FEBS Letters 580 (2006) 3883–3888

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

Uwe Remminghorst, Bernd H.A. Rehm*

Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

Received 10 April 2006; revised 24 May 2006; accepted 29 May 2006

Available online 16 June 2006

Edited by Gianni Cesareni

Abstract Here the putative alginate biosynthesis gene *alg44* of *Pseudomonas aeruginosa* was functionally assigned. Non-polar isogenic *alg44* deletion mutants of *P. aeruginosa* were generated and did neither produce alginate nor released free uronic acids. No evidence for alginate enrichment in the periplasm was obtained. Alginate production was restored by introducing only the gene *alg44*. PhoA fusion protein analyses suggested that Alg44 is a soluble protein localized in the periplasm. Hexahistidine-tagged Alg44 was detected by immunoblotting. The corresponding 42.6 kDa protein was purified and identified by MALDI/TOF-MS analysis. Alg44 might be directly involved in alginate polymerization presumably by exerting a regulatory function.
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Keywords: Alginate biosynthesis; Alginate; Alginate polymerase; *Pseudomonas aeruginosa*

1. Introduction

Bacterial alginates consist of β-1,4-linked monomers of β-D-mannuronic acid and its C5 epimer α-L-guluronic acid. Alginate is an important virulence factor produced by the opportunistic human pathogen *Pseudomonas aeruginosa*. Primarily synthesized as polymannuronate from GDP-mannuronic acid, the nascent polymer chain undergoes modification in the periplasm by acetylation and epimerization [1]. A protein scaffold might guide the alginate chain through the periplasm towards the outer membrane export channel [2–5]. Although initial alginate polymerization and cytoplasmic membrane transfer are still not understood, it has been recently shown that Alg8 seems to be a bottleneck in alginate production. Increased copy number of *alg8* resulted in an increased alginate production, suggesting that the putative glycosyltransferase Alg8 is a catalytic subunit of the alginate polymerase [6]. So far only the isogenic *alg8* deletion mutant did neither produce alginate nor released uronic acids.

In this study, the *alg44* gene localized in the alginate biosynthesis gene cluster of *P. aeruginosa* was functionally assigned by generation of a non-polar *alg44* deletion mutant, functional expression as well as by subcellular localization, purification and identification of the Alg44 protein.

*Corresponding author. Fax: +64 6 350 5688.
E-mail address: B.Rehm@massey.ac.nz (B.H.A. Rehm).

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were cultivated as previously described [6]. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Construction of isogenic alg44 deletion mutants

Two regions of the *alg44* gene were amplified using *Taq* polymerase and primers alg441N-Ec5, alg441N-Ba, alg442C-Ba and alg442C-Ec5 (Table 1). Region alg44N (362 bp) comprises the first 358 nucleotides of the designated *alg44* open reading frame [7] including 4 nucleotides upstream of the start codon. Region alg44C (332 bp) comprised bases 820–1151 relative to the *alg44* coding region [7], respectively. Both PCR products represented the flanking region for homologous recombination and isogenic mutants were generated and confirmed as previously described [6].

2.3. Complementation of isogenic alg44 deletion mutants

For complementation of *alg44* deletion mutants, the coding region of the *alg44* gene of *P. aeruginosa* PAO1 was amplified by PCR using primers alg44N(HiSDNd) and alg44C(Ba) and subcloned into broad-host-range vector pBBR1MCS-5 [8], resulting in plasmid pBBR1MCS-5:alg44. Additionally, the 3′-end primer alg44C(HisBa) was used to generate an *alg44* gene encoding a C-terminally hexahistidine-tagged Alg44, which was inserted into vector pBBR1MCS-5 as described above (Table 1). All inserts cloned into the multiple cloning site of vector pBBR1MCS-5 are under control of the *lac* promoter.

2.4. Subcellular localization of Alg44 using either PhoA or LacZ fusions

The gene *alg44* was amplified by PCR using *Pfx* polymerase and primers alg44N(HiSDNd) and alg44C(Δstop). The corresponding 1202 bp PCR product was used to construct plasmid pBBR1MCS-5:alg44(Δstop). *Xba*I–*Bam*HI fragments of vectors pPHO7 [9] and pJE608 [10] were inserted into *Xba*I–*Bam*HI restricted pBBR1MCS-5:alg44(Δstop) to generate translational fusions with either PhoA or LacZ. Subcellular fractionation was performed as described previously [6]. Osmotic shock was used to isolate periplasmic extracts [11].

2.5. Alkaline phosphatase/β-galactosidase activity assays

Alkaline phosphatase and β-galactosidase enzymatic assays were performed according to the methods of Miller [12] and Manoil [13], respectively.

2.6. Heterologous production and purification of Alg44

Plasmid pBBR1MCS-5:alg44His was hydrolyzed with *Nde*I and *Bam*HI and the resulting 1.2 kbp fragment was cloned into *Nde*I and *Bam*HI hydrolyzed vector pT7-7 [14], creating plasmid pT7-7:alg44His. *E. coli* BL21 (DE3) pLysS was used for heterologous expression. Crude extracts were subjected to immobilized metal ion chromatography (Ni-NTA agarose, Qiagen) using denaturing conditions (6 M GuHCl). The hexahistidine-tagged protein was purified using buffers as described in the manufacturers manual (Qiagen).

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doi:10.1016/j.febslet.2006.05.077

Table 1
Bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
P. aeruginosa		
FRD1	Cystic fibrosis isolate; Alg$^+$	[31]
FRDΔalg44Gm	Δ*alg44*::*aacC1*; Alg$^-$	This study
FRDΔalg44	Δ*alg44*; Alg$^-$	This study
PAO1	Prototrophic wild-type strain; Alg$^-$	[32]
PDO300	Δ*mucA22* variant of PAO1; Alg$^+$	[33]
PDO300Δalg44Gm	Δ*alg44*::*aacC1*; Alg$^-$	This study
PDO300Δalg44	Δ*alg44*; Alg$^-$	This study
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi*-1; *proA*, *hsd*R17 (r_k^-, m_k^+), *recA1*, *tra*-gene of plasmid RP4 integrated in chromosome	[34]
MC4100	F$^-$ *araD*139 Δ(*argF-lac*)U169 *rpsL*150 (Str^r) *relA1 flbB*5301 *deoC*1 *ptsF*25*rbsR*	[35]
DH5α	F′/*endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYAargF*) U169 *deoR* (Φ80*dlac*Δ(*lacZ*)M15)	Life Technologies
BL21(DE3) pLysS	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cm^r)	Novagen
Plasmids		
pBBR1MCS-5	Gm^r; broad-host-range vector; $P_{(lac)}$	[8]
pBBR1MCS-5:alg44	*Hin*dIII–*Bam*HI fragment comprising gene *alg44* inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44His	*Hin*dIII–*Bam*HI fragment encoding C-terminally hexahistidine-tagged Alg44 inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44(Δstop)	*Hin*dIII–*Bam*HI fragment encoding Alg44 without stop codon inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44lacZ	Translational Alg44-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44phoA	Translational Alg44-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^r; Cb^r, gene replacement vector containing *sacB* gene for counterselection	[36]
pEX100TΔalg44Gm	Ap^r, Cb^r, Gm^r; vector pEX100T with *Sma*I inserted *alg44* deletion construct	This study
pPS856	Ap^r; Gm^r; source of 1100 bp *Bam*HI-fragment comprising *aacC1* gene flanked by FRT signal sequences	[36]
pPFLP2	Ap^r; Cb^r; broad-host-range vector encoding Flp recombinase	[36]
pPHO7	Ap^r; *phoA* without signal sequence	[9]
pJE608	LacZ lacking the first 8 amino acids with promoter $P_{(tac)}$ in pMMB67EH	[10]
pT7-7	Ap^r, T7Φ10 expression vector	[14]
pT7-7:alg44His	*Nde*I–*Hin*dIII fragment of pBBR1MCS-5:alg44His inserted into vector pT7-7	This study
Oligonucleotides		
alg441N-Ec5	GCGTCGATATCCACCATGAATACAGCCGTCAACG	
alg441N-Ba	TCACGGATCCCCAGGTAGGAGGTGATCAGGTAG	
alg442C-Ba	CTACGGATCCCAACTGGTAGCCGACGGGCAATAC	
alg442C-Ec5	GTGTCGATATCGTCACGGCCTTGTTCAGCAG	
alg44up	TGATGGATCCGTTCACCATGCTGGTGCTGTTC	
alg44down	AACTCTGCAGCAGGCTGACGGTG	
alg44N(HiSDNd)	CCGCCAAGCTTAGGAGCCCGACCATATGAATACAGCCGTCAACGTCAACG	
alg44C(Ba)	AACGGATCCTCAGCGAGCGGTGGCCAGGGTCAC	
alg44C(HisBa)	AACGGATCCTCAATGGTGATGGTGATGGTGACGAGCGGTGGCCAGGGTCAC	
alg44C(Δstop)	AAAAAGGATCCCGAGCGGTGGCCAGGGTCAC	

2.7. SDS–PAGE and immunoblot analyses

Proteins from *P. aeruginosa* or *E. coli* containing plasmids encoding Alg44His$_6$ were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [15]. Proteins were electroblotted onto nitrocellulose membrane (Protran BA 83, Schleicher & Schuell) and then incubated with HisProbe™-horseradish peroxidase conjugate (HisProbe™-HRP, Pierce). Immunoblots were developed using a chemiluminescence protocol according to the manufacturers manual (SuperSignal® West HisProbe™, Pierce).

2.8. Alginate production and uronic acid assays

Alginate production assays and uronic acid assays were performed as described previously [6].

2.9. Electron microscopy

Cells were fixed as described elsewhere [16], and electron microscopy was performed on a Philips CM201c transmission electron microscope [17].

3. Results

3.1. Construction of an isogenic alg44 knock out mutant

To investigate the function of the *alg44* gene in alginate biosynthesis by *P. aeruginosa*, marker-free and non-polar *alg44* deletion mutants of alginate-overproducing strains *P. aeruginosa* PDO300 and *P. aeruginosa* FRD1 were generated, respectively (Fig. 1). Both deletion mutants showed a non-mucoid and alginate-negative phenotype when cultivated on agar plates. To investigate whether the non-mucoid phenotype is caused by alginate lyase (AlgL) mediated degradation of an exposed unprotected alginate chain, culture supernatants of *P. aeruginosa* PDO300Δalg44 were analysed with respect to alginate and free uronic acids. Neither alginate nor free uronic acids were detected (Table 2). Recently, Jain and Ohman showed that alginate lyase is required for alginate production

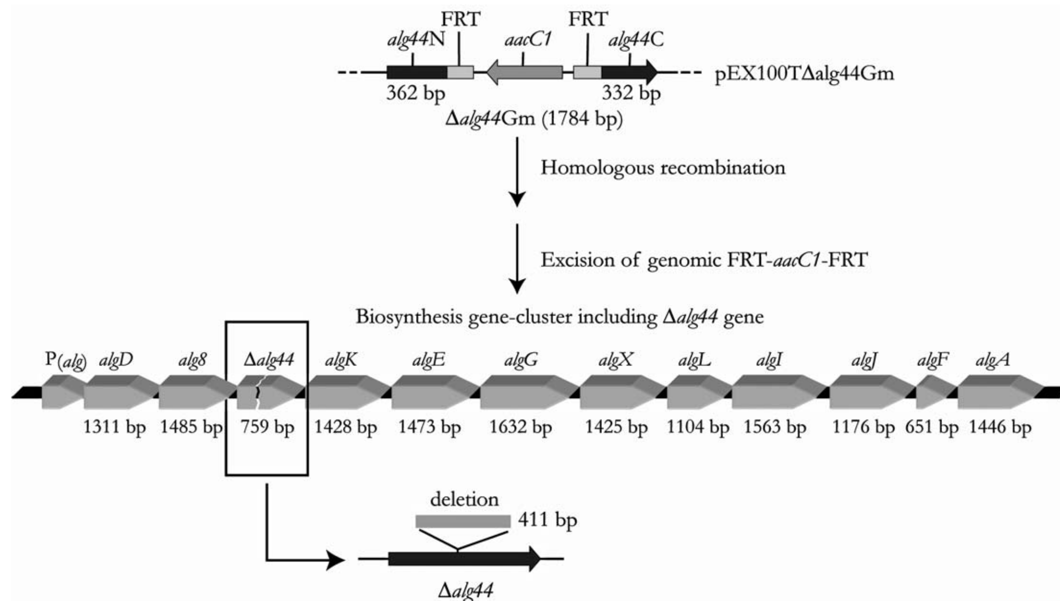

Fig. 1. Schematic view of *alg44* knock out construct of plasmid pEX100TΔalg44Gm used for homologous recombination and the alginate biosynthesis operon after replacement of native *alg44* gene with Δ*alg44*.

Table 2
Alginate and cellular dry mass production by different *P. aeruginosa* strains harboring various plasmids

Strain	Mean ± S.D.	
	Alginate production (g/g CDM)	Cellular dry mass (CDM [g])
PDO300	0.131 ± 0.037	0.171 ± 0.027
PDO300(pBBR1MCS-5)	0.629 ± 0.026	0.196 ± 0.006
PDO300(pBBR1MCS-5:alg44)	0.731 ± 0.122	0.171 ± 0.015
PDO300Δalg44	ND[a]	0.127 ± 0.006
PDO300Δalg44(pBBR1MCS-5)	ND[a]	0.173 ± 0.003
PDO300Δalg44(pBBR1MCS-5:alg44)	1.187 ± 0.721	0.158 ± 0.034
PDO300Δalg44(pBBR1MCS-5:alg44His)	0.786 ± 0.257	0.159 ± 0.124
PDO300Δalg44(pBBR1MCS-5:alg44lacZ)	0.392 ± 0.031	0.157 ± 0.003
PDO300Δalg44(pBBR1MCS-5:alg44phoA)	1.343 ± 0.914	0.145 ± 0.063

[a]ND, not detectable. *P* values were determined using Kruskal–Wallis one way analysis of variance on ranks. All mean values and S.D.s were based on four independent experiments.

and that AlgL deficient mutants accumulated alginate in the periplasm [4]. No uronic acids were detected when analyzing periplasmic extract of the *alg44* deletion mutant, and electron microscopy analysis of cells of *P. aeruginosa* PDO300 wild-type and *alg44* deletion mutant showed neither swelling of the periplasm nor differences in cell shape and structure between wild-type and mutant (data not shown).

3.2. Restoration of alginate biosynthesis using the alg44 open reading frame

To verify that disruption of *alg44* had no downstream effects on the alginate biosynthesis operon and to functionally assign the putative *alg44* gene, a plasmid containing only the designated *alg44* ORF (pBBR1MCS-5:alg44) was used to complement the *P. aeruginosa* PDO300Δalg44 mutant. This plasmid mediated restoration of alginate production in the *alg44* mutant. Alginate production of complemented *alg44* mutants carrying either the native *alg44* gene or a gene encoding C-terminally tagged Alg44 in vector pBBR1MCS-5, had no statistically significant influence ($P > 0.05$) on alginate yield (Table 2). Since, *P. aeruginosa* PDO300 harboring gentamycin-resistance mediating vector pBBR1MCS-5 showed already a presumably antibiotic stress mediated increased alginate production when compared with the wild-type strain; this recombinant strain was used as control (Table 2). The cellular dry mass (CDM) produced by the various strains showed no significant difference ($P > 0.05$) (Table 2).

3.3. Analysis of translational fusion proteins of Alg44

Topology prediction analysis (SMART [18], TMHMM [19]) of Alg44 suggest one transmembrane domain (amino acids 159–178) with the N-terminus exposed to the cytosol. No signal sequence could be predicted [18,19]. To investigate the localization and topology of Alg44, C-terminal translational fusions to reporter enzymes LacZ and PhoA were generated, respectively. Both reporter enzyme fusions had no impact on Alg44 function, as indicated by complementation studies (Table 2). Reporter enzyme assays revealed a specific alkaline phosphatase activity of 5.036 ± 0.648 U/mg of CDM and a β-galactosidase activity of 0.834 ± 0.215 U/mg, respectively. The reporter enzyme assays were also performed in strains of *E. coli* DH5α and MC4100, revealing an alkaline

phosphatase activity of 2.973 ± 0.282 U/mg CDM and a β-galactosidase activity of 0.906 ± 0.112 U/mg CDM. Cellular fractionation experiments were performed with strain *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44phoA) in order to investigate the subcellular localization of Alg44. Interestingly, higher alkaline phosphatase activity was detected in the soluble supernatant, but not in the insoluble membrane fraction. The membrane free supernatant revealed an alkaline phosphatase activity of 17.577 ± 1.843 U/mg protein, whereas the membrane fraction showed only an activity of 4.086 ± 0.510 U/mg protein. Isolated periplasmic extract showed an alkaline phosphatase activity of 8.302 ± 0.720 U/mg protein.

3.4. Immunological detection and purification of $Alg44His_6$

To detect and verify the expression of *alg44* and to demonstrate the presence of Alg44, the production of hexahistidine-tagged Alg44 protein was investigated in the native host *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44His) as well as in *E. coli* BL21 (pLysS, pT7-7:alg44His). $Alg44His_6$ was detected by immunoblotting using anti-His_6-antibodies. The immunoblot showed specific antibody binding to a protein with the apparent molecular weight of 42.4 ± 0.4 kDa, which was consistent with the predicted molecular weight of $Alg44His_6$ of 42.6 kDa (Fig. 2A and B). To verify that the detected protein is encoded by the designated *alg44* ORF, crude extracts of *E. coli* BL21 (pLysS, pT7-7:alg44His) were subjected to affinity chromatography. SDS–PAGE and MALDI/TOF-MS analysis showed that $Alg44His_6$ could be purified from crude extracts (Fig. 2C).

4. Discussion

Here the first marker-free non-polar *alg44* deletion mutant of *P. aeruginosa* was generated by using homologous recombination and the loss of alginate production suggested that Alg44 is required for alginate production (Fig. 1, Table 2). Uronic acid monomer and oligomer analysis suggested that the alginate-negative phenotype was not due to extensive degradation of alginate by the alginate lyase as reported for *algG*, *algK* and *algX* mutants [20–22] and that Alg44 is involved in alginate polymerization. AlgG, AlgK and AlgX are proposed periplasmic scaffold proteins. AlgX might also interact with a serine-protease homologue MucD, which is involved in alginate biosynthesis gene regulation [23]. *P. aeruginosa* PDO300-Δalg44 did not accumulate alginate in the periplasm, as was found in *algL* mutants [4].

Only the 1170 bp ORF of *alg44* [24] encoding a putative 41.78 kDa protein mediated restoration of wild-type level alginate production suggesting that *alg44* encodes a protein required for alginate production. Production of Alg44 with the

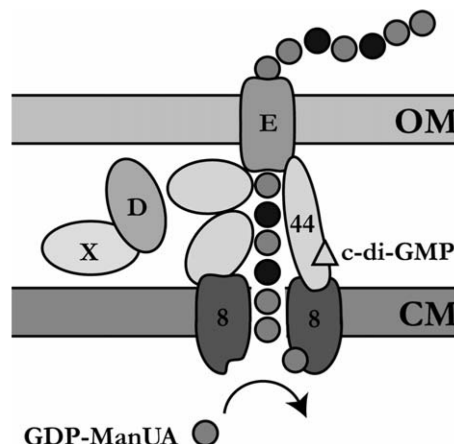

Fig. 3. Model of the alginate polymerization, modification and export. CM, cytoplasmic membrane; OM, outer membrane, 8, Alg8; 44, Alg44, E, AlgE; X, AlgX; D, MucD; c-di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; GDP-ManUA, GDP-mannuronic acid.

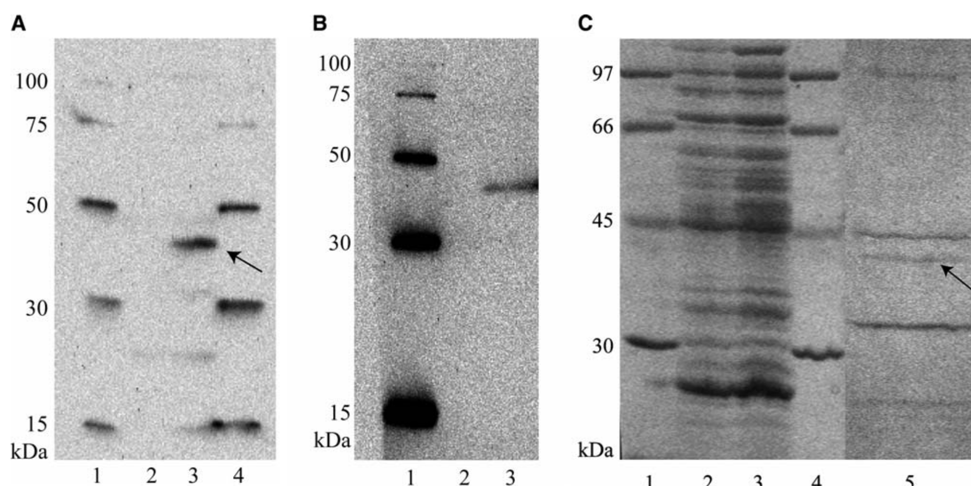

Fig. 2. (A) Immunoblot analysis of cell extracts of *E. coli* BL21 (pLysS) harboring overexpression plasmids. Lanes 1 and 4, hexahistidine tagged molecular weight standard; lane 2, *E. coli* BL21 (pLysS) (pT7-7); lane 3, *E. coli* BL21 (pLysS) (pT7-7:alg44His); arrow indicates $Alg44His_6$. (B) Immunoblot analysis of cell extracts of *P. aeruginosa* PDO300Δalg44 harboring various plasmids. Lane 1, hexahistidine tagged molecular weight standard; lane 2, *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5); lane 6, *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44His). (C) SDS–PAGE analysis of $Alg44His_6$ purified by affinity chromatography. Lanes 1 and 4, molecular weight standard; lane 2, crude extract of *E. coli* BL21 (pLysS) (pT7-7); lane 3, crude extract of *E. coli* BL21 (pLysS) (pT7-7::alg44His); lane 5, partially purified $Alg44His_6$; arrow indicates protein subjected to MALDI/TOF-MS analysis.

corresponding molecular weight was confirmed by immunoblot analysis of the hexahistidine-tagged Alg44, which was also purified and identified by MALDI-TOF/MS analysis.

C-terminal fusions to Alg44 did not interfere with protein functionality, suggesting that the C-terminus is not directly involved in Alg44 function (Table 2). The activity of the alkaline phosphatase and the lack of β-galactosidase activity supported the constrained topology prediction using HMM-based topology tool Phobius [25], which suggested a cytosolic N-terminus, a transmembrane domain (amino acid 159–178) and a periplasmic localization of the C-terminus. However, the highest specific alkaline phosphatase activity was not associated with the insoluble membrane, but with the soluble membrane-free fraction as well as the periplasmic extract, which suggested a periplasmic localization of Alg44. This finding did not support the presence of the predicted transmembrane domain. Protein export into the periplasm is usually depending on a N-terminal signal sequence. The detection of 42.6 kDa hexahistidine tagged protein Alg44 in *P. aeruginosa* indicated the presence of an unmodified mature protein (Fig. 2), which is consistent with the lack of a signal sequence. Other proteins such as, e.g., the dehalogenases LinA and LinB from pseudomonads/sphingomonads, have been localized to the periplasm without N-terminal processing [26].

Homology searches [27] revealed a 16.8% similarity (Jscore: 106.71) of the C-terminal half of Alg44 (aa 202–389) to MexA (PDB structure 1t5eA/1vf7a). MexA is a periplasmic membrane fusion protein (MFP) component of the multidrug transporter complex MexAB-OprM in *P. aeruginosa*, linking the cytoplasmic membrane component MexB with the outer membrane export protein OprM [28]. These similarities together with the periplasmic localization of protein Alg44, might indicate a role of Alg44 bridging the cytoplasmic-membrane associated polymerase (only protein shown to be in the cytoplasmic membrane is Alg8) with the outer membrane export channel AlgE (Fig. 3). The C-terminal part of Alg44 might be involved in connecting or colocalizing Alg8 and AlgE, but scaffold proteins AlgKGXL might be required to protect the nascent alginate chain against degradation. Recently, a PilZ domain (PDB structure 1yln/1ywu) in the N-terminal region of Alg44 was identified [29], which might be involved in binding the novel regulatory molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) [30]. Thus Alg44 might function as a regulatory membrane fusion protein (Fig. 3).

Acknowledgements: This study was supported by a research grant to B.H.A.R. from the Institute of Molecular Biosciences at Massey University and the Deutsche Forschungsgemeinschaft (Re 1097/6-1 to B.H.A.R.). U.R. received a doctoral scholarship from Massey University. Proteomic analysis (MALDI-TOF/MS) was performed by Dr. Simone König (University of Münster, Germany) and electron microscopy analysis was performed by Aaron Hicks (Massey University, Palmerston North, New Zealand).

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