Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
Polymerisation and export of alginate in *Pseudomonas aeruginosa*:
Functional assignment and catalytic mechanism of Alg8/44

A thesis presented to Massey University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy in Microbiology

Uwe Remminghorst

2007
Acknowledgements

First and foremost I would like to thank my supervisor Prof. Bernd Rehm for the provision of the exciting research topic, the ongoing support and the continuous encouragement during the course of my thesis work. Bernd, I would like to express my deep gratitude to you for giving me the opportunity to do “Operation New Zealand” with you!

I would also like to thank my co-supervisor Dr. Jan Schmid for the support, encouragement, time and effort he invested in me. Thanks Jan!

Furthermore, I would like to thank Verena for her unconfined support and help especially during hard times. Thanks that I could always rely on you and your advice (not only scientifically). Worte sind nicht genug um meine Dankbarkeit auszudrücken – Danke, dass Du Dein Leben mit mir teilst und danke, dass es Dich gibt!!!

Special thanks to my workmates and especially to Minion (Iain) for working with me (and depleting all my solutions), to Jane, Katrin, Andrea, Helen, Anika, and MaoSheng for putting up with me day after day – and, I know it can get difficult at times.

Moreover, I would very much like to thank all those in the IMBS who have helped with so many different things that I can’t even name them all without going beyond the scope of this thesis. I would like to specially thank Chris and Paul for the almost weekly Friday “management”-meetings (Bottoms up guys); Ann, Cynthia, Tracy, Katrina and especially Pat for helping me out on so many occasions and for explaining to me the strange ways things go (sometimes) at Massey.

I feel deeply indebted to a few people back in Germany who never gave up phoning and writing me, including but not limited to Mark, the Werner-Crew (Susan, Bernd, Jenny, Stefan, Anja, Swatty, and Sonja) and old colleagues and friends from IMBS (Henning) and the IMMB Münster (Ingo, Nils, Christian E., Christian B., Frank, Herbert, Andrea, Katja). Thanks for keeping in contact; you often gave me the impression New Zealand is just around the corner.

I would also like to thank Massey University for its financial assistance by providing me with a doctoral scholarship.

Last but not least, special thanks to my parents. Vielen Dank Euch beiden für die immerwährende Unterstützung die Ihr mir gegeben habt. Trotz 18.500 Kilometer Entfernung konnte ich mich immer auf Euch verlassen. Allerherzlichsten Dank – ohne Euch wäre dieses alles nicht möglich gewesen!
Preface

This thesis is based on the latest version (DRC 06/139 from 31/08/2006) of the “Submission of a thesis based on publications” format as described on page 53 of the “Handbook for Doctoral Study” of Massey University.

The following parts and chapters of this thesis have been published or are submitted for publication in internationally peer-reviewed journals. The publications used in this thesis do not appear in chronological order.

Chapter I (General introduction)


Chapter II


Chapter III


Chapter IV


Chapter V


Chapter VI

The following experiments of the publications/chapters were performed by Uwe Remminghorst:

**Chapter I:** The review article was mainly written by Uwe Remminghorst.

**Chapter II:** The in vitro alginate polymerisation assay, including the enzymatic synthesis and purification of GPD-mannuronic acid, and the development of the threading model was performed by Prof. Bernd H. A. Rehm. Prof. Gudmund Skják-Brek performed the 1H-NMR analyses of the alginate samples. All other experiments described in this chapter were performed by Uwe Remminghorst.

**Chapter III:** All experiments were performed by Uwe Remminghorst. As stated in the acknowledgement section of the publication, the proteomic analysis (MALDI-TOF/MS) was performed by Dr. Simone König (University of Münster, Germany) and the electron microscopy analysis was performed by Mr. Aaron Hicks (Massey University, New Zealand).

**Chapter IV:** All experiments were performed by Uwe Remminghorst.

**Chapter V:** The PHA content analysis of the different strains was performed by Uwe Remminghorst.

**Chapter VI:** All primer and the cloning strategy for the knockout generation were designed by Uwe Remminghorst. The construction of the β-galactosidase and alkaline phosphatase fusion constructs and their respective reporter gene assays were performed by Uwe Remminghorst.

This is to certify that the above mentioned experiments and/or tasks have been conducted by Mr. Uwe Remminghorst.

29/06/07 [Signature]

Prof. Dr. Bernd H. A. Rehm

29/06/07 [Signature]

Uwe Remminghorst
Abstract

Alginate biosynthesis is not only a major contributor to pathogenicity of *P. aeruginosa* but also an important factor in colonization of adverse environmental habitats by biofilm formation. The requirement of proteins Alg8 and Alg44, encoded by their respective genes in the alginate biosynthesis gene cluster, for alginate biosynthesis of *P. aeruginosa* was demonstrated, since deletion mutants were unable to produce or polymerise alginate. AlgX deletion mutants failed to produce the alginate characteristic mucoid phenotype, but showed low concentrations of uronic acid monomers in the culture supernatants. Complementation experiments using PCR based approaches were used to determine the complementing ORF and all deletion mutants could be complemented to at least wildtype levels by introducing a plasmid harbouring the respective gene. Increased copy numbers of Alg44 did not impact on the amount of alginate produced, whereas increased copy numbers of the *alg8* gene led to an at least 10 fold stronger alginate production impacting on biofilm structure and stability. Topological analysis using reporter protein fusions and subsequent subcellular fractionation experiments revealed that Alg8 is located in the cytoplasmic membrane and contains at least 4 transmembrane helices, 3 of them at its C terminus. Its large cytosolic loop showed similarities to inverting glycosyltransferases and the similarities were used to generate a threading model using SpsA, a glycosyltransferase involved in spore coat formation of *B. subtilis*, as a template. Site-directed mutagenesis confirmed the importance of identified motifs commonly detected in glycosyltransferases. Inactivation of the DXD motif, which has been shown to be involved in nucleotide sugar binding, led to loss-of-function mutants of Alg8 and further replacements revealed putative candidates for the catalytic residue(s). Contradicting the commonly reported prediction of being a transmembrane protein, Alg44 was shown to be a periplasmic protein. The highest specific alkaline phosphatase activity of its fusion protein could be detected in the periplasmic fraction and not in the insoluble membrane fraction. Bioinformatical analysis of Alg44 revealed structural similarities of its N terminus to PilZ domains, shown to bind cyclic-di-GMP, and of its C terminus to MexA, a membrane fusion protein involved in multi-drug efflux systems. Thus, it was suggested that Alg44 has a regulatory role for alginate biosynthesis in bridging the periplasm and connecting outer and cytoplasmic membrane components. AlgX was shown to interact with MucD, a periplasmic serine protease or chaperone homologue, and is suggested to exert its impact on alginate production via MucD interaction. *In vitro* alginate polymerisation assays revealed that alginate production requires protein components of the outer and cytoplasmic membrane as well as the periplasm, and these data were used to construct a model describing a multi-enzyme, membrane and periplasm spanning complex for alginate polymerisation, modification and export.
# Table of contents

Acknowledgements
Preface
Abstract ........................................................................................................................... I
Table of contents ........................................................................................................... II
List of tables ................................................................................................................... V
List of figures ................................................................................................................. VI
Abbreviations ................................................................................................................ VII

## Chapter I (General introduction):

**Bacterial extracellular polysaccharides and their biosynthesis .........................1**

- Bacterial exopolysaccharides..................................................................................2
- Dextran ....................................................................................................................3
- Xanthan ....................................................................................................................4
- Cellulose / Chitin ...................................................................................................5
- Glycosyltransferases ............................................................................................7
- References ..............................................................................................................8

**Bacterial alginites: from biosynthesis to application ........................................11**

- Abstract .............................................................................................................12
- Introduction .........................................................................................................13
- Genetics of alginate biosynthesis .....................................................................14
- Biosynthesis of alginate ....................................................................................15
- Applications of alginites .................................................................................22
- Conclusions and future perspectives ...............................................................23
- References ...........................................................................................................24

**Aim and scope of this thesis .................................................................31**
### Chapter II:

**In vitro alginate polymerisation and functional role of Alg8 in alginate production by Pseudomonas aeruginosa**

- **Abstract**: 34
- **Introduction**: 35
- **Materials and Methods**: 36
- **Results**: 42
- **Discussion**: 47
- **Acknowledgements**: 50
- **References**: 50

### Chapter III:

**Alg44, a unique protein required for alginate biosynthesis in Pseudomonas aeruginosa**

- **Abstract**: 57
- **Introduction**: 58
- **Materials and Methods**: 58
- **Results**: 61
- **Discussion**: 64
- **Acknowledgements**: 66
- **References**: 66

### Chapter IV:

**Membrane topology and site-specific mutagenesis of Alg8, a putative glycosyltransferase, involved in alginate polymerisation by Pseudomonas aeruginosa**

- **Abstract**: 71
- **Introduction**: 72
- **Materials and Methods**: 74
- **Results and Discussion**: 77
- **Acknowledgements**: 84
- **References**: 84
### Chapter V:
**Attachment and biofilm architecture of a supermucoid**
*Pseudomonas aeruginosa*

- Abstract .......................................................... 89
- Introduction ....................................................... 90
- Experiments ....................................................... 90
- Discussion ........................................................ 93
- Acknowledgements ............................................. 93
- References ......................................................... 94

### Chapter VI:
**Biochemical analysis of alginate biosynthesis protein AlgX from**
*Pseudomonas aeruginosa:* purification of an AlgX-MucD protein complex

- Abstract .......................................................... 97
- Introduction ....................................................... 98
- Materials and Methods ....................................... 98
- Results and Discussion ....................................... 102
- Conclusions ....................................................... 107
- Acknowledgements ............................................. 107
- References ......................................................... 107

### Chapter VII:
**Comprehensive summary and discussion**

- Project aims ...................................................... 111
- Alg8 – Knock out and Complementation .................. 112
- Alg8 – Glycosyltransferases ................................. 115
- Alg8 – Topology and Localization ......................... 121
- Alg8 – Impacts on biofilms ................................. 123
- Alg8 – Outlook ................................................. 123
- Alg44 – Knock out and Complementation ............... 124
- Alg44 – Topology and Localization ....................... 125
- Alg44 – Outlook ................................................. 127
- AlgX – Knock out and Complementation ............... 127
- AlgX – Outlook ................................................. 129
- References ......................................................... 130
List of tables

Chapter II:
Table 1 Bacterial strains, plasmids and oligonucleotides .......................................................... 37
Table 2 In vivo alginate synthesis using subcellular fractions of P. aeruginosa FRD1 ..... 43
Table 3 Alginate and cellular dry mass production by various P. aeruginosa strains...... 44

Chapter III:
Table 1 Bacterial strains, plasmids and oligonucleotides .......................................................... 59
Table 2 Alginate and cellular dry mass production various by P. aeruginosa strains....... 63

Chapter IV:
Table 1 Bacterial strains, plasmids and oligonucleotides .......................................................... 75
Table 2 Enzymatic activities of Alg8 fusions to LacZ and PhoA .............................................. 79
Table 3 In vivo alginate polymerase activity of site-specific Alg8 mutants ...................... 82

Chapter VI:
Table 1 Bacterial strains, plasmids and oligonucleotides .......................................................... 99
Table 2 Peptide fragments of AlgX and MucD identified by MALDI-TOF/MS ...... 104
List of figures

Chapter I:

Figure 1 Chemical structures of alginate oligomers and monomers ................................ 13
Figure 2 Regulation network of alginate biosynthesis ........................................................ 14
Figure 3 Alginate biosynthesis pathway in *P. aeruginosa* ...................................................... 16
Figure 4 Model of the putative polymerisation/modification/export complex .................. 18
Figure 5 Chemical structure of cyclic-di-GMP .................................................................. 19

Chapter II:

Figure 1 Disruption of *alg8* by homologous recombination ............................................. 43
Figure 2 Membrane topology and threading model of Alg8 .................................................. 46

Chapter III:

Figure 1 Disruption of *alg44* by homologous recombination ............................................. 61
Figure 2 Immunoblot analysis and purification of Alg44 ..................................................... 64
Figure 3 Model of the putative polymerisation/modification/export complex .................. 65

Chapter IV:

Figure 1 Predicted membrane topology of Alg8 using various algorithms ....................... 78
Figure 2 Secondary structure prediction of Alg8 using program jPred ............................... 78
Figure 3 Phenotypical characterization of various site-specific mutants of Alg8 ............... 80
Figure 4 Threading model of Alg8 showing amino acid replacements ............................ 81

Chapter V:

Figure 1 Solid surface assay analysis of various *P. aeruginosa* strains ............................ 91
Figure 2 Biofilm formation of *P. aeruginosa* in continuous culture flow cell ................. 92

Chapter VI:

Figure 1 SDS-PAGE analysis of purified AlgX by affinity chromatography .................... 103
Figure 2 Hydrophobicity plot of AlgX using program ProtScale ........................................ 105
Figure 3 Immunoelectron microscopic localization of AlgX ............................................. 105

Chapter VII:

Figure 1 Model of the putative polymerisation/modification/export complex .................. 112
Figure 2 Proposed glycosyltransfer by inverting mechanism ............................................. 116
Figure 3 Model of processive polymerisation of polysaccharides .................................... 117
Figure 4 Threading model of Alg8 showing localization of the DXD motifs .................... 119
Figure 5 Predicted membrane topology of Alg8 ................................................................. 121
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Azotobacter</td>
<td>LB</td>
</tr>
<tr>
<td>°C</td>
<td>M</td>
</tr>
<tr>
<td>C or Cys</td>
<td>MALDI-TOF</td>
</tr>
<tr>
<td>e-di-GMP</td>
<td>bis-(3'-5')-cyclic dimeric guanosine monophosphate</td>
</tr>
<tr>
<td>CM</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>D or Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease A</td>
</tr>
<tr>
<td>E or Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>E. Escherichia</td>
<td>NAD⁺[H]</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolymeric substance</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>G</td>
<td>Guluronic acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-(trihydrogen diphosphate)</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GT</td>
<td>Glycosyltransferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H or His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]-piperazine-N'-[2-ethane sulfonic acid]</td>
</tr>
<tr>
<td>HMM</td>
<td>Hidden Markov model</td>
</tr>
<tr>
<td>K or Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KDO</td>
<td>2-Keto-3-deoxyoctanoate</td>
</tr>
<tr>
<td>LacZ</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>M</td>
<td>Mannuronic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PhoA</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>PIA</td>
<td>Pseudomonas isolation agar</td>
</tr>
<tr>
<td>PIM</td>
<td>Pseudomonas isolation medium</td>
</tr>
<tr>
<td>PronaseE</td>
<td>Proteinase E</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
</tbody>
</table>