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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

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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)

Uwe Remminghorst and Bernd H. A. Rehm (2006). Bacterial alginates: from biosynthesis to applications. Biotechnology Letters **28** (21), 1701-1712.

Chapter II

Uwe Remminghorst and Bernd H. A. Rehm (2006). *In vitro* alginate polymerisation and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. Applied and Environmental Microbiology **72** (1): 298-305.

Chapter III

Uwe Remminghorst and Bernd H. A. Rehm (2006). Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Letters **580** (16): 3883-3888.

Chapter IV

Uwe Remminghorst and Bernd H. A. Rehm (2007). Membrane topology and site-specific mutagenesis of Alg8, a putative glycosyltransferase involved in alginate polymerisation by *Pseudomonas aeruginosa*. Journal of Bacteriology – submitted for publication.

Chapter V

Andrea Campisano, Zoe Jordens, Uwe Remminghorst and Bernd H. A. Rehm (2007). Attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa*. Journal of Bacteriology - submitted for publication.

Chapter VI

Jana Gutsche, Uwe Remminghorst and Bernd H. A. Rehm (2006). Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie 88 (3-4): 245-251.

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 Skjåk-Bræk performed the ^{1}H-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 _____

(Date, Signature)

Prof. Dr. Bernd H. A. Rehm

29/06/07 _____

(Date, 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.

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Abbreviations

A.	*Azotobacter*
°C	Degrees Celsius
C or Cys	Cysteine
c-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate
CM	Cytoplasmic membrane
CoA	Coenzyme A
D or Asp	Aspartate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease A
E or Glu	Glutamate
E.	*Escherichia*
EPS	Exopolymeric substance
Fig.	Figure
G	Guluronic acid
GDP	Guanosine 5'-(trihydrogen diphosphate)
GFP	green fluorescent protein
GT	Glycosyltransferase
GTP	Guanosine triphosphate
H or His	Histidine
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethane sulfonic acid]
HMM	Hidden Markov model
K or Lys	Lysine
kDa	Kilo Dalton
KDO	2-Keto-3-deoxyoctanoate
LacZ	β-Galactosidase
LB	Luria-Bertani
M	Mannuronic acid
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
MS	mass spectrometry
Mw	Molecular weight
NAD^+[H]	Nicotinamide adenine dinucleotide [reduced form]
ND	not detectable
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
ORF	open reading frame
P.	*Pseudomonas*
PAGE	Polyacrylamide gel electrophoresis
PhoA	Alkaline Phosphatase
PIA	*Pseudomonas* isolation agar
PIM	*Pseudomonas* isolation medium
PronaseE	Proteinase E
RNase	Ribonuclease A
SDS	Sodium dodecyl sulphate
TCA	Tricarboxylic acid cycle
TLC	Thin layer chromatography
TM	Transmembrane