A novel gene, *algK*, from the alginate biosynthetic cluster of *Pseudomonas aeruginosa*

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Colonization of the cystic fibrosis lung by *Pseudomonas aeruginosa* is greatly facilitated by the production of an exopolysaccharide called alginate. Many of the enzymes involved in alginate biosynthesis are clustered in an operon at 34 min on the *P. aeruginosa* chromosome. This paper reports the nucleotide sequence of a previously uncharacterized gene, *algK*, which lies between the *alg44* and *algE* genes of the operon. DNA sequencing data for *algK* predicted a protein product of approximately 52.5 kDa which contains a putative 27 amino acid N-terminal signal sequence and a consensus cleavage and lipid attachment site for signal peptidase II. Expression of *algK* using either T7 or *tac* promoter expression systems, and *in vivo* labelling studies with [³⁵S]methionine, indicated that *algK* encodes a polypeptide of approximately 53 kDa which is processed to a mature protein of approximately 50 kDa when expressed in *Escherichia coli* or *P. aeruginosa*, in agreement with the nucleotide sequence analysis. Results from an AlgK- β -lactamase fusion survey support this interpretation and also provide evidence that mature AlgK is entirely periplasmic and is probably membrane-anchored.

Keywords: AlgK, alginate, exopolysaccharide, *Pseudomonas aeruginosa*, cystic fibrosis

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which can cause severe infections of the respiratory tract, of burn wounds and of the urinary tract. In patients with cystic fibrosis (CF), *P. aeruginosa* causes a pulmonary infection which generally remains intractable both to antibiotic therapy and to elimination by the immune system.

The persistence of *P. aeruginosa* in the CF lung depends heavily on its ability to form microcolonies, embedded within a polyanionic biofilm of alginate exopolysaccharide (Lam *et al.*, 1980). The pattern of microcolony formation has profound implications for the course of the disease, as the alginate biofilm protects *P. aeruginosa* from phagocytosis, impedes antibiotic pen-

etration and also facilitates bacterial adherence to the respiratory tract. The progressive emergence of mucoid strains therefore allows the establishment of chronic *P. aeruginosa* infection within the CF lung, ultimately leading to irreversible lung damage, respiratory failure and death. As a result, this pathogen has proved to be a major cause of morbidity and mortality amongst CF patients.

Alginate from *P. aeruginosa* is composed of a linear copolymer of 1,4- β -linked D-mannuronic acid and its C-5 epimer, L-guluronic acid, in which the mannuronic acid residues are variably modified with O-acetyl groups (Skjåk-Bræk *et al.*, 1986). Much of the pathway for alginate biosynthesis has been defined. The majority of the enzymes involved in alginate biosynthesis are clustered in an 18 kb operon (Chitnis & Ohman, 1993) at 34 min on the *P. aeruginosa* chromosomal map. The operon encodes proteins such as the dual-function enzyme phosphomannose isomerase-GDP mannose pyrophosphorylase (*algA*; Shinabarger *et al.*, 1991) and also GDP-mannose dehydrogenase (*algD*; Deretic *et al.*, 1987). Other genes identified in the alginate biosynthetic

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Abbreviation: CF, cystic fibrosis.

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cluster encode a C5-mannuronate epimerase which is responsible for the incorporation of L-guluronate residues into alginate (*algG*; Franklin *et al.*, 1994), an O-acetylase which esterifies mannuronate residues (*algF*; Shinabarger *et al.*, 1993), a D-mannuronate-specific alginate lyase (*algL*; Boyd *et al.*, 1993; Schiller *et al.*, 1993) and an outer-membrane pore which is considered to mediate alginate excretion (*algE*; Chu *et al.*, 1991; Rehm *et al.*, 1994). Despite this knowledge, however, the mechanism by which alginate polymerization and export occur remains poorly understood.

Two regions remain uncharacterized within the 34 min biosynthetic cluster. The first is a region of approximately 2 kb between *alg44*, a gene which encodes a putative hydrophobic protein of undefined function (Maharaj *et al.*, 1993), and *algE*. The second region (approximately 2.5 kb) lies between *algL* and *algF*, and is thought to encode an acyl-carrier protein (May & Chakrabarty, 1994). In this paper we describe the cloning and sequencing of the first of these two regions and report the identification of a new gene which we have called *algK*. We show, in both *Escherichia coli* and *P. aeruginosa*, that *algK* encodes a poorly expressed pre-protein of approximately 53 kDa which is processed to form a smaller mature polypeptide. We also provide evidence, obtained using fusion proteins, that mature AlgK adopts an entirely periplasmic location and probably undergoes an N-terminal post-translational modification.

METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* and *P. aeruginosa* were routinely cultured in LB broth or on minimal agar containing M9 medium (per litre: 1 g NH₄Cl, 3 g KH₂PO₄, 1.6 g Na₂HPO₄, 1 ml 1 M MgSO₄, 50 µl 1 M CaCl₂) supplemented with 0.08% (w/v) glycerol, 0.4% (w/v) glucose, 0.04% (w/v) thiamin and antibiotics, where appropriate. The latter medium was used to select for *P. aeruginosa* following triparental matings with *E. coli*. Antibiotics were used at the following concentrations (per ml) unless otherwise stated: ampicillin, 50 µg (200 µg for *P. aeruginosa*); tetracycline, 15 µg; kanamycin, 50 µg; chloramphenicol 5 µg. Alginate production was assessed visually from colonies grown on LB agar or MAP medium (Franklin *et al.*, 1994).

Triparental matings were used to mobilize plasmids from *E. coli* to *P. aeruginosa* using the conjugation helper plasmid pRK2013, as described previously (Darzins & Chakrabarty, 1984).

DNA manipulations and nucleotide sequencing. General DNA manipulations were performed as described by Sambrook *et al.* (1989). The plasmid pRM812 (Table 1) contains a 9 kb *Hind*III fragment encompassing a region of the alginate gene cluster from *algD* to *algG* in phagemid pUC119. A *Sma*I-*Eco*RI digest of pRM812 yielded a 2.0 kb DNA fragment which was initially subcloned into pBluescript to form pAA1. DNA sequences were determined by the dideoxy chain-termination procedure (Sanger *et al.*, 1977). Both DNA stands were completely sequenced using fragments from pAA1 (see Fig. 1), subcloned into M13mp19, or following exonuclease III and nuclease S1 digestion of the pJBS633

derivatives, pAA26 or pAA22, which contain the entire *Sma*I-*Eco*RI fragment from pRM812 (Fig. 1) in opposite orientations. pAA26 carries a *Bam*HI-*Eco*RV fragment which was subcloned from pAA1. Construction of pAA22 is described later.

Construction of plasmids for expressing *algK*. Expression vectors pAA13 and pAA15 were constructed by subcloning of *algK* on a *Bam*HI-*Hind*III fragment from pAA1 into the vectors pT7-3 and pT7-5, respectively. pAA20, which carries the complete *algK* ORF, was constructed by PCR amplification, using pAA1 as a template and primers 1 (5'-GGTACCCGCATATGAAGATGCCCATC-3') and 2 (5'-GGGGATCCTCATAGGTTTCTGGCTCTTCTT-3'). The primers were synthesized such that the amplified fragment contained an *Nde*I site overlapping the translational start codon of the *algK* ORF, and also a *Bam*HI site at the 3' end (C-terminus) to facilitate cloning of the PCR product into the *Nde*I-*Bam*HI sites of pT7-7.

pAA23 was constructed by subcloning PCR-amplified *algK* together with the strong pT7-7 ribosome-binding site (RBS; from vector pAA20), using primers 2 and 3 (5'-CAACGATATCCCTCTAGAAAA-3'), into pMMB66(EH). The 5' end of primer 3 contains an *Eco*RV site to facilitate cloning of the PCR product into the *Sma*I and *Bam*HI sites of pMMB66(EH). The amplified product contains the ATG from the strong RBS of pT7-7 together with the immediate 44 upstream bases from this vector (Tabor, 1990).

Expression of AlgK in *E. coli* and *P. aeruginosa*. AlgK was expressed under the control of the T7 promoter in *E. coli* BL21(DE3)[pLysS] cells, as described by Studier & Moffatt (1986). Following overnight growth in LB broth at 37 °C, 50 µl of culture was inoculated into 5 ml aliquots of LB broth containing the appropriate antibiotics and incubated for a further 2 h at 37 °C. Aliquots (1 ml) of uninduced culture were harvested by microfugation and stored frozen until further analysis. The remaining culture was induced by the addition of 1 mM IPTG and 1 ml aliquots of induced culture were subsequently sampled at hourly intervals and stored until analysis. Conditions for expression of pMMB66 (EH) derivatives were essentially as described for pT7 vectors, with the exception that HB101 and PA8873 were used as the plasmid hosts.

In vivo labelling of AlgK with [³⁵S]methionine. The T7 expression system described by Tabor (1990) was used to preferentially radiolabel *algK* products expressed from pAA20. Cultures of BL21(DE3)[pLysS] containing pAA20 were grown to mid-exponential phase at 37 °C in LB broth, and 1 ml samples were collected by centrifugation, washed twice in M9 medium and resuspended in 1 ml M9 medium supplemented with glucose (0.4%, w/v), thiamin (0.04%, w/v), 18 common amino acids (lacking methionine and cysteine; each at 40 µg ml⁻¹), and appropriate antibiotics. Cells were incubated for a further 1 h and then IPTG was added (1 mM) to permit transcription from the T7 RNA polymerase gene. The incubation was continued for a further 45 min or alternatively, after 15 min rifampicin (200 µg ml⁻¹) was added and the incubation was continued for a further 30 min at 37 °C. The [³⁵S]methionine [10 µCi, 800 Ci (370 kBq) mmol⁻¹; Amersham] was added, and cells were incubated at 37 °C for a further 5, 10 or 20 min before harvesting.

In pulse-chase experiments, in which *algK* was expressed under the *tac* promoter in *P. aeruginosa* PA8873(pAA23), bacteria were grown for 18 h in LB broth at 37 °C. Cells from 0.75 ml culture were collected by centrifugation (5000 g,

Table 1. Bacterial strains, plasmids and phages

Strain/plasmid/ phage	Relevant characteristics†	Source/reference
Bacteria		
<i>E. coli</i>		
BL21(DE3)	F ⁻ <i>omp</i> ⁺ r _B ⁻ m _B ⁻ (DE3 is a λ lysogen bearing <i>lacI lacUV5 gene1</i>)	Studier <i>et al.</i> (1990)
HB101	F ⁻ <i>hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA13 ara-14 proA2 rpsL20</i> (Str ^R) <i>xyl-5 mtl-5 supE44 λ</i> ⁻	Maniatis <i>et al.</i> (1982)
JM101	<i>thi</i> Δ (<i>lac-proAB</i>) [F'- <i>traD36 proAB lacI</i> ^q Z Δ M15]	Yanisch-Perron <i>et al.</i> (1985)
<i>P. aeruginosa</i>		
PA8873	<i>alg-A, his-1</i>	Darzens & Chakrabarty (1984)
PAB	Constitutively secretes alginate	A. Linker
PABK	Carries a chromosomal <i>algK</i> ' : pBR325 insertion	This study
Plasmids		
pAA1	pBluescript derivative, containing region from <i>alg44</i> to <i>algE</i> on <i>SmaI-EcoRI</i> fragment	This study
pAA13	pT7-3 derivative, containing subcloned region from <i>alg44</i> to <i>algE</i> on <i>BamHI-HindIII</i> fragment from pAA1	This study
pAA15	pT7-5 derivative containing subcloned region from <i>alg44</i> to <i>algE</i> on <i>BamHI-HindIII</i> fragment from pAA1	This study
pAA20	pT7-7 derivative containing <i>NdeI-BamHI</i> fragment amplified from pAA1 by primers 1 and 2 encoding AlgK	This study
pAA22	pJBS633 derivative containing <i>EcoRV-BamHI</i> fragment amplified from pAA20 by primers 3 and 4 encoding AlgK	This study
pAA23	pMMB66(EH) derivative containing <i>EcoRV-BamHI</i> fragment amplified from pAA20 by primers 3 and 2 encoding AlgK	This study
pAA26	pJBS633 derivative subcloned region from <i>algE</i> to <i>alg44</i> on <i>BamHI-EcoRV</i> fragment from pAA1	This study
pAA31	pBR325 carrying a 410 bp <i>PstI</i> fragment of <i>algK</i>	This study
pAA32	pMMB66(EH) carrying a <i>SmaI-HindIII</i> <i>algKE</i> fragment	This study
pAA33	pAA32 carrying a <i>HindIII</i> <i>algA</i> fragment	This study
pAA35	pAA22 derivative expressing AlgK- β -lactamase fusion protein	This study
pMPG32	pJBS633 with the <i>PvuII</i> site replaced by a <i>SmaI</i> site	This study
pBluescript	<i>E. coli</i> cloning plasmid, ColE1 Amp ^R <i>lacZα</i>	Short <i>et al.</i> (1988)
pJBS633	Tet ^R Kan ^R ' <i>blaM</i>	Broome-Smith & Spratt (1986)
pLysS	Cml ^R , pACYC184 derivative containing T7 lysozyme gene	Studier <i>et al.</i> (1990)
pMMB66(EH)	Broad-host-range expression vector, Amp ^R <i>lacI</i> ^q <i>tac</i>	Fürste <i>et al.</i> (1986)
pRK2013	ColE1 Tra ⁺ -(RK2) Kan ^R	Figurski & Helinski (1979)
pRM812	pUC119 derivative containing 9 kb <i>HindIII</i> <i>algD-algG</i> fragment	Provided by R. Maharaj
pT7-3	<i>E. coli</i> expression vector, Amp ^R ColE1 <i>p</i> _{T7}	Tabor (1990)
pT7-5	<i>E. coli</i> expression vector, Amp ^R ColE1 <i>p</i> _{T7}	Tabor (1990)
pT7-7	<i>E. coli</i> expression vector, Amp ^R ColE1 <i>p</i> _{T7} and RBS	Tabor (1990)
Phages		
M13mp19	<i>E. coli</i> cloning vector, <i>lacZα lacI</i>	Yanisch-Perron <i>et al.</i> (1985)
R408	Helper phage, <i>gene2</i>	Russel <i>et al.</i> (1986)

† Alg⁻, non-mucoid; Tra⁺, transfer by conjugation; Amp^R, ampicillin resistance; Tet^R, tetracycline resistance; Kan^R, kanamycin resistance; Cml^R, chloramphenicol resistance; RBS, ribosome-binding site.

10 min), washed with M9 medium, resuspended in 3 ml M9 medium supplemented as before with glucose and thiamin and then incubated at 37 °C for 2 h. IPTG was added, followed by incubation for 30 min. [³⁵S]Methionine [10 μ Ci; 800 Ci (29.6 TBq) mmol⁻¹; Amersham] was added, and cells were incubated at 37 °C for 2 min. Unlabelled L-methionine (0.1 %, w/v) was then added, and samples were withdrawn at suitable time intervals. Samples were analysed by SDS-PAGE and autoradiography.

Construction of AlgK'- β -lactamase fusion proteins. pAA22 was used for construction of fusion proteins; it was prepared by amplification of *algK* from pAA20 with primer 3 and primer 4 (5'-CGGGATCCAGCTGTAGGCTTTCTGGC-TCTTCTT-3'). The amplified *algK* product contains the pT7-7-derived RBS at the 5' end and a *PvuII* restriction site at the 3' end (replacing the TGA stop codon), which is followed by a *BamHI* site downstream. The amplified fragment was cloned into the *EcoRV-BamHI* sites of pMPG32 (see below)

Fig. 1. The construction of vector pAA1 from pRM812 and the respective internal restriction sites present in this region are shown at the top. The sequencing strategy applied to the unsequenced region between *alg44* and *algE* is indicated. The flat-headed arrows show the extent and direction of the sequence obtained from specific M13mp19 subclones and the barbed arrows indicate sequence obtained following exonuclease III deletion of plasmids pAA22 and pAA26. The regions of DNA sequence reported previously (Chu *et al.*, 1991; Maharaj *et al.*, 1993) are indicated by black bars. pUC119 vector DNA of pRM812 is indicated by hatched shading.

to form pAA22. (pAA35 was constructed by deletion of a *PvuII*–*SmaI* fragment from pAA22.)

Unidirectional digestion of pAA22, using exonuclease III and S1 nuclease, was carried out as described by Henikoff (1987), with appropriate modifications described for pJBS633 (Broome-Smith & Spratt, 1986). Essentially, pAA22 was completely digested with *SphI* and *BamHI* to allow unidirectional digestion into the *algK* gene from the *BamHI* position by exonuclease III followed by S1 nuclease. The reaction products were ligated and transformed into *E. coli* JM101, selecting for kanamycin resistance. Recombinants which were predicted to have an 'in-frame' AlgK– β -lactamase fusion were identified by their ability to grow when 6 μ l of overnight culture was spotted onto agar containing 200 μ g ampicillin ml⁻¹ (Broome-Smith & Spratt, 1986).

The minimum inhibitory concentration (MIC) for single cells of *E. coli* JM101 containing pAA22-derived plasmids was subsequently determined by spotting 4 μ l of a 10⁻⁵ dilution of an overnight culture (about 40 bacteria) onto LB agar plates containing a range of concentrations of ampicillin. The MIC represented the lowest concentration of ampicillin that prevented growth of the bacterial colonies after overnight incubation at 37 °C (Broome-Smith & Spratt, 1986).

Dideoxy sequencing of *algK'*–*blaM* fusion junctions was carried out on single-stranded plasmid DNA, obtained from pJBS633 derivatives by infecting cells with helper phage R408 (Russel *et al.*, 1986), using the oligonucleotide 5'-CTGTGCACCCAACTGA-3', which primes from codons 14 to 18 of the mature form of TEM β -lactamase across the fusion

junction (Broome-Smith & Spratt, 1986). The pJBS633 used is a variant of that previously mentioned (Broome-Smith & Spratt, 1986) in which the linker sequence 5'-CCCGGGCCCCGGG-3', which contains a *SmaI* site, has been inserted within the *PvuII* site of the vector, forming pMPG32.

Insertional inactivation of *algK* and complementation studies. An *algK* fragment of approximately 460 bp was amplified from pAA22 using primer 7 (5'-CGCTGCAGGACCCGCGCAAGGC-3'), which contains a *PstI* site towards the 5' end, and primer 4, which primes the complementary strand from the *algK* TGA stop codon. A natural *PstI* site occurs in *algK* (nucleotides 1614–1619, Fig. 2) and therefore digestion of the PCR product with *PstI* gave rise to a 410 bp fragment which was subcloned into the corresponding site of the *blaM* gene of pBR325. HB101 cells carrying the resulting plasmid, pAA31, were triparentally mated with *P. aeruginosa* strain B (PAB) which constitutively secretes alginate. Single homologous recombinants were selected on minimal plates, containing 200 μ g tetracycline ml⁻¹, and plasmid integration into the chromosome was confirmed by Southern hybridization (Southern, 1975), by probing with a fragment of the *cml* gene from pBR325. The resulting isolates (PABK) were examined visually for alginate production on LB agar or on MAP medium, which promotes alginate production (Franklin *et al.*, 1994).

Complementation studies were carried out in PABK with plasmid-encoded *algKEA*. A *SmaI*–*HindIII* fragment which spans from the 3' end of *alg44* into *algG*, and contains the entire *algKE* region, was initially cloned from pRM812 into the broad-host-range vector pMMB66(EH), forming pAA32. The *HindIII* fragment from pAD4038 which carries the *algA* gene was then inserted into the corresponding site in pAA32. The resulting plasmid, pAA33, was mobilized into PABK by triparental mating, as before and exconjugants were selected on minimal agar containing tetracycline and ampicillin (each at 200 μ g ml⁻¹). Expression of *algKEA* was induced from the *ptac* promoter of pAA33 on LB plates or MAP plates, with 1 mM IPTG, and colonies were examined for alginate production.

SDS-PAGE and immunoblotting. Overnight cultures bearing the required plasmid were harvested and electrophoresed in the presence of SDS (Laemmli, 1970). Following electrophoresis, proteins were either stained with Coomassie brilliant blue, visualized by autoradiography, or transferred by electroblotting to a nitrocellulose membrane (Towbin *et al.*, 1979). Immunoblotting procedures were carried out with anti- β -lactamase antisera (5 prime to 3 prime Inc.) and alkaline-phosphatase-conjugated anti-rabbit IgG (whole molecule; Sigma). The detection of bound antisera by alkaline phosphatase staining was carried out as described by Knecht & Dimond (1984).

RESULTS

Identification of a new gene, *algK*, within the 34 min alginate gene cluster of *P. aeruginosa*

The uncharacterized region upstream of *algE* (which encodes the alginate-permeable outer-membrane pore) within the 34 min alginate gene cluster of *P. aeruginosa* was selected for sequence analysis. Studies were confined to a *SmaI*–*EcoRI* fragment (approximately 2 kb) which contains the entire uncharacterized segment between *alg44* and *algE*, together with some flanking DNA (see Fig. 1). This fragment was isolated from pRM812 and

SmaI
 cccgggtgaacttcacggtcgccggcgacggtcacaacgcagcggccgcatcgtaacac 60
 (alg44)G *
 cgccggtggagcggcagcctgtctcgcgaatccgcgtgcagatccagcccgaccagcc 120
 gctcgacgccagtcacgcccgcgcggcgaggtcagcatcgccggcctgcccgcggcg 180
 caccctgtgaacaagcgctgaccccgccacgcgctcgctgacgggtaccgacccatga 240
 M K 2
 agatgccatcctcctcctcctcctcctcctcctcctcctcctcctcctcctcctcctc 300
 M P I L P P L P L A S R H L L L A S A I 22
 tcgcccggccgcccgtgctgcgggactgcggcagcagcctcggccaggagggccctgg 360
 A L A A G C A G L P D Q R L A Q E A L E 42
 agcggcgacactcgcacccgcccagagcaactaccagccctggccgcatgggttacg 420
 R G D L A T A Q S N Y Q A L A A M G Y A 62
 ccgacgccaggtcgccctcgcgcacatcgaggtgcgacggcgacagtcgcccagcagg 480
 D A Q V G L A D M Q V A S G D S A Q Q A 82
 ccaaggccgaagaagctctaccgagggccgcgacgctcgcggcgcccgccgcccgcgg 540
 K A E K L Y R E A A Q T S P R A R A R L 102
 ttggcgaagtggctggcgccgcaagcggcgccgacgagccgacgagccgagggcgagc 600
 G K W L A A K P G A S D A E H R E A E R 122
 gctgctcagcagggcctcgaacaggggcgaggacagtcgcccgtggcgctcatctgtc 660
 L L S Q A F E L D G E D S A L V P L I V L 142
 tctacctgcagtcacgcagtcctggcgggaactccagcagcagcagcagtcagcagcag 720
 Y L Q Y P Q S W P E I D P Q Q R I D Q W 162
 ggcgccgctgagtcgcccagggcgacgtggcgagatcatcctctaccgacccaggg 780
 R A R G L P Q A D L A Q I I L Y R T Q G 182
 gcacctacgcccagcactggggcgagatcgagcaggtctgcagcgcctggctgcggcgca 840
 T Y A Q H L G E I E Q V C Q R R W L R R M 202
 tggatgtgctggtcgaactcgcccggtgaactccagatcgagggcaacgagcgaagcag 900
 D V C W Y E L A T V Y Q M Q G N A E K Q 222
 agaaggtcctgctcgaacagctcgcccgccctacaagccggcgccgggtgcccggcgagc 960
 K V L L E Q L R A A Y K A G R V P G E R 242
 gggctgactcgggtggcggggtgctcgcgcagcggcgaaactcggccagccgaccccgaga 1020
 V D S V A G V L A D G P E L G Q L P D P Q T 262
 ccgcccagggcgctgctggaagagatcgcccgagctaccgccggcctgggtcagcctgg 1080
 A Q A L L E E I A P S Y P A A W V S L A 282
 ccaagctgctctagactatcccgaccagggcgacctggaaagatgctcggtcactctga 1140
 K L L Y D Y P D Q G D L E K M L G Y L K 302
 agaacgccaggacgcgcggccagccagcgcgaactgctcctcggggcgccctcactacg 1200
 N A Q D A A Q P R A E L L L G R L Y Y D 322
 accgcaagtgggcgccgagcccgccgcaagcgccgagcgccgacccgctcgaagccggcg 1260
 G K W A P Q D P R K A E R H L L K A A A 342
 ccagcgaacccagcccaactactacctggggcgagatctaccgcgcggcctcctcggca 1320
 S E P Q A N Y Y L G Q I Y R R G F L G K 362
 aggtctctccgcagagggcgctgcagcactgactcctcgcgcggcgccggcgccagccca 1380
 V Y P Q K A V D H L I L A A R A G Q A S 382
 gcgcccagatggccctggccagctctggtcgccggccgcccagcagccagccggcg 1440
 A D M A L A Q L W S Q G R G I Q P N R V 402
 tcaacgctactgctcctcgccagctcgccgctgcagcagcaggtaccgacggccagcgacc 1500
 N A Y V F G Q L A V Q Q Q V P Q A S D L 422
 tgcctgcagcatcgaaagcgcaactcgccggcgccgcaacgcagcagcgccagcagcttcg 1560
 L G Q I E A Q L P P A E R S Q A Q Q L L 442
 tcaagcgcaacaacagagccggcgcaacaaactggcagggccacgctcagcgtcgagca 1620
 K R E Q Q S R G N N W Q A T V S L L Q S 462
 gccaggactcgccgatcaacgaagaagagcctatga
 Q D S P I N E E E P E S L *
 M (alg8)

Fig. 2. Nucleic acid sequence and predicted amino acid sequence of the *algK* ORF. The sequence is shown from the *SmaI* site (lower-case and underlined) of the C-terminal end of *alg44* to the translation initiation codon of *algE*. Amino acids are shown by the standard one-letter abbreviations beneath the nucleotide sequence. Termination codons are designated by asterisks. The proposed cleavage signal recognized by leader peptidase II is underlined and the adjacent cysteine (C) residue required for lipid attachment is boxed.

was then subcloned into pBluescript to form vector pAA1. Sequence analysis revealed a single ORF (Fig. 2) of 1425 bp, in the same transcriptional orientation as the other genes of the operon, which encoded a putative protein of 475 amino acids with a predicted molecular mass of 52470 Da. We have named this gene *algK*. The ORF starts at an ATG codon 227 bp downstream from the stop codon of *alg44* (Maharaj *et al.*, 1993) and terminates at a TGA stop codon, which overlaps with the ATG translational start codon of *algE* (Chu *et al.*, 1991), suggesting that these two genes are translationally coupled. Analysis of codon usage showed a high G + C content (approximately 70 mol%) with a particularly high G + C bias in the third position (93 mol%), which

is a typical characteristic of *P. aeruginosa* DNA (Minton *et al.*, 1984). The inferred amino acid sequence of AlgK predicted that the protein contained 46%, 31%, 11% and 11% non-polar, hydrophilic, acidic and basic residues, respectively. Overall the protein has a predicted isoelectric point of 5.64. The AlgK amino acid sequence was used to perform a homology search using the GenBank database, but no significant similarities were identified. We also observed that the amino acid sequence of AlgK contained a putative signal peptide at the N-terminus (underlined in Fig. 2). This signal sequence contained the consensus pattern for cleavage and lipidation, associated with lipoproteins which are processed by leader peptidase II (Hayashi *et al.*, 1984; Pugsley, 1993) with the cleavage site (Ala Gly¹Cys) at the 3' end of the signal sequence. This suggests that AlgK may be initially synthesized as a precursor (pre-AlgK) of about 53 kDa and then processed by signal peptidase II to a 50 kDa mature, membrane-anchored, lipoprotein.

Expression of *algK* in *E. coli* and *P. aeruginosa*

In order to substantiate our findings, we examined whether the region between *algE* and *alg44* produced a polypeptide of the predicted molecular size. The initial expression studies of *algK* were performed using the vectors pT7-3 and pT7-5. A *Bam*HI–*Hind*III DNA fragment from pAA1 (containing the entire *SmaI*–*Eco*RI fragment from pRM812) was therefore subcloned into pT7-3 and pT7-5 to form plasmids pAA13 and pAA15, respectively. These plasmids were transformed into *E. coli* BL21(DE3)[pLysS] and T7 RNA polymerase was induced with IPTG (Sambrook *et al.*, 1989). Protein samples from BL21(DE3)[pLysS, pAA13] or BL21(DE3)[pLysS, pAA15] were separated by SDS-PAGE and stained with Coomassie brilliant blue. β -Lactamase was over-expressed from pAA13 but no novel protein bands were seen from samples containing pAA15 (data not shown). This suggested that the T7 RNA polymerase was functioning properly but that AlgK might be translated inefficiently.

In order to evaluate this possibility and to confirm that the ORF was correctly determined, *algK* was subcloned into vector pT7-7 in order to increase the efficiency of translation. pT7-7 differs from pT7-3 and pT7-5 in having a strong RBS containing an *Nde*I sequence, and a translational start site to enhance expression of adjacent coding information. The *algK* gene was therefore amplified using PCR technology, with pAA1 as the DNA template, and inserted into pT7-7. Synthetic oligonucleotide primers were designed, such that the upstream primer (primer 1) contained an *Nde*I site encompassing the predicted ATG translational start codon of *algK* and the downstream primer (primer 2) contained a *Bam*HI site adjacent to the complementary sequence of the *algK* TGA stop codon. The amplified fragment was cloned into the corresponding sites of pT7-7 (forming pAA20), placing *algK* under the inducible T7 promoter in such a manner that the complete *algK* ORF was expressed from the vector-encoded RBS. Cultures of BL21(DE3)[pLysS, pAA20] were induced as

Fig. 3. SDS-PAGE analysis of the expression of AlgK from pAA23 in *P. aeruginosa* PA8873, and *E. coli* HB101. Lanes 1 and 2 show HB101(pMMB66 EH) treated with IPTG for 4 h to induce expression from the *tac* promoter; lanes 3–7 contain HB101(pAA23) treated with IPTG for 0, 1, 2, 3 and 4 h, respectively. Lanes 8 and 9 contain PA8873 and PA8873(pMMB66 EH) treated with IPTG for 4 h; lanes 10–14 contain PA8873(pAA23) treated with IPTG for 0, 1, 2, 3 and 4 h, respectively. Cells were lysed, and proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Positions of molecular size markers are shown on the left; the protein expressed from *algK* is indicated by the arrow.

before and subjected to SDS-PAGE. A broad band of 50–53 kDa was observed which was not found in the control samples. This protein band corresponded well with the size of AlgK predicted from the DNA sequence (data not shown).

To determine whether AlgK is expressed in *P. aeruginosa* as it is in *E. coli* the *algK* gene was cloned into the broad-host-range expression vector pMMB66(EH). Since the previous experiments predicted that translation may be inefficient, the RBS of pT7-7 was incorporated into the amplified fragment which was cloned into pMMB66(EH). A further synthetic oligonucleotide was designed (primer 3) which primes upstream of both the RBS and the ATG start of *algK* in pAA20 and also contains an *EcoRV* restriction site towards the 5' end of the primer. PCR with primer 3 and primer 2 therefore results in the amplification of the *algK* gene, containing the strong RBS from pT7-7 with an *EcoRV* site at the 5' end, and a *Bam*HI site at the 3' end. This DNA fragment was cloned into the *Sma*I–*Bam*HI restriction sites of pMMB66(EH) to form plasmid pAA23. This placed *algK* under the control of the inducible *ptac* promoter. Overexpression was carried out in both *E. coli* HB101 and *P. aeruginosa* PA8873, following the induction protocol used previously with the pT7 vectors. In both *E. coli* and *P. aeruginosa*, an AlgK polypeptide was observed which migrated as seen previously with the pT7 system as a broad band at approximately 50–53 kDa (Fig. 3, lanes 4–7 and 11–14, respectively). This indicated that production of AlgK occurs in a similar manner in both organisms.

In vivo processing of AlgK

Analysis of the nucleotide sequence of *algK* suggested that the protein product may undergo processing by leader peptidase II, resulting in removal of a signal

Fig. 4. Autoradiogram of proteins expressed from the T7 promoter in *E. coli* strains BL21(DE3)[pLysS]. Cultures were labelled with [35 S]methionine for various periods, denatured in LSB (Laemmli, 1970) and separated by SDS-PAGE. Samples were as follows: lane 1, BL21(DE3)[pLysS, pT7-7] treated with IPTG and labelled with [35 S]methionine for 5 min; lanes 2–4, BL21(DE3)[pLysS, pAA20] treated with IPTG and labelled with [35 S]methionine for 1, 5 and 20 min respectively; lanes 5–7, BL21(DE3)[pLysS, pAA20] treated with IPTG and rifampicin and labelled with [35 S]methionine for 1, 5, and 20 min, respectively; lane 8, BL21(DE3)[pLysS, pT7-7] treated with IPTG and rifampicin and labelled with [35 S]methionine for 5 min. Putative AlgK preprotein and mature protein are arrowed. The positions of molecular size markers are shown on the left.

sequence and lipid attachment at the N-terminus of the mature protein. This may explain why a broad band was observed following SDS-PAGE (Fig. 3). In order to examine whether post-translational processing of AlgK was occurring, a series of radiolabelling experiments was performed. Labelling of BL21(DE3)[pLysS, pAA20] with [35 S]methionine revealed two polypeptides of approximately 53 and 50 kDa (Fig. 4, lanes 2–4 and 5–7)

Fig. 5. Autoradiogram of pulse-labelled proteins in *P. aeruginosa* cells expressing *algK*. Cells were labelled with [35 S]methionine and then the decay in their proteins was monitored for intervals between 0 and 30 min. Cells were lysed and proteins were separated by SDS-PAGE. Lanes 1, 2, and 3 show PA8873(pMMB66 EH) labelled with [35 S]methionine for 2 min and then chased for 0, 5 and 30 min, respectively, with unlabelled methionine. Lanes 4, 5, 6, 7 and 8 show PA8873(pAA23) labelled with [35 S]methionine for 2 min and then chased for 0, 3, 5, 15 and 30 min, respectively, with unlabelled methionine. The positions of molecular size markers are shown on the left. Pre-AlgK and AlgK indicate the precursor and mature proteins expressed by *algK*, respectively.

that were not observed in the control samples (Fig. 4, lanes 1 and 8). The size of these correlated well with the inferred sequence of pre-AlgK and mature AlgK.

To provide further evidence for processing, a pulse-chase analysis was carried out in *P. aeruginosa*. Cultures of PA8873(pAA23) were initially labelled with [35 S]methionine and then incubated in an excess of non-radioactive L-methionine to establish the processing pattern of newly synthesized proteins. Samples were withdrawn at various intervals and analysed by SDS-PAGE and autoradiography (Fig. 5). The 53 kDa pre-AlgK band was observed at the initial (2 min) time point (Fig. 5, lane 4) and then gradually faded in intensity during the chase period. This decrease correlated with a concomitant increase in intensity of the 50 kDa protein (Fig. 5, lanes 5–8). Overall, this pattern suggested that

Fig. 6. Construction of pAA35 and the pAA22 series. The *algK* gene was amplified and subcloned into pMPG32 between the *EcoRV* and *BamHI* sites. A directed, in-frame fusion between the full-length *algK* gene and *blaM* was constructed (pAA35) by restriction with *PvuII* and *SmaI*, followed by ligation. A random series of fusions between the coding region for mature TEM β -lactamase and fragments of the *algK* gene was produced by exonuclease deletion from the 3' end of *algK*, as described in the text. *algK'*-*blaM* represents an in-frame fusion between part of the *algK* gene and the coding region for the mature form of TEM β -lactamase.

AlgK is produced as a preprotein which is subsequently cleaved in both *E. coli* and *P. aeruginosa*, in a manner consistent with processing by signal peptidase II, to give a mature polypeptide of approximately 50 kDa.

Analysis of the subcellular location of AlgK

Sequence analysis and the above studies provided evidence for the processing and possible secretion of AlgK. In order to confirm this interpretation, the subcellular location of AlgK was examined by fusing N-terminal fragments of AlgK to mature β -lactamase (Fig. 6). Fusion of mature β -lactamase (which lacks its native signal sequence) to secreted proteins or to domains of

membrane proteins that are normally orientated on the periplasmic side of the cytoplasmic membrane generally results in the translocation of this enzyme to the periplasm and thereby provides individual cells of *E. coli* with a high degree of resistance to ampicillin (Broome-Smith & Spratt, 1986). Fusion to a region of polypeptide which does not normally cross the cytoplasmic membrane fails to provide individual cells with resistance to ampicillin as the cytoplasmic β -lactamase is inappropriately located to protect the penicillin-binding proteins. In this system, single-cell resistance must be assessed, since multicellular droplets of bacteria which express in-frame fusions can normally tolerate 200 μ g ampicillin ml^{-1} independently of the subcellular location of the fusion polypeptide. This results from cross-protection arising from limited cell lysis and enzyme release.

In the present study, a derivative of the vector pJBS633, pMPG32 (Broome-Smith & Spratt, 1986), was used to construct in-frame fusions between the coding region of mature TEM β -lactamase and that of the *algK* gene which was partially deleted from the 3' end. Initially, *algK* was cloned into pMPG32 to form plasmid pAA22. The *algK* fragment was amplified by PCR using pAA20 as the DNA source and primers 3 and 4. Primer 3 was used to allow amplification of *algK* together with an efficient (pT7-7) RBS. Primer 4 contains *Bam*HI and *Pvu*II restriction sites at the 5' end of the oligonucleotide and primes immediately downstream and overlapping the complementary sequence of the *algK* TGA stop codon. The resulting PCR product (*algK* with flanking *Eco*RV and *Bam*HI restriction sites) was cloned into the respective sites of pMPG32. This allows the retention of the tetracycline promoter of pJBS633 which mediates expression of the *algK'*-*blaM* fusions.

Initially a directed translational fusion was generated by digesting pAA22 with *Pvu*II and *Sma*I, followed by religation. This resulted in plasmid pAA35, in which the entire *algK* gene was fused in-frame to *'blaM'*. Additionally, plasmid pAA22 was digested with *Bam*HI and *Sph*I and then treated with exonuclease III for varying periods, followed by S1 nuclease, to generate a unidirectional set of nested deletions in the 3' region of *algK*. The samples were then treated with Klenow polymerase to produce 'blunt ends' and digested with *Sma*I prior to religation. Kanamycin-resistant, tetracycline-sensitive transformants that expressed putative 'in-frame' AlgK- β -lactamase fusions were identified by their ability to grow from multicellular droplets when spotted onto LB-agar containing 200 μ g ampicillin ml^{-1} . Single-stranded plasmid DNA was subsequently prepared from constructs with 'in-frame' fusions and the positions of the *algK'*-*blaM* fusion junctions were determined by sequencing (Table 2).

The single-cell MICs were then determined and were used to predict the subcellular locations of the β -lactamase moiety of each of the fusions (Broome-Smith & Spratt, 1986). From Table 2 it can be seen that fusion proteins which contained 42 or more residues of AlgK

Table 2. In-frame junctions of each *algK'*-*blaM* fusion as determined by nucleotide sequencing

The MIC of ampicillin required to lyse single cells of JM101 expressing individual fusion proteins is indicated and the position of the β -lactamase moiety of each fusion as inferred from the single cell MIC is shown alongside.

Position of fusion junction	MIC of ampicillin ($\mu\text{g ml}^{-1}$)	Proposed subcellular location of domains
Pro ⁷	< 5	Cytoplasmic
Glu ⁴²	> 800	Periplasmic
Gln ⁵⁰	> 800	Periplasmic
Gln ⁸⁰	> 800	Periplasmic
Lys ⁸⁶	> 800	Periplasmic
Ser ¹²⁵	> 800	Periplasmic
Leu ²⁸⁴	> 800	Periplasmic
Gln ²⁹¹	> 800	Periplasmic
Ala ³⁸⁸	> 800	Periplasmic
AlgK ⁴⁷⁶	> 800	Periplasmic

conferred substantial ampicillin resistance upon single cells of *E. coli*, suggesting that the β -lactamase moiety of each of these fusions is located in the periplasm. This result predicts that the carboxy terminus of AlgK, from residues 42 onwards, is located on the periplasmic side of the cytoplasmic membrane. In contrast, *E. coli* cells expressing fusion protein with only seven residues of AlgK showed no increase in single-cell MIC, indicating that the β -lactamase moiety of this fusion protein was not translocated to the periplasm. The latter cells did, however, produce enzymically active fusion proteins since they could withstand 200 μ g ampicillin ml^{-1} when plated as multicellular droplets. These data are consistent with the production of an AlgK pre-protein which is secreted into the periplasm together with the removal of a signal sequence.

Immunodetection of AlgK- β -lactamase fusion proteins produced in *E. coli*

As further evidence for the continuity of the *algK* ORF and also that the interpretation of the MIC data was correct, the in-frame AlgK- β -lactamase fusion proteins were examined by immunoblotting, using antisera against TEM- β -lactamase and an anti-IgG antibody-alkaline phosphatase detection system. In each case, fusion proteins which contained 42 or more residues of AlgK were observed to migrate to positions approximately 3 kDa smaller than would be predicted from the molecular mass of the unprocessed fusion protein (Fig. 7). In contrast, the fusion containing only seven amino acids from the N-terminus of AlgK ran as predicted. This result is consistent with the cleavage and removal of a signal peptide within the first 42 amino acids of

Fig. 7. Detection of AlgK and truncated AlgK- β -lactamase fusion proteins. Cultures of strain JM101, containing pAA35 or pAA22 derivatives carrying in-frame *algK'*-*blaM* translational fusions, were grown overnight. Cells were harvested, denatured with sample buffer (Laemmli, 1970) and equal loadings were run on a 10% SDS-polyacrylamide gel. The gel was immunoblotted using anti- β -lactamase antibodies, followed by detection with alkaline phosphate-conjugated goat anti-rabbit IgG. The positions of molecular size markers are indicated on the left.

AlgK. In samples containing pAA35 (Fig. 7, lane 1), two high-molecular-mass AlgK- β -lactamase fusion proteins were clearly visible, with the upper band being approximately 3 kDa larger than the lower band. This suggests that both the unprocessed and the mature forms of the fusion protein are present.

Insertional inactivation of *algK* results in loss of alginate production

To evaluate whether *algK* was required for alginate production the chromosomal *algK* gene was disrupted by insertional inactivation, using pBR325 as a suicide vector. An *algK* fragment of 460 bp was amplified from pAA22 using primer 7, which contains a *Pst*I site towards the 5' end, and primer 4, and then digested with *Pst*I before subcloning into the corresponding site of pBR325. Single homologous recombinants were selected on minimal plates, containing 200 μ g tetracycline ml⁻¹, following transfer of pAA31 into *P. aeruginosa* strain B (PAB), which constitutively secretes alginate, by triparental mating. The resulting isolates (PABK) were non-mucoid when plated on LB agar or MAP medium, which promotes alginate production (Franklin *et al.*, 1994).

Previous studies (Chitnis & Ohman, 1993) indicated that insertional inactivation of genes in the alginate gene cluster may cause polar effects on downstream gene expression and result in loss of mucoidy. Since the *algE* and *algA* genes which lie downstream from *algK* are essential for alginate production (Chu *et al.*, 1991; Darzins *et al.*, 1986), the possibility that absence of mucoidy observed with PABK reflected a polar effect of the *algK* disruption was explored. A *Sma*I-*Hind*III fragment which spans from the 3' end of *alg44* into *algG*, and contains the entire *algKE* region, was therefore cloned from pRM812 into the broad-host-range vector pMMB66(EH), forming pAA32. The *Hind*III fragment from pAD4038 which carries the *algA* gene was then inserted into the corresponding site in pAA32. The resulting plasmid, pAA33, was mobilized into PABK by triparental mating and exconjugants were selected on minimal agar containing tetracycline and ampicillin. Expression of *algKEA* was induced from the *ptac* promoter of pAA33 on LB plates or MAP plates containing IPTG. However, no evidence for restoration of a mucoid phenotype was observed.

DISCUSSION

The present study describes the cloning and sequencing of a new gene, *algK*. This gene is found in a 2 kb *Sma*I-*Eco*RI fragment located between *alg44* and *algE*. The cloned DNA contains a 1425 bp ORF that codes for a 475 amino acid hydrophilic protein of 52470 Da and is transcribed in the same orientation as the other genes of the operon.

Expression of *algK* from its natural RBS in *E. coli*, with vector pT7-5, did not result in detectable AlgK polypeptide. However, when a strong RBS was utilized for expression, a broad band of 50–53 kDa was observed, suggesting that the RBS of *algK* is relatively weak. However, it cannot be excluded that the short intergenic region (227 bp) which lies between *alg44* and *algK* may influence polypeptide production. Sequence analysis also revealed overlap between the translational stop codon of *algK* and the start codon of *algE* and suggests that translational coupling may occur. It seems likely therefore that the product of *algE* would also be produced in relatively low abundance. This is in agreement with previous studies on AlgE (Grabert *et al.*, 1990) and therefore may indicate that the intergenic region or other factors play a role in controlling the level of AlgK production.

Analysis of the putative amino acid sequence revealed a consensus lipoprotein cleavage site and predicted that a pre-protein of approximately 53 kDa is synthesized which would undergo removal of an N-terminal signal sequence, together with lipidation of the mature polypeptide (approximately 50 kDa) and targeting of the protein outwith the cytoplasm. However, high-level expression of the AlgK protein in *E. coli* or *P. aeruginosa* revealed only a single, broad protein band of 50–53 kDa (Fig. 3). [³⁵S]Methionine labelling of the protein product

(Figs 4 and 5) provided evidence that post-translational cleavage of AlgK did indeed occur and suggested that the single broad band observed previously represented a mixture of the unprocessed and processed forms of AlgK.

Construction of AlgK- β -lactamase fusion proteins and detection by immunoblotting predicted a periplasmic orientation for the AlgK protein and provided further evidence for the presence of a cleavable signal sequence within the N-terminal 42 amino acids and also, for processing of larger fusion proteins (Fig. 7). However, although the AlgK- β -lactamase fusion survey predicted that mature AlgK was entirely periplasmic, it gave no clue as to whether this lipoprotein was anchored to the outer face of the inner membrane or the inner face of the outer membrane. However, the studies of Grabert *et al.* (1990) suggest that AlgK is most likely anchored to the inner membrane. In an extensive survey on mucoid and non-mucoid revertants of *P. aeruginosa* strains, they found one novel protein band in the outer membrane of mucoid strains. Sequencing of *algE* (Chu *et al.*, 1991) showed that this novel 54 kDa outer-membrane protein corresponded to the AlgE protein. AlgK was not detected in the outer membrane (Grabert *et al.*, 1990), suggesting that AlgK is anchored to the cytoplasmic membrane.

Immediately upstream of *algK* lie the *alg44* and *alg8* genes. The amino acid sequences of the proteins encoded by these genes (as predicted from their nucleotide sequences) previously led to the suggestion that they have a cytoplasmic membrane orientation and play a role in mannuronate polymerization (Maharaj *et al.*, 1993). More recently, hydrophobic cluster analysis (Saxena *et al.*, 1995) revealed that Alg8 was similar in overall structure and thus possibly function to the putative polymerization region of AcsAB, the *Acetobacter xylinum* cellulose synthase, and thus belonged to a family of β -glycosyltransferases. No inner-membrane transport protein for cellulose synthesis has been identified but the model proposed by Ross *et al.* (1991) suggests that the cellulose synthase protein may be associated with both polymerization and transport of the polymer across the inner membrane. Whether such a dual role exists for the other proposed members of this family, chitin synthase of *Saccharomyces cerevisiae* (Bulawa *et al.*, 1986), hyaluronic acid synthase of group A streptococci (Dougherty & van de Rijn, 1994) and NodC of *Azorhizobium caulinodans* (Geremia *et al.*, 1994), or whether additional components are required which may mediate polymer transport, remains to be determined.

The location of genes encoding the periplasmic-oriented AlgK protein and the outer-membrane alginate-permeable pore (AlgE) directly downstream of genes encoding a putative mannuronate polymerase/cytoplasmic polymannuronate transporter is remarkably similar to the genetic arrangements of other polysaccharide export systems from other organisms. These include the *acs* locus (Saxena *et al.*, 1994), the cellulose synthase and transport operon of *A. xylinum*,

the *bex* (b capsule expression) operon of *Haemophilus influenza* (Kroll *et al.*, 1990) and the *ctr* (capsule polysaccharide transport region) operon of *Neisseria meningitidis* (Frosch *et al.*, 1991). In each case, these operons encode a single periplasmic protein component which is considered to be associated with the periplasmic transport of the polysaccharide. It therefore seems highly likely that AlgK may also function similarly, and as such may act as a periplasmic facilitator to channel alginate through the periplasmic space to the outer-membrane pore of *P. aeruginosa*, AlgE.

In order to gain preliminary information on the function of AlgK and also to evaluate its importance for alginate production, we examined the effect of gene disruption on exopolysaccharide synthesis. Disruption of *algK* by insertion of a selectable cassette which conveys antibiotic synthesis was observed to result in loss of mucoidy. However, the possibility that this approach also abolished expression of an essential downstream gene of the operon cannot be excluded. Efforts to restore mucoidy by simultaneous complementation with *algK*, *algE* and *algA* have been unsuccessful; however, the studies of Darzins *et al.* (1985) on *alg60* have suggested that the latter gene is also necessary for alginate production and this may explain our observations.

At present, the most likely role of AlgK would therefore seem to be to act as a facilitator to translocate alginate across the periplasm. Whether such a facilitator function would require that the alginate remains accessible for acetylation during this process, or whether this modification may occur prior to translocation across the periplasm, remains to be determined.

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REFERENCES

- Boyd, A., Ghosh, M., May, T. B., Shinabarger, D., Keogh, R. & Chakrabarty, A. M. (1993). Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene* 131, 1–8.
- Broome-Smith, J. K. & Spratt, B. G. (1986). A vector for the construction of translational fusions to TEM β -lactamase and the analysis of protein export signals and membrane topology. *Gene* 49, 341–349.
- Bulawa, C. E., Slater, M., Cabib, E., Auyoung, J., Sburlati, A. & Adair, W. L. (1986). The *S. cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. *Cell* 46, 213–225.
- Chitnis, C. E. & Ohman, D. E. (1993). Genetic analysis of the alginate biosynthetic cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol Microbiol* 8, 583–590.
- Chu, L., May, T. B., Chakrabarty, A. M. & Misra, T. K. (1991). Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. *Gene* 107, 1–10.
- Darzins, A. & Chakrabarty, A. M. (1984). Cloning of genes

- controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. *J Bacteriol* 166, 9–18.
- Darzins, A., Wang, S.-K., Vanags, R. I. & Chakrabarty, A. M. (1985). Clustering of mutations affecting alginic acid biosynthesis in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* 167, 516–524.
- Deretic, V., Gill, G. F. & Chakrabarty, A. M. (1987). *Pseudomonas aeruginosa* infection and cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. *Nucleic Acids Res* 15, 4567–4581.
- Dougherty, B. A. & van de Rijn, I. (1994). Molecular characterisation of *hasA* from an operon required for hyaluronic acid synthesis in group A streptococci. *J Biol Chem* 269, 169–175.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid pRK2 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci USA* 76, 1648–1652.
- Franklin, M. J., Chitnis, C. E., Gacesa, P., Sonesson, A., White, D. C. & Ohman, D. E. (1994). *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. *J Bacteriol* 176, 1821–1830.
- Frosch, M., Edwards, V., Bousset, K., Kraube, B. & Weisgerber, C. (1991). Evidence for a common molecular origin of the capsule gene loci in Gram-negative bacterial expressing group II capsular polysaccharides. *Mol Microbiol* 5, 1251–1263.
- Fürst, J. P., Pansegrau, W., Frank, R., Blöcker, H., Sholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in multi-host-range *tacP* expression vector. *Gene* 48, 119–131.
- Geremia, R. A., Mergaert, P., Geelen, D., Vanmontagu, M. & Holsters, M. (1994). The NodC protein of *Azorhizobium caulinodans* is an N-acetylglucosaminyltransferase. *Proc Natl Acad Sci USA* 91, 2669–2673.
- Grabert, E., Wingender, J. & Winkler, U. K. (1990). An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 68, 83–88.
- Hayashi, S., Chang, S.-Y., Chang, S. & Wu, H. C. (1984). Modification and processing of *Bacillus licheniformis* prepenicillinase in *Escherichia coli*. Fate of mutant penicillinase lacking a lipoprotein modification site. *J Biol Chem* 259, 10448–10454.
- Henikoff, S. (1987). Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol* 155, 156–165.
- Knecht, D. A. & Dimond, R. L. (1984). Visualisation of antigenic proteins on Western Blots. *Anal Biochem* 136, 180–184.
- Kroll, J. S., Loynds, B., Brophy, L. N. & Moxon, E. R. (1990). The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. *Mol Microbiol* 4, 1853–1862.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lam, J., Chan, R. & Costerton, J. W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun* 28, 546–556.
- Maharaj, R., May, T. B., Wang, S.-K. & Chakrabarty, A. M. (1993). Sequence of *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. *Gene* 136, 267–269.
- Maniatis, T., Fritsch, E. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- May, T. B. & Chakrabarty, A. M. (1994). *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. *Trends Microbiol* 2, 151–156.
- Minton, N. P., Atkinson, T., Brunton, C. J., Sherwood, R. F. (1984). The complete nucleotide sequence of the *Pseudomonas* gene coding for carboxypeptidase-G2. *Gene* 31, 31–38.
- Pugsley, A. P. (1993). The complete general secretory pathway in Gram-negative bacteria. *Microbiol Rev* 57, 50–108.
- Rehm, B. H. A., Boheim, G., Tommassen, J. & Winkler, U. K. (1994). Overexpression of *algE* in *Escherichia coli*: subcellular localisation, purification and ion channel properties. *J Bacteriol* 176, 5639–5647.
- Ross, P., Mayer, R. & Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. *Microbiol Rev* 55, 35–38.
- Russel, M., Kidel, S. & Kelley, M. R. (1986). An improved filamentous helper phage for sequencing single stranded plasmid DNA. *Gene* 45, 333–338.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitions. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Saxena, I. M., Kudlicka, K., Okuda, K. & Brown, R. M. (1994). Characterisation of genes in the cellulose synthesising operon (*acs* operon) of *Acetobacter xylinum*: implications for cellulose crystallisation. *J Bacteriol* 176, 5735–5752.
- Saxena, I. M., Brown, M. B., Fevre, M., Geremia, R. A. & Henrissat, B. (1995). Multidomain architecture of β -glycosyl transferases: implications for mechanisms of action. *J Bacteriol* 177, 1419–1424.
- Schiller, N. L., Monday, S. R., Boyd, C. M., Keen, N. T. & Ohman, D. E. (1993). Characterisation of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing and expression in *Escherichia coli*. *J Bacteriol* 175, 4780–4789.
- Shinabarger, D., Berry, A., May, T. B., Rothmel, R., Fialho, A. & Chakrabarty, A. M. (1991). Purification and characterisation of phosphomannose isomerase guanosine diphospho-D-mannose pyrophosphorylase. *J Biol Chem* 266, 2080–2088.
- Shinabarger, D., May, T. B., Boyd, A., Ghosh, M. & Chakrabarty, A. M. (1993). Nucleotide sequence and expression of the *Pseudomonas aeruginosa* *algF* gene controlling acetylation of alginate. *Mol Microbiol* 9, 1027–1035.
- Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988). λ ZAP: a bacteriophage λ expression vector with *in-vivo* excision properties. *Nucleic Acids Res* 16, 7583–7600.
- Skjåk-Bræk, G., Grasdalen, H. & Larsen, B. (1986). Monomer sequence and acetylation patterns in some bacterial alginates. *Carbohydrate Res* 154, 239–250.
- Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98, 503–517.
- Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective, high-level expression of cloned genes. *J Mol Biol* 189, 113–130.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorf, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185, 60–89.
- Tabor, S. (1990). Expression using the T7 RNA polymerase promoter system. In *Current Protocols in Molecular Biology*, pp. 16.0.1–16.2.11. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. M. Moore, J. G. Seidman, J. A. Smith & K. Strahl. New York: Greene Publishing Associates.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13

phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. *Gene* **33**, 103–109.

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