the tubes five times, then added 10µl of protease solution to each tube and mixed by inverting. 350µl of neutralization solution was added after 5minutes and mixed by inverting. The mixture was then centrifuged for 10minutes at 13000rpm. The supernatant from one tube for each culture is transferred to marked columns and centrifuged for 1minutes and discarded the supernatant. The supernatant from the other tube belonging to the same culture was transferred to the same column and centrifuged for 1minutes. Again the supernatant was discarded. Then the 750µl of washing solution containing ethanol was added to each column and centrifuged 1minutes to throw away the supernatant. The column was again washed with 250µl of washing solution and centrifuged 2minutes to throw away supernatant. After washing the column is transferred to clean eppendrof tube and eluted with 60µl of DNase free water. Then the tube with plasmid DNA was stored at -20°C.(28)

2.6 Restriction digestion with endonuclease

Restriction endonucleases are bacterial enzymes that cut duplex DNA at specific site to produce desired fragments. The recognition site of most of restriction enzymes are short palindromic sequences (29) . The amount of DNA which needs to be cut determines the amount of restriction enzymes going to be used in digestion. T4 DNA polymerase or klenow DNA polymerase can be

used for removal of 3' overhang and 5' overhang fill in (http://www.addgene.org/plasmid_protocols/restriction_digest/). For any restriction digestion it requires DNA, buffer and enzyme. Usually the volume of restriction digestion is 20µl and the reaction mixture is DNA 3-17µl, buffer 0.5µl, BSA 0.5µl and restriction enzyme 0.5µl.

2.7 Separation of DNA on Agarose Gel Electrophoresis

Agarose gel electrophorersis is the best method to separate DNA fragments based on their varying sizes. Due to the phosphate backbone of the DNA molecule, which is negatively charged, DNA fragments will migrate to positively charged anode. The separation is based upon mass/charge ratio. The mobility of DNA molecule on agarose gel is dependent on the size of DNA molecule, agarose concentration, DNA conformation, voltage applied, types of agarose, presence of ethidium bromide and electrophoresis buffer(30).

Agarose gel is prepared by dissolving agarose in 1× TAE buffer till boiling point and poured into a gel chamber for solidification. The comb is placed in the gel chamber before the gel is poured off. Usually 0.7-5µl of standard (ladder) is used to compare band sizes. Before applying DNA molecules into a gel it is mixed with 1-2µof loading dye so that it can be visualized under uv light.

2.8 Extraction and purification of DNA fragments from gel

QIAquick gel extraction kit was used to extract and purify DNA from gel. It is one of the fast and convenient methods for extraction and purification of DNA fragments from gel.

The required DNA fragment was viewed under uv light and excised from the agarose gel with clean and sharp scalpel. The excised gel slice was then weighed and added 3volumes buffer QG to 1 volume gel and incubated at 50°C for 10 minutes or more until the gel slice has completely dissolved. The gel slice was vortexed in every 2-3 minutes to dissolve the gel properly. After the gel was mixed completely it was checked that the color of the mixture is yellow. Then 1volume of isopropanol was added to the sample and mixed. The DNA sample was then applied on QIAquick spin column and centrifuged for 1minutes (13000rpm). The flow was discarded and the tube was placed back and added 500µl of buffer QG and centrifuged for 1minutes

(13000rpm). The flow was discarded and the same tube was placed back. To wash the column 750µl of buffer PE was added on the column and centrifuged for 1minutes (13000rpm). The flow was discarded and placed the same column back to the same tube. Then the empty column was centrifuged for 1 minute to remove all the washing solutions properly. Then the column was placed on the clean sterile eppendorf tube and added 30µl of elution buffer and waited for 1-2minutes and then centrifuged for 1minute (13000rpm) and the eluted DNA was on the ependorf tube (31).

2.9 Ligation

DNA ligation was the process of joining gene or fragments of interest into a compatibly digested vector backbone with the help of enzyme DNA ligase. DNA ligase is an enzyme capable of repairing nicks in double stranded DNA molecule and catalyzes the formation of phosphodiester linkages holding the nucleotides together.

DNA ligation is performed by using the enzyme T4 DNA ligase. This DNA ligase is isolated from bacteriophase T4 that forms a covalent bond between the 5' phosphate at the end of one strand and the 3' hydroxyl of the other strand. DNA ligation is mainly performed with the fragments generated after restriction digestion that digests DNA asymmetrically across their recognition sequence generating sticky ends which allows the vector part and the insert part stick together (32).

During ligation it is necessary to know the amount of vector and insert going to be used. Usually the insert is smaller than the vector and the ratio should be 3insert: 1vector.

Reaction Mixture

14µl insert

3µl vector

2µl ligase buffer

1µl DNA ligase

2.10 Transformation

Transformation is the process by which cell uptake foreign naked DNA and undergoes genetic alteration. Bacterial transformation refers to the genetic change brought about by taking up and expressing DNA. Cells capable of undergoing transformation are called competent cells. There are two types of competence, natural and artificial competence. Natural competence occurs in bacterial species carrying sets of genes responsible for transformation machinery. Artificial competence is induced by chilling cells in the presence of divalent cation such as $CaCl_2$ making the cell walls permeable to plasmid DNA (33).

Preparation of RbCl competent cells

1% of overnight culture *E.coli* S17.1. λ pir (10µl) was innoculated in Psi-medium (10ml) in shaking incubator at 37°C until OD_{600} is 0.4. Optical density was measured using spectrophotometer using psi-medium as a blank. The cells were then incubated on ice for 15 minutes and harvested the cells by centrifugation for 5minutes at 4500rpm. The supernatant was discarded. The cells were then resuspended in cold TFB1 and incubated for 5minutes. The cells were then harvested by centrifugation for 5minutes at 4500rpm and discarded supernatant as before. The harvested cells were then resuspended in 3ml cold TFB2. Aliquots of about 100µl was transferred to a clean sterile eppendrof tubes and frozen immediately on ethanol or dryice. The cells were then stored at -80°C.

Heat shock transformation

100µl of competent cells were placed on ice. The ligation mixture of 10µl was added to 100µl of competent cells and mixed gently. The mixture was incubated on ice for 30minutes to 1hour. After incubating on ice the cells were heat shocked at 37°C for 2-3 minutes and added 900µl of SOC medium prewarmed at 37°C. And incubated at 37°C in shaking incubator for 1-2hours. The transformed cells were then plated on LA plate containing selective antibiotic for plasmid and incubated at 37°C overnight. A positive and negative controls were used that indicated either the ligation reaction worked or not, or if there is any contamination.

The next morning the plates were checked for transformants. If there was transformants in the plates they were picked with sterile tooth pick and grown overnight. At least four colonies were

picked to check the plasmid of interest. The transformants were then subjected to restriction digestion to check either the transformants contained the expected plasmids or not. The enzymes should be choosen in such a way that it gives banding pattern other than that of vector or insert that were ligated.

2.11 Conjugation

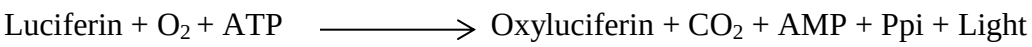
Conjugation is a process in which a DNA is transferred from a donar cell to a recipient cell with a means of bridge like connection between two cells. Donar cell (F^+ cell) consists of fertility factor called F-factor which is responsible for generation of F-pilus. Pilus attaches to recipient cell (F^- cell) and F-plasmid is transferred to recipient cell. The F factor is integrated to bacterial chromosome by site specific recombination. A cell that carries integrated F-factor is called Hfr cell (34).

Procedure

E. coli S17.1(pHE327) was a donar cell carrying plasmid of interest and *A. Vinelandii* ATCC12518 was a recipient cell. ATCC12518 were innoculated in BM and grown for 3days at 30°C in shaking incubator. The donar cell was innoculated in LB with selective antibiotic ampicillin or apramycin overnight at 37°C in shaking incubator. The next day transferred 100µl of donar cells to 10ml LB and grown for 2-3hours. 3ml of ATCC12518 was centrifuged and washed with 10% sterile glycerol to remove sliminess. Glycerol and slime was poured off. 3ml of donar E.coli was mixed with 3ml of recipient ATCC12518 and centrifuged for 10 minutes at 5000rpm. The supernatant was removed and remaining 200µl cells were resuspended. The resuspended cells were placed as a drop on LA plates without antibiotics. Then the plates were incubated at 30°C for overnight. The grown cells from LA plates were then removed with sterile spatula and transferred into 1ml BM and serially diluted upto 10^{-4}. from each dilution mixure, 200µl of cells were plated on burks agar containing 25µg/l apramycin and incubated for 3days at 30°C. The grown colonies were then inoculated in BM for 3days at 30°C in shaking incubator and stored for further analysis.

2.12 Luciferase assay

Luciferase reporter assay is a tool to study expression of gene and cellular events coupled to gene expression. Luciferase assay has become one of the convenient method in measuring expression of a cloned gene. Luciferase is a class of oxidative enzyme found on several species used for bioluminesce. The overall chemical reaction during this process is



During this assay the regulatory region of gene of interest is cloned upstream of luciferase gene in expression vector which results ector DNA into cells and the cells were grown for specific time period. After the cells were grown, the cells were collected and breaked to remove all proteins including luciferase. The luciferin is then added with necessary cofactor to measure enzymatic activity. Luminometer is used to measure the luminiscence.

Luciferase based genetic reporter assay can be widely used in molecular biology and has great application in promoter structural analysis, functional analysis of promoter SNPs and studying cell signalling pathways (35).

Procedure

The luciferase assay was done following Promega Technical Bulletin: Luciferase Assay System. At first 40µl of non transformed cells were mixed with 50µl transformed culture and added 10µl of K_2HPO_4 (pH 7.8), 20mM EDTA and stoed at -80°C. For lucifersae assay the cells are brought to room temperature by placing the tube in a room temperature water bath. 300µl of freshly prepared lysis mix was then added, mixed and incubated for 10 minutes at room temperature. 100µl of luciferase assay reagent was dispensed to eppendrof tubes, one tube per sample. The the luminometer was programmed. 20µl of cell lysate was added to eppendrof tube containing luciferase assay reagent. The tube was then placed in luminometer and the reading was initiated. The whole assay was performed under low light condition.

2.13 Alginate Assay

Alginate assay was dome to check the amount of alginate production. For this at first 250µl culture was transferred to a tube and diluted with 1.25ml 0.2M NaCl and mixed well.Then centrifuged for 10 minutes at 13000rpm. 1ml supernatant was transferred to new 1.5ml tubes. Pellet was discarded. 33µl 3M NaOH was added to the each tube with 1ml supernatant. The tube was stored at -20°C for further analysis.

Procedure

The frozen samples were thawed and deacetylated by shaking for 1hour. Then it was centrifuged for 10minutes at 13000rpm. 100µl supernatant was transferred to new tubes and added 300µl 0.05M Tris- HCl,1.5% NaCl (pH7.5). Alginate was dissolved and made standards with concentrations 0, 01, 0.2, 0.3, 0.4, 0.5 g/l. After all the standards are ready, 150µl .05M Tris HCl,1.5% NaCl (pH7.5) was added to each well of 96 well UV-plate. 75µl of sample or standard added three parallels of each with six of the no alginate standards. All the mixtures in the each well were mixed well. The UV plate was then subjected to spectrophotometer to measure optical density (OD_{230}). After measuring the OD_{230}, 8µl 1u/ml M-lyase and 8µl 1u/ml G-lyase was added to each well except to the three well of those no alginate standards and mixed well by pipetting. Then incubated at room temperature and measured optical density (OD_{230}) after 3 and 4 hours.

2.14 m-cherry

m-cherry is a fluorescent protein which matures very rapidly which makes it possible to see results very soon after transcription. m-cherry is a red monomer which is highly photostable and resistant to photobleaching. m-cherry can be widely used in molecular biology for studying gene expression. It is very less expensive than that of luciferase assay and does not need ATP as well (http://www.clontech.com/US/Products/Fluorescent_Proteins_and_Reporters/Fluorescent_Proteins_by_Name/mCherry_Fluorescent_Protein).

CHAPTER 3

3 Result

3.1 Insertion of apramycin resistant gene

pTA92 contains a kanamycin resistance gene, but kanamycin is not regarded as a good marker for *A.vinelandii* (Ertesvag). In order to replace it with an apramycin resistance gene pTA92 and insert plasmid POA2 were both digested with PstI enzyme (fig 3.1.1). The purification was done using QIA quick Gel Purification kit. The 1.4kb insert was ligated to 6.8 kb PTA92. *E.coliS17.1λ* pir were used as competent cells to transform the ligation mixture using heat shock transformation. The transformed cells were on LA plate with apramycin which was incubated overnight at 37°C. Four colonies were picked from LA plate and inoculated in LB with apramycin at 37°C in shaking incubator overnight. Then the plasmids were isolated and digested with XhoI and PspoMI. Since the insert could have been inserted both ways, the expected fragments would be 6.0kb+2.2kb or 5.4kb+2.8kb. As shown in fig 3.1.2 plasmids with the insert in both orientation were isolated. One of each was chosen and designated as pJS1 and pJS2 respectively (lane1 pJS1 and lane3 pJS2). pJS1 was used for creating control vector for M-cherry promoter probe and pJS2 was used for making promoter probe vector.

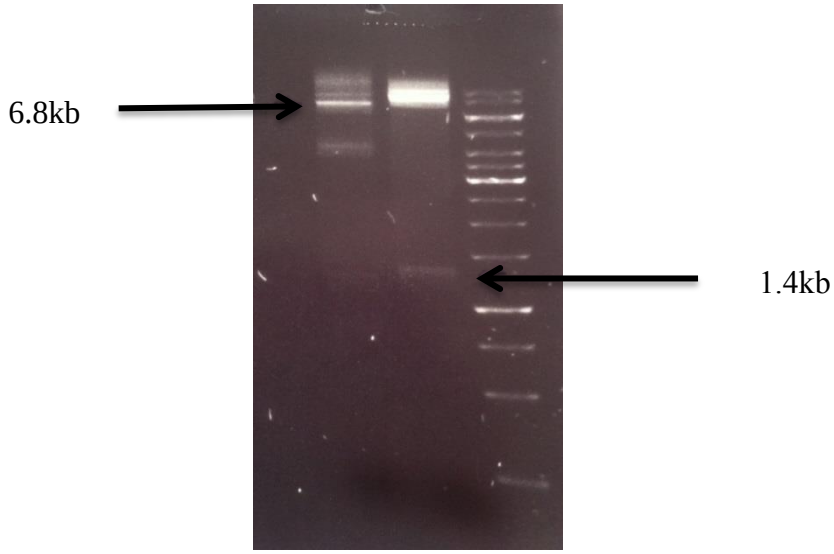


Figure 3-1 Agarose gel electrophoresis of pTA92 and pOA2 digested with pstI.

Gene ruler 1kb ladder was used as standard.

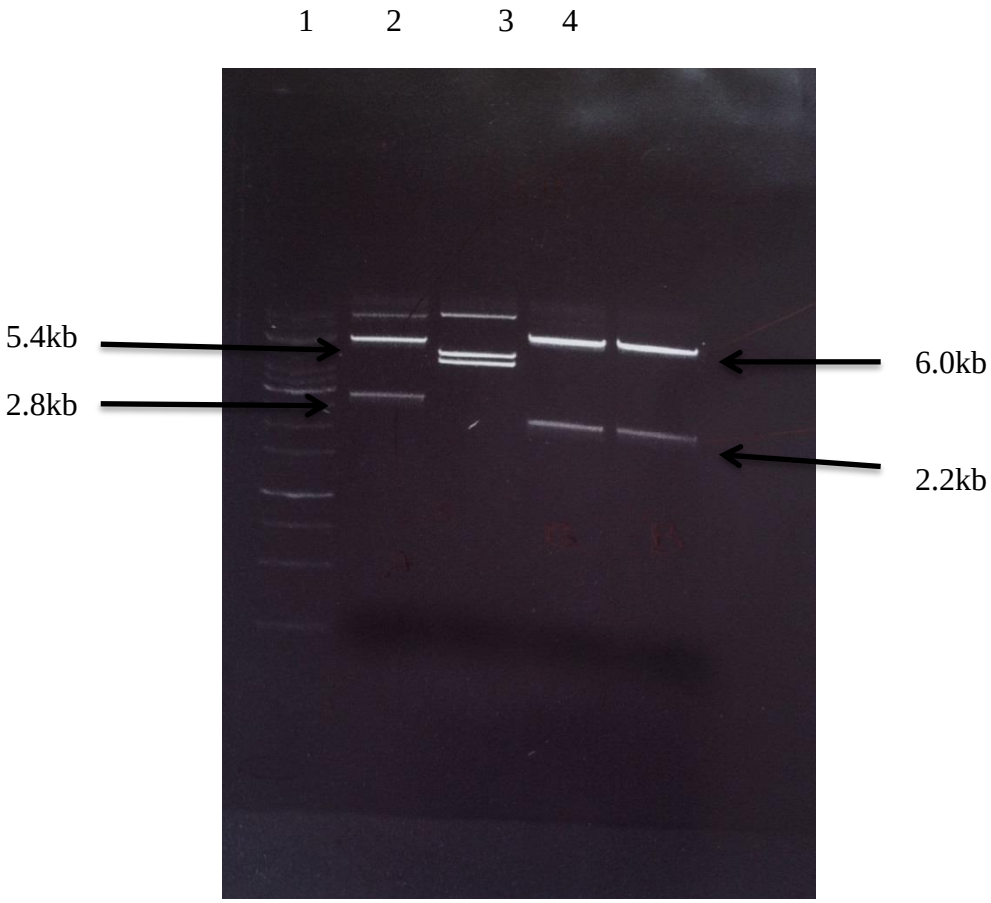


Figure 3-2 Agarose gel electrophoresis of plasmids digested with xhoI and PspoMI. Gene ruler 1kb ladder was used as standard.

3.2 Create control vector for Mcherry-promoter by pm-promoter

pJS1 has mcherry controlled by T7 RNA polymerase promoter, and *A.vinelandii* doesnot encode this polymerase. So it is needed to express mcherry from the Pm promoter. In order to be able to check that mcherry can be expressed in *A.vinelandii* the plasmid pOA3 and pJS1 was digested using NdeI and AvrII. The 6.4kb part of pOA3 and 2.8kb part of PJS1 is extracted from gel and then purified using QIA quick Gel extraction kit and ligated. The ligation mixture was transformed in competent cell *E.coliS17.1λ* pir and plated in LA plate containing ampicillin. Four colonies were picked and inoculated in LB with ampicillin at 37°C in shaking incubator overnight for isolating plasmid PJS3. To check the constructed plasmid it was cut with BglII and HindIII. All the cut plasmids were found to be correct plasmid as shown in gel figure 3.1.3 with

band sizes 5.8kb and 2.1kb. pJS3 could later be used to conjugate with A.vinelandii to test mcherry.

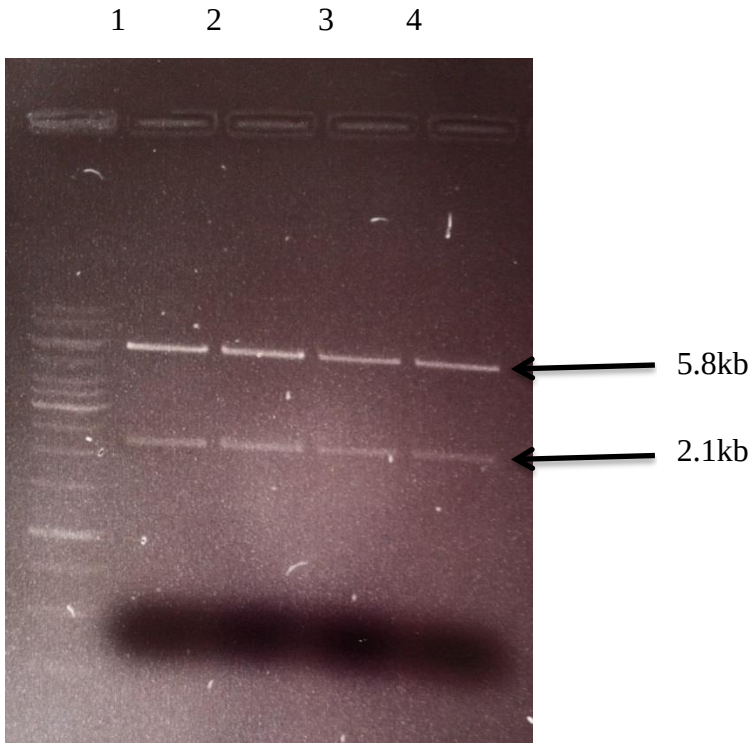


Figure 3-3 Agarose gel electrophoresis of pJS3 digested with BglII and HindIII. Gene ruler 1kb ladder was used as a standard.

3.3 Controlled expression of AlgW

AlgW is shown to be important for alginate production in *P.aeruginosa*, and in *A.vinelandii algW* mutant also produce less alginate (Mærk et al, unpublished). So, complementation of strain algW is needed and the effect of overexpression in the wild type is tested. To examine this algW needs to be cloned into a vector that can be transferred to *A.vinelandii*. pHE364 is an *E.coli* vector containing *algW*. To facilitate cloning BglII linker was inserted into the SalI site of PHE364. For this at first PHE364 was digested with Acc65I and SalI separately. For both of these klenow DNA polymerase was used before applying on gel. In both digestion, a single band was excised and purified and ligated to BglII linker. The ligation mixture was transformed in *E.coliS17.1λ*pir using heat shock transformation and plated in LA plates with ampicillin and incubated at 37°C overnight. The transformed cells were then picked and inoculated in LB containing ampicillin for plasmid isolation in shaking incubator at 37°C overnight. The plasmids isolated were then digested with NdeI+ BglII and NcoI separately to verify the plasmid. The expected band was

3.0kb+1.3kb+0.9kb when digested with NdeI+BglII and 3.4kb+1.7kb when digested with NcoI. The plasmid was then verified.

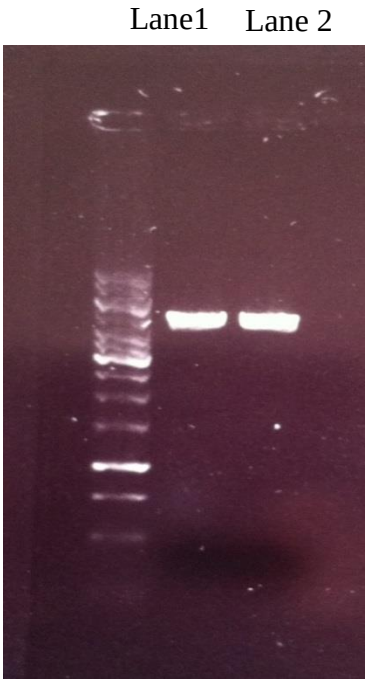


Figure 3-4 Agarose gel electrophoresis of pHE364 digested with SalI (lane1) and Acc65I (lane2)

Figure 3-5 Agarose gel picture of pJS5 after digesting with NdeI+BglII and NcoI . Plasmid in the lane3 was selected

3.4 Construction of promoter probe vector:

Mcherry is an easier and cheaper way to measure gene expression. To show this the insert PHE365 and vector PJS2 both was digested using PspoMI and AgeI and the expected band sizes were 0.45kb+0.042kb +3.2kb and 1.0kb+7.2kb respectively. The 0.45kb part from insert and 7.2kb from vector was excised from gel and purified using QIA quick Gel Extraction kit. The 0.45kb insert was then ligated to 7.2kb vector and left overnight. The ligation mixture was then transformed to *E.coliS17.1λ*pir using heat shock transformation and plated in LA plate with ampicillin and incubated overnight at 37°C. Four transformed cells were then picked and inoculated in LB containing ampicillin in shaking incubator at 37°C overnight for plasmid isolation. The isolated plamid PJS4 were then digested with BamHI and AgeI and was verified. The expected band size was 2.2kb and 5.4kb. As seen in figure 3.4.1 all the plasmids isolated were the correct plasmids. One was chosen and designated pJS4. This plasmid can later be used for probing a promoter.

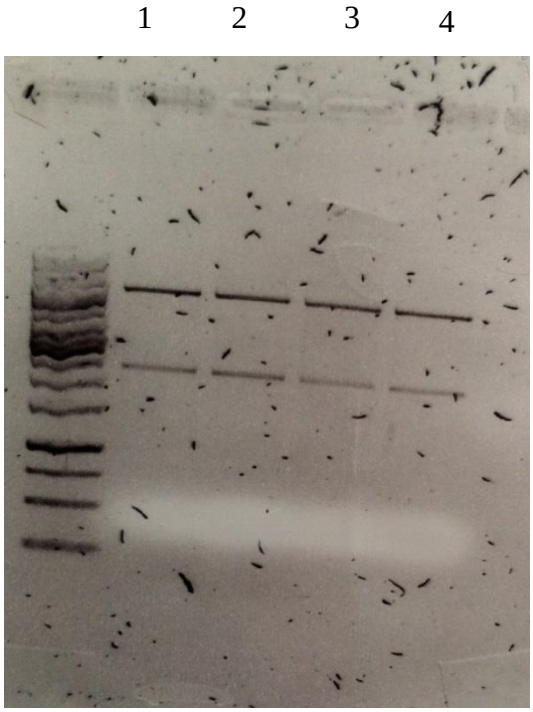


Figure 3-6 Agarose gel picture of pJS4 digested with BamHI and AgeI.

Gene ruler 1kb plus ladder was used as a standard.

3.5 Measurement of algD expression in AlgB strain, *A. vinelandii* 12518Tc and 12518

The experiment was done to see the effect of the algB inactivation on *algD* expression. For this the preculture of algB strain, 12518Tc and 12518 was grown for 3days and 100µl of preculture was inoculated in 10ml burks media. The *E.coliS17.1*(PHE327) was also inoculated in LB with ampicillin or apramycin overnight. Next day 100µl of *E.coliS17.1*(PHE327) was inoculated in fresh 10ml LB and grown for 2-3hours. 3ml of *A.vinelandii* was centrifuged and washed with 10% sterile glycerol to remove sliminess. Glycerol with slime was then poured off. 3ml of E.coli was then added to it and mixed and centrifuged to remove most supernatant. The left supernatant was then resuspended and the whole drop was placed on LA plates as a drop and incubated at 30°C overnight. The drop was then resuspended in 1ml burks media and 200µl was plated on burks agar containing 25µg/ml apramycin with dilution series 0,-1,-2,-3,-4. 200µl of each sample was spread on plate. The apramycin resistant colonies were then grown and the strains were frozen.

A.vinelandii strains *algB*, 12518Tc and 12518, three parallels of each were grown and the samples after 24, 48 and 72 hours were measured for luciferase activity. The luciferase activity of *algB* was found to be lower (fig 3-7). The less expression of *algB* resulted in less transcription from *algD* which results in less alginate production. There is a more activity in wild type *A.vinelandii ATTC12518Tc.* It was also found that pHE364 could be used with other A.vinelandii strains as well.

3.6 Measurement of algD expression in mutant strains algW and amrZ

Likewise in the next study *A.vinelandii* strains ATTC12518 Tc, *algB* and two mutant strains *algW* and amrZ were grown to measure *algD* expression. In contrast to previous experiment, in this experiment the activity of control strain 12518Tc was extremely low. The activity of *algB* was almost same in both experiments. Activity in mutant strain *algW* was also low but activity in mutant strain *amrZ* was found to be much higher than control strain in 24 hours (fig 3-8).

In the next part, *A.vinelandii* strains 12518Tc, algB and mutant strains algW and amrZ , three parallels of each strains were subjected to measure absorbance at OD_{600} to measure growth. There was a significant gowth all strains in 48hours than 24 and 72 hours. There was more growth in algB strain than control strain 12518Tc and other two mutant strains. Also all three parallels of each strain have almost same growths (fig3-8).

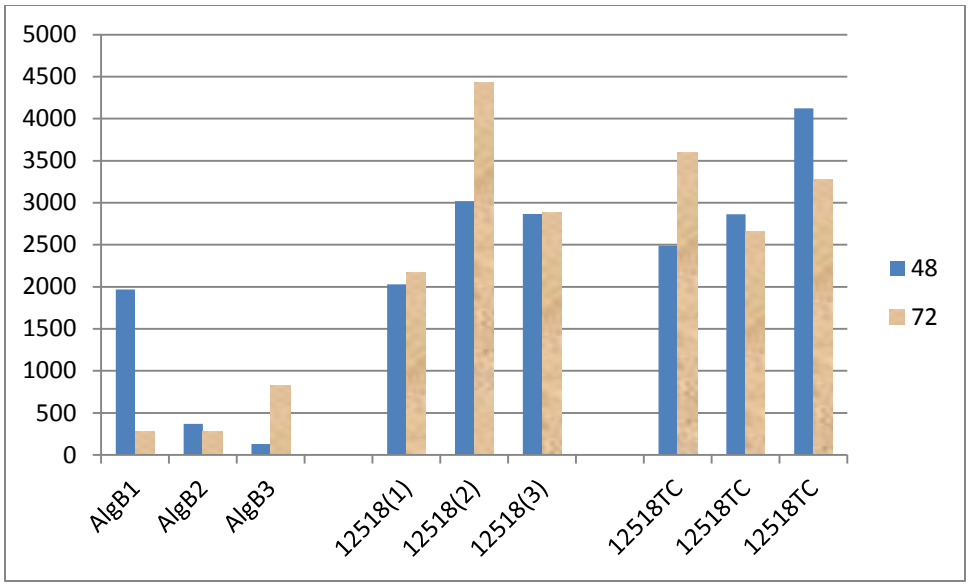


Figure 3-7 Luciferase activity of A.vinelandii ATCC12518 (pHE327) strains AlgB, 12518 and 12518Tc..

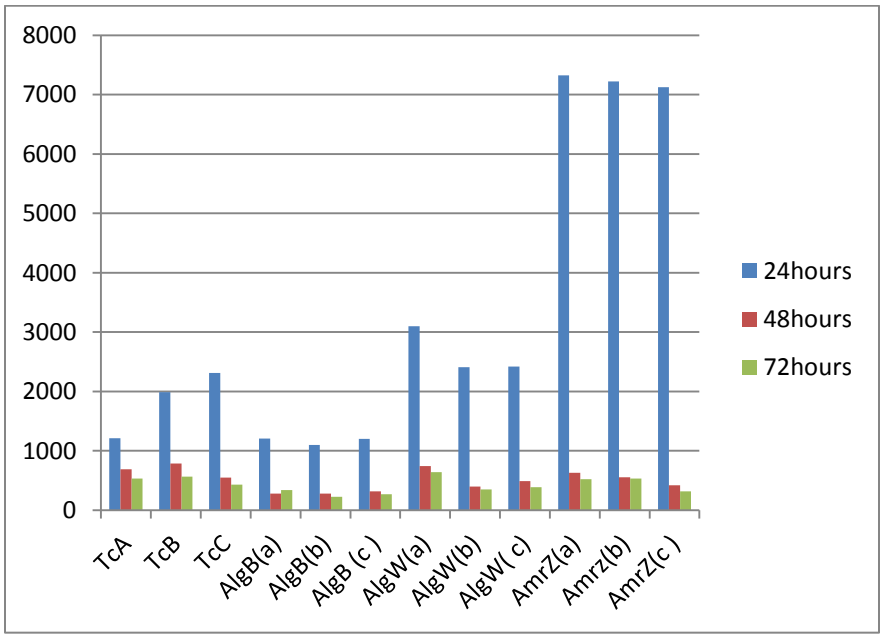


Figure 3-8 Luciferase activity of A.vinelandii strains 12518TC, AlgB, AlgW and AmrZ.

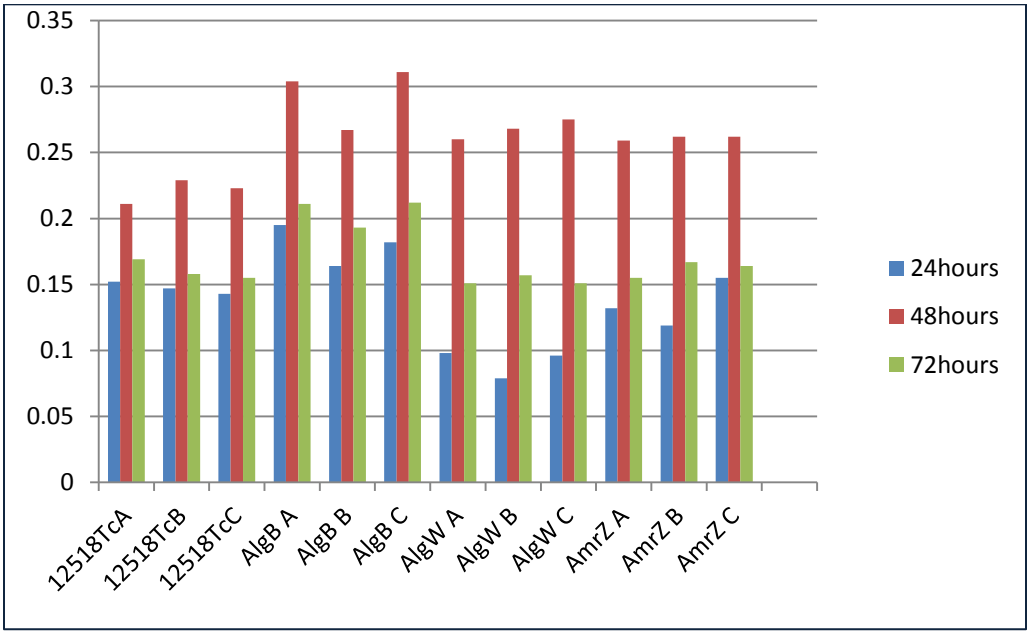


Figure 3-9 Line graph showing growth of A.vinelandii strains 12518Tc, AlgB, AlgW and Amrz. The growth was found to be higher after after 48 hours than 24 and 72 hours

3.7 Effect of succinate on alginate and growth

It has previously (Mærk et al, unpublished) been shown that adding succinate to the medium increased alginate production in wild type *A.vinelandii*. This could be caused by the increased amount of carbon used in that experiment. For measuring an effect of sodium succinate since sodium succinate was added, wild type was grown in RAI medium containing the same amounts of carbon as in the previous experiment.

To check the effect of succinate on alginate production and growth at first the wild type *A.vinelandii 12518* was grown on BA plate with no antibiotics. Then the three flasks were precultured. Then it was inoculated to 9new flasks with RAI medium in which 3 flasks were with fructose only(A), 3with fructose and sodium(B), and 3with fructose and succinate(C). In all three types of flask there were same C- concentrations from sugars. After 48 and 72 hours absorbance was measured at OD_{600} using spectrophotometer.

As expected there was a significant growth in the medium supplemented with sodium succinate than the medium supplemented with fructose only and fructose and sodium (Fig 3-11).

Also, after 48 and 72 hours of growth all the samples, three parallels of each were subjected for alginate assay. But there was no measurable alginate production. Alginate production was less than 0.1g/l.

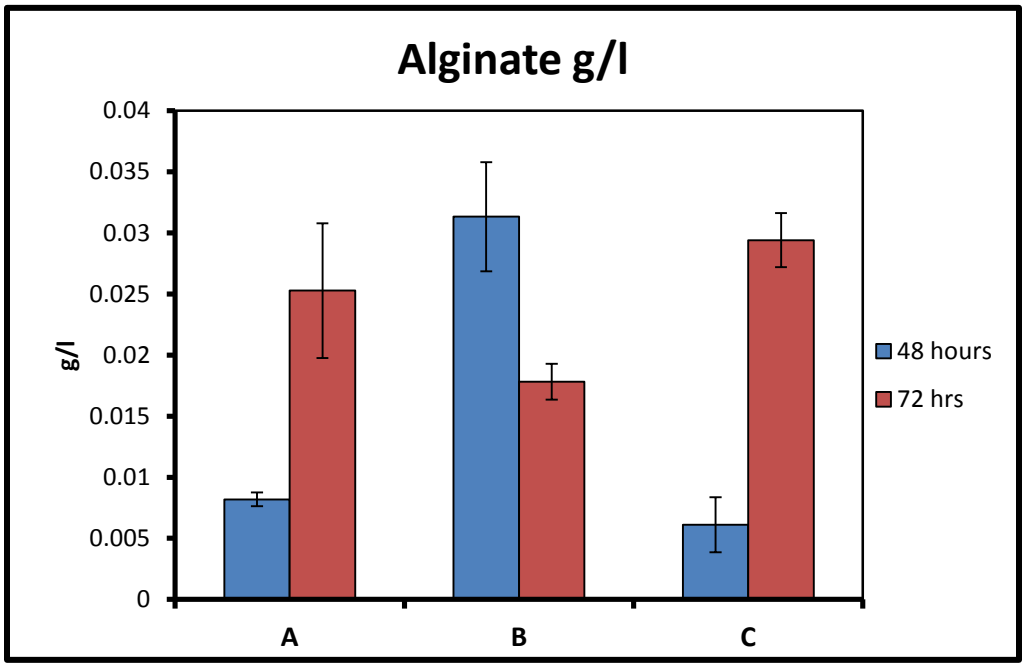


Figure 3-10Alginate production by wildtype *A.vinelandii* in RAI mediums supplemented with A) fructose only B) fructose and sodium C) fructose and succinate

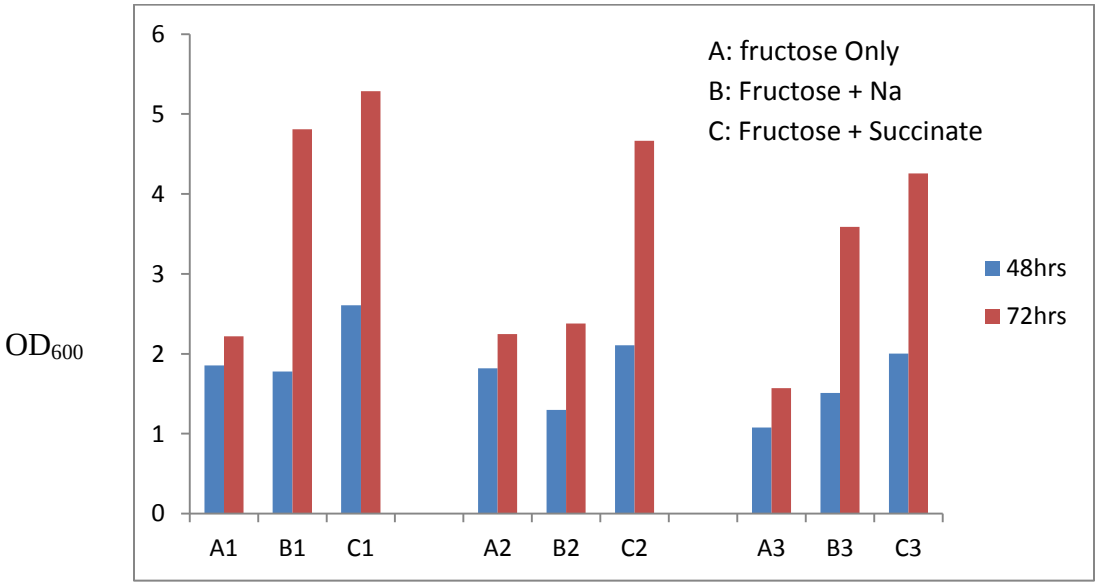


Figure 3-11 Bar diagram showing growth of wild type *A.vinelandii12518* in RAI medium supplemented with fructose, fructose+sodium and fructose + succinate respectively

4 Discussions

In the first part of study control vector for mcherry promoter probe pJS3 was created. pJS3 could be later used to conjugate with *A.vinelandii* to test mcherry. For measurement of *algD* expression in *A.vinelandii* ATTC12518Tc, *algB* and 12518 strains luciferase assay was carried out. It was the initial experiment to learn how to measure luciferase. During luciferase assay three parallels were measured for each strain. In *algB* strain one parallel was shown to have more activity than other two parallels. Likewise in 12518 strain as well one parallel shown to have high activity in 48hours than the other two parallels. The control strain 12518Tc has almost the same activity in all three parallels.

In the second step luciferase assay of *A.vinelandii* strains ATTC12518Tc, *algB* along with mutant strains *algW* and *amrZ* was carried out. pHE327 was grown with these strains since it contained luciferase gene. The luciferase activity of control strain ATTC12518Tc was found to be extremely low whereas the activity of *algB* was almost same as previous luciferase assay. The low activity of control strain may be because there was a great problem while growing the control strain. The control strain was grown but may have lost the plasmid but somehow became resistant. So, the control strain 12518Tc may not contain that luciferase plasmid. Also the *algW* activity was found to be low. But in *amrZ* very high activity was found. Promoter was really active as wild type. This experiment was needed to be repeated but due to time limitations couldn't do so.

The other part of study was to check the effect of succinate on alginate and growth. The wild type *A.vinelandii* was grown in RAI mediums with fructose only, fructose and sodium, and fructose and sodium succinate three parallels of each. The growth was found to be much higher in RAI medium with fructose and sodium succinate which was as expected. It is due to higher amount of carbon in the medium. Since, strain on fructose and sodium, and fructose only were remarkably same except on two parallels, it can be said that sodium have no any characteristically significance in growth.

The luciferase data obtained in the second part of study in A.vinelandii strains 12518Tc, algB, algW and amrZ was found to be unreliable since there was very less activity in control strain 12518Tc. If there was more time this experiment should have been repeated. For further work, pJS3 which is a derivative plasmid of pJS2 in which mcherry is controlled by Pm promoter can be used to conjugate with A.vinelandii to test mcherry. Also, for further studies plasmid pJS4 which is a derivative of pHE365 can be used for probing a promoter. In the study to know the effect of succinate in alginate and growth, which was previously done by Maerk et al, (unpublished) there was more alginate production by wild type *A.vinelandii* in the medium supplemented with succinate. But in this study, there was a significant growth of wildtype *A.vinelandii* in the medium supplemented with succinate but there was no any characteristic alginate production. So, alginate assay should be repeated again to get reliable results.

5 Conclusions

In the first part of study, plasmids with apramycin resistant gene were obtained which were later used for making control vector for m-cherry promoter probe. m-cherry promoter probe under control of Pm promoter was constructed. Also, the expression vector was created for overexpression of gene.

In the second part, promoter probe vector was constructed using mcherry as a reporter gene to measure gene expression. *AlgD* expression was measured in three *A.vinelandii* strains ATTC12518Tc, 12518 and *algB* strain using luciferase assay. The luciferase assay of *algB* strain was found to be lower which was as expected. The less expression of algB resulted in less transcription from *algD* which resulted in less alginate production. The luciferase activity on control strain 12518Tc was higher. In the next part, *A.vinelandii* strains 12518Tc and algB along with mutant strains *algW* and *algB* were grown to measure *algD* expression. In contrast to previous experiment, in this experiment the activity on control strain was much lower, *algB* has almost same activity as previous one. Mutant strain algW has also very low activity but mutant strain *amrZ* has much more activity than control strain 12518TC. From this we can conclude that promoter in *amrZ* is really active as wildtype.

In part three, effect of succinate on alginate and growth was measured. It was found that wild type A.vinelandii ATTC12518Tc has maximum growth in RAI medium supplemented with sodium succinate than that of RAI medium supplemented with fructose only and fructose and sodium.

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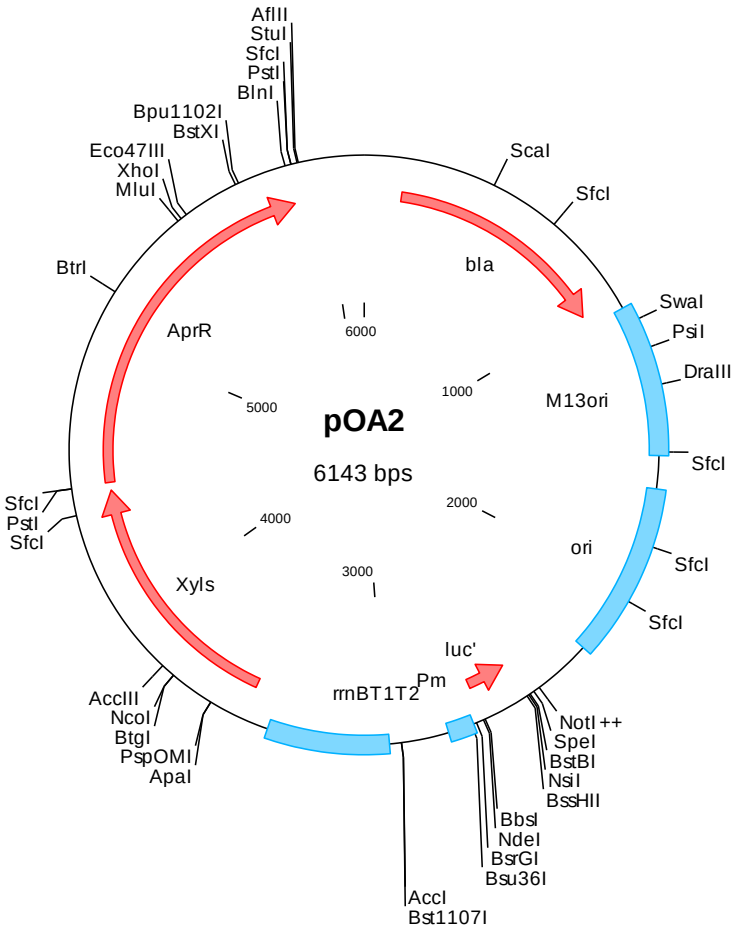
7 List of abbreviations

Amp	Anpicillin
Apr	apramycin
BA	Burks agar
BSA	Bovine Serum Albumin
Ca^{++}	Calcium
H^{+}	Hydrogen
Kb	kilobase
LA	Luria-Bertani agar
LB	Luria-bertani medium
MOPS	The 3-(N-morpholino)-propane-sulfonic acid
OD	Optical Density
bp	Base pair
PCR	Polymerase chain reaction
pH	The power of Hydrogen
PHB	poly-β-hydroxybutyric acid
SOC	Super Optimal Broth with Catabolic repression
UV	ultraviolet

8 Appendix

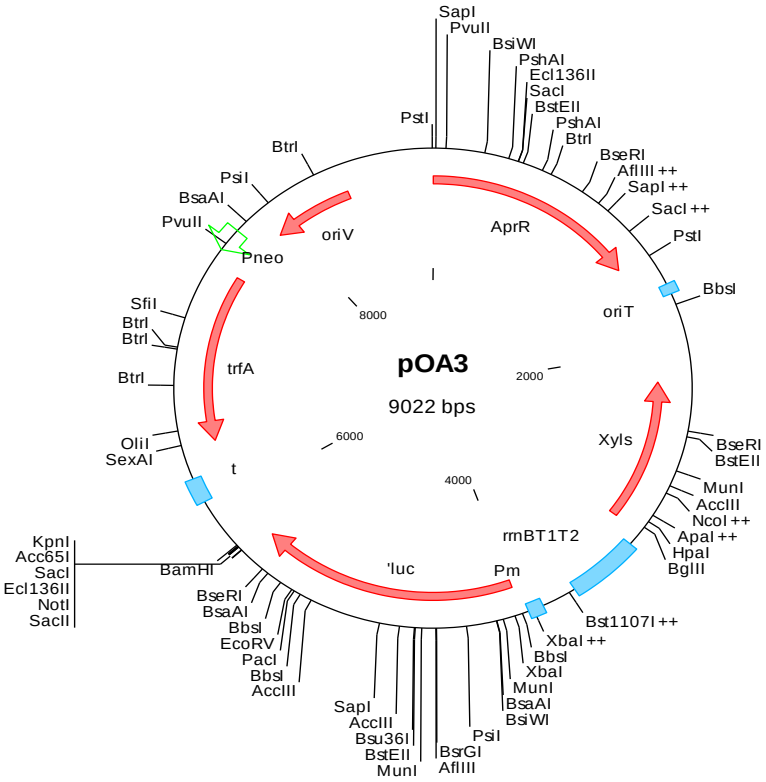
8.1 Plasmid maps of parental plasmids and designated plasmid ih this work; pOA2, pOA3, pTA92, pHE364, pHE365, pJS1, pJS2, pJS3, pJS4, pJS5



A

FigA.1: Plasmid physical map of pOA2 with its restriction sites

B



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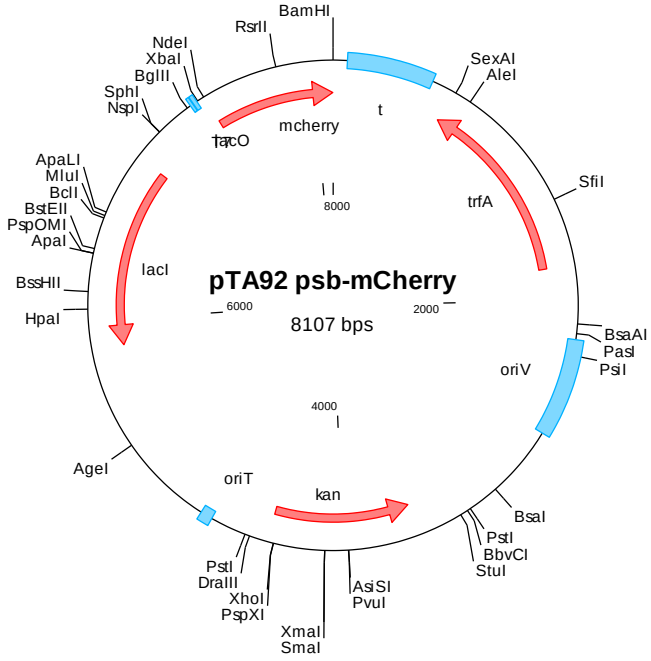
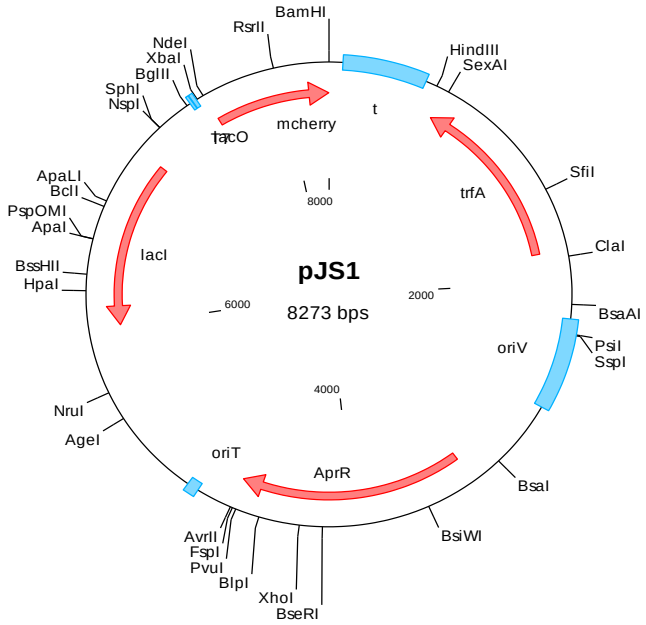
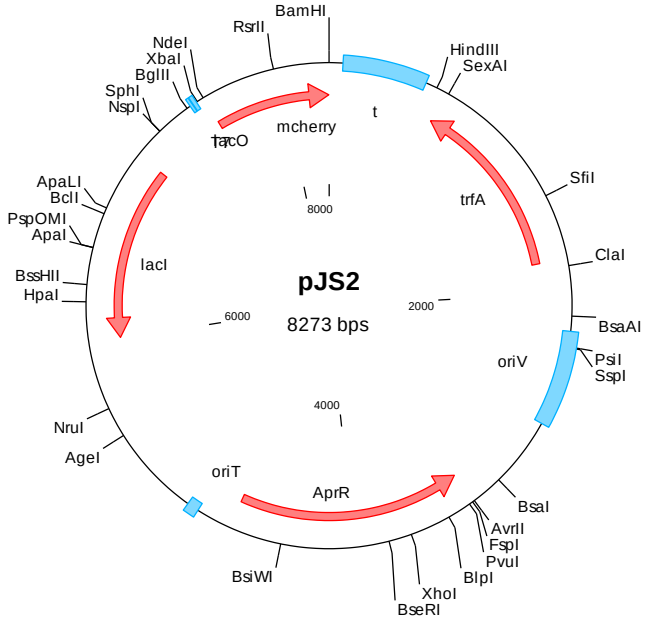


Fig A.1: Plasmid physical map with their restriction sites B)pOA3 C)pTA92

D

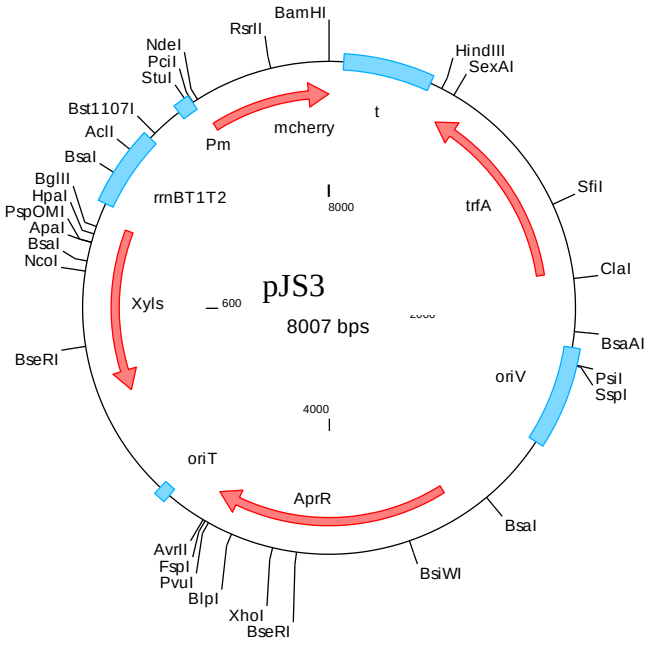


E



FigA.1: Plasmid physical map with their restriction sites D) pJS1 E) pJS2

F



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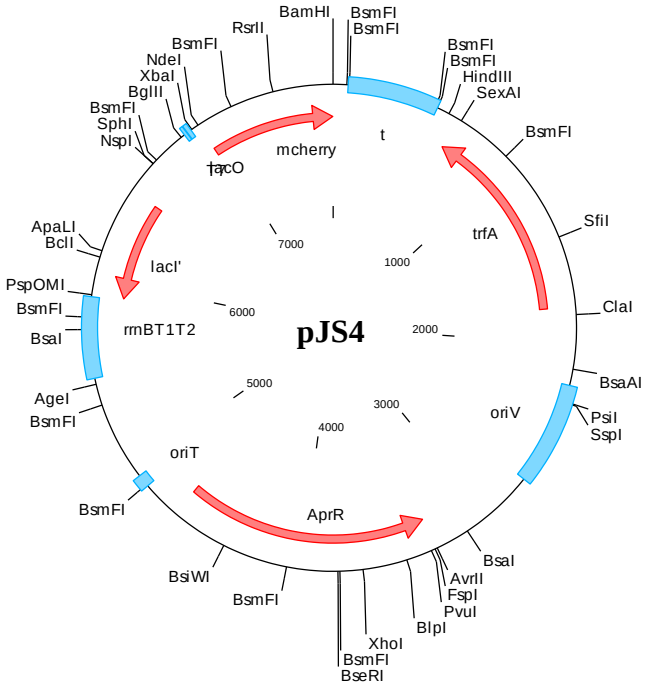
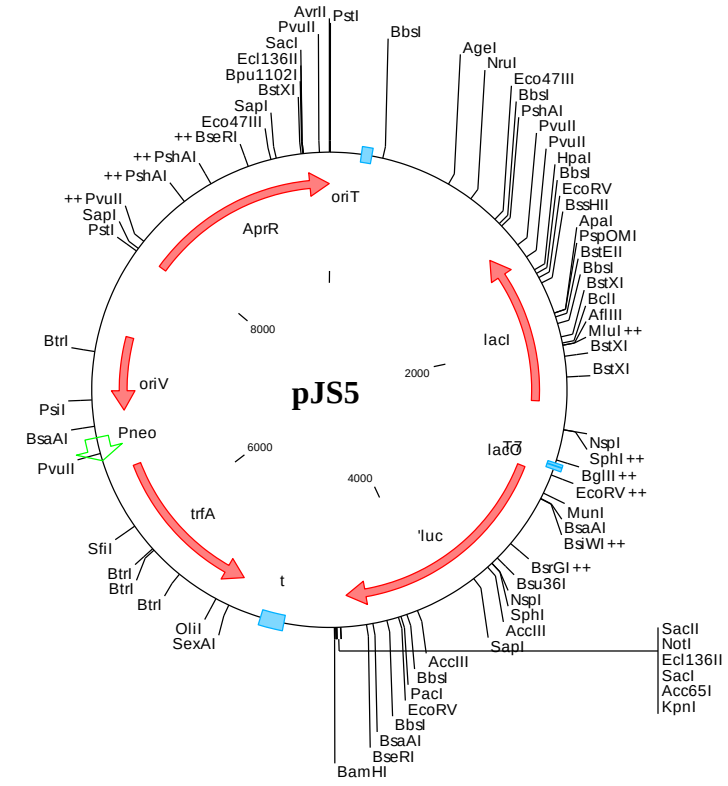
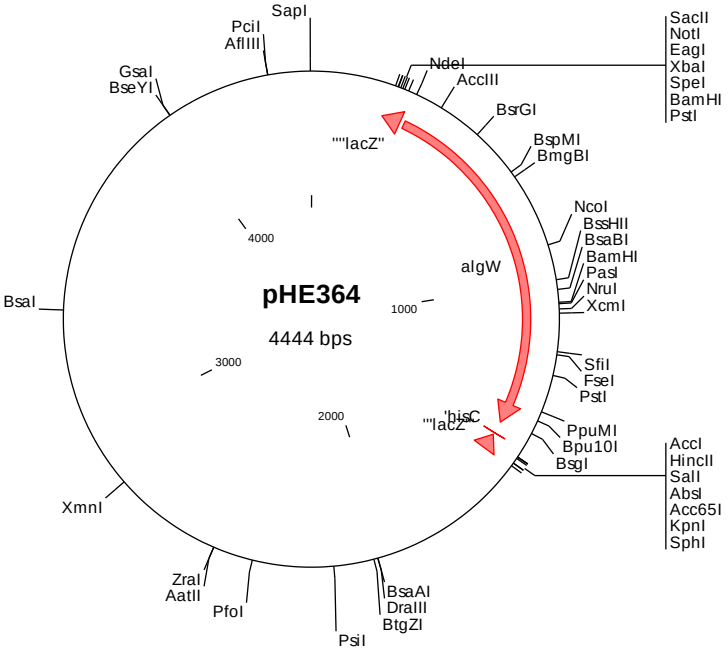


FigA.1: Plasmid physical map with their restriction sites F)pJS3 G)pJS4

H

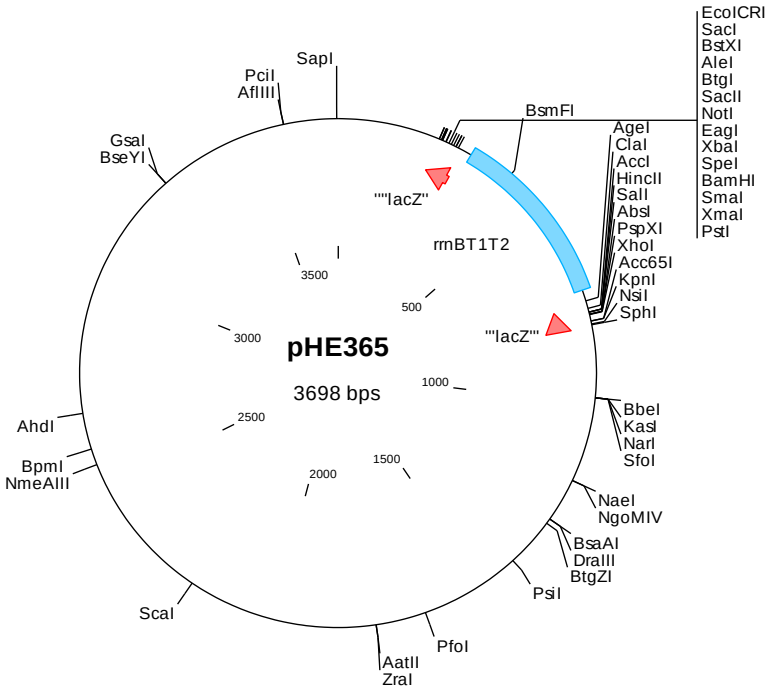


I



FigA.1: Plasmid physical map with their restriction sites H)pJS5 I)pHE364

J



FigA.1: Plasmid physical map with tits restriction sites J) pHE365

8.2 DNA standadrd for Gel Electrophoresis

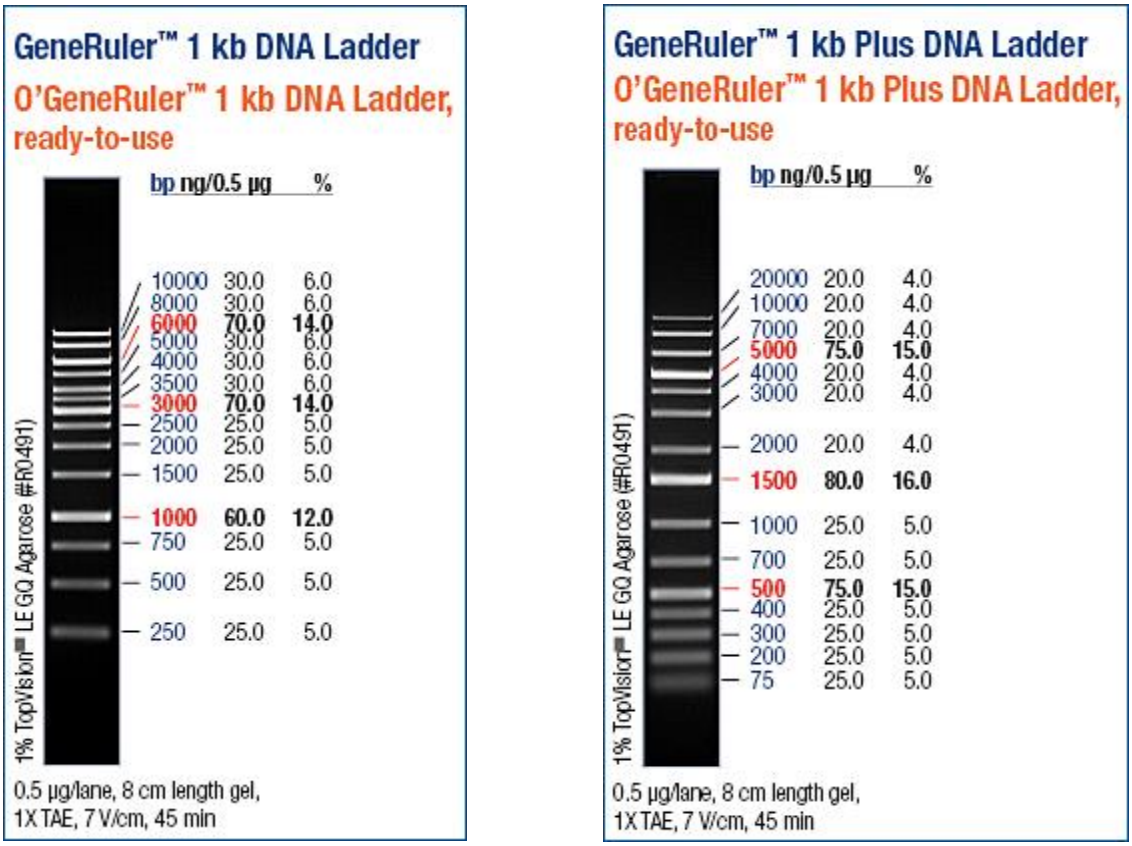


Fig B.1: Gene Ruler^{TM} 1 kb DNA Ladder and Gene Ruler^{TM} 1 kb plus DNA Ladder

8.3 Data for Luciferase activity

Table C.1 Data for luciferase activity of *A.vinelandii* strains ATTC12518Tc, 12518 and AlgB strains (three parallels of each)

A.vinelandii strains	48hours	72hours
12518TcA	2491	3593
12518TcB	2863	2656
12518TcC	4122	3273
12518A	2028	2171
12518B	3018	4429
12518C	2865	2884
AlgB A	1968	278
AlgB B	368	274
AlgB C	129	822

Table C.2 Luciferase activity of A.vinelandii strains 12518Tc, 12818, AlgB, AmrZ and AlgW (three parallels of each)

A.vinelandii strains	24 hours	48hours	72 hours
12518Tc A	1213	692	534
12518Tc B	1987	784	564
12518Tc C	2312	551	432
AlgB A	1208	282	337
AlgB B	1101	279	224
AlgB C			
AlgW A	3101	742	641
AlgW B	2407	398	352
AlgW C	2422	492	387
AmrZA	7324	631	524
AmrZB	7223	554	532
AmrZC	7125	418	315

8.4 Data for absorbance OD_{600} to measure growth of wild type *A.vinelandii* 12518Tc grown in RaI medims supplemented with fructose only, fructose and sodium, and fructose and succinate to know effect of succinate on growth (three parallels of each)

RaI Medium	1	2	3
48 hours			
A	1853	1817	1076
B	1779	1298	1509
C	2606	2105	2003
72hours			
A	2217	2247	1571
B	4811	2379	3589
C	5287	4665	4256

A : Fructose only

B: Fructose and sodium

C : Fructose and succinate

A. Data for absorbane OD_{600} to measure growth of different *A.vinelandii* strains 12518Tc, AlgB, AlgW, AmrZ (three parallels of each)

A.vinelandii strains	24hours	48hours	72hours
15818Tc A	152	211	169
12518Tc B	147	229	158
12518Tc C	143	223	155
AlgB A	195	304	211
AlgB B	164	267	193
AlgB C	182	311	212
AlgW A	98	260	151
AlgW B	79	268	157
AlgW C	96	275	151
AmrZ A	132	259	155
AmrZ B	119	262	167
AmrZ C	155	262	164

8.5 Alginate assay data

Table 2 Alginate assay data of wildtype *A.vinelandii ATTC12518Tc* in RAI medium with different supplements

A.vinelandii stains		**T_0 hours (Before adding lyases)**	**After adding lyases (After 3 hours)**	**After adding enzymes (After 4hours)**
Standard	**I**	0.143	0.149	0.148
	II	0.124	0.124	0.123
	III	0.124	0.130	**0.129**
	I	0.124	1.343	**1.342**
	II	0.126	1.379	**1.369**
	III	0.127	1.369	**1.358**
0.1	**I**	0.144	3.084	**3.085**
	II	0.144	3.086	**3.087**
	III	0.145	3.072	**3.074**
0.2	**I**	0.163	3.526	**3.525**
	II	0.160	3.598	**3.599**
	III	0.163	3.563	**3.565**
0.3	**I**	0.166	3.958	**3.958**
	II	0.165	3.599	**3.589**
	III	0.168	3.947	**3.949**
0.4	**I**	0.178	4.303	**4.305**
	II	0.176	4.226	**4.228**
	III	0.174	4.204	**4.216**

0.5	**I**	0.184	4.746	**4.748**
	II	0.182	4.719	**4.746**
	III	0.185	4.718	**4.719**
A_{48}	**I**	0.30	1.711	**1.713**
	II	0.313	1.681	**1.683**
	III	0.315	1.680	**1.683**
B_{48}	**I**	0.312	1.696	**1.697**
	II	0.334	1.682	**1.689**
	III	0.381	1.723	**1.731**
C_{48}	**I**	0.398	1.654	**1.657**
	II	0.451	1.723	**1.724**
	III	0.491	1.738	**1.742**
A_{72}	**I**	0.412	1.945	**1.947**
	II	0.395	1.861	**1.865**
	III	0.411	1.820	**1.839**
B_{72}	**I**	0.481	1.89	**1.892**
	II	0.442	1.858	**1.859**
	III	0.398	1.837	**1.839**
C_{72}	**I**	0.501	2.058	**2.059**
	II	0.531	2.135	**2.139**
	III	0.593	2.165	**2.163**

Standard curve

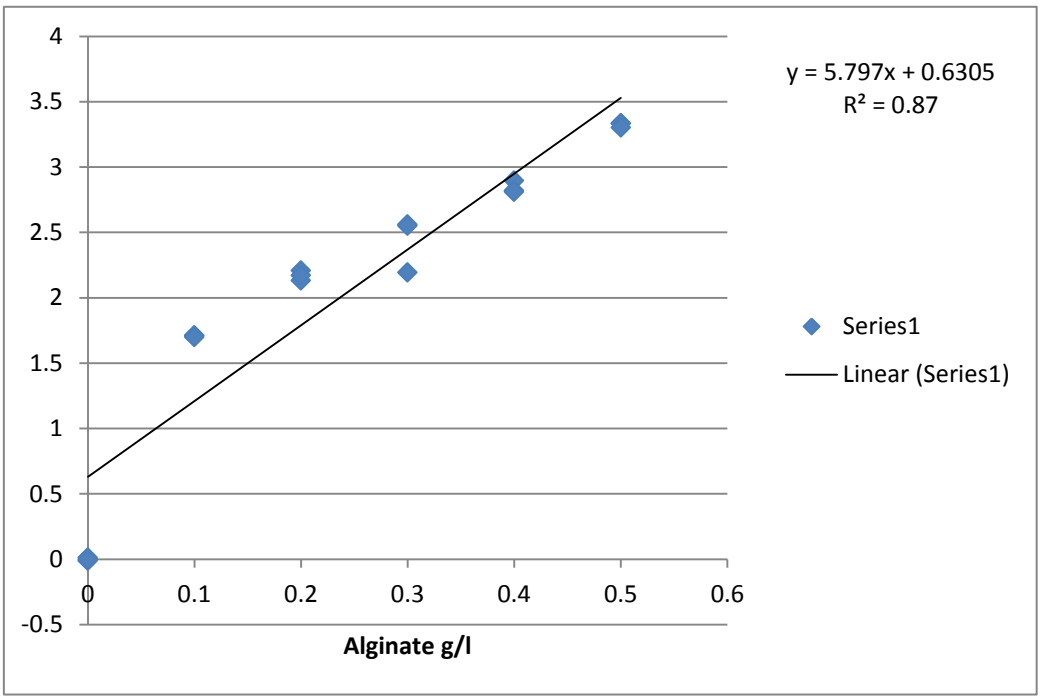


Figure 8-1 standard curve used to calculate alginate production after absorbance