The *Azotobacter vinelandii* gene *algJ* encodes an outer-membrane protein presumably involved in export of alginate

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The *algJ* gene from *Azotobacter vinelandii* was cloned using a labelled RNA probe representing the coding region of the *algE* gene from *Pseudomonas aeruginosa*. DNA sequencing revealed an ORF of 1452 bp encoding a protein of 484 amino acid residues with a calculated molecular mass of 54 611 Da. An RNA probe corresponding to *algE* was also used for Southern hybridization of chromosomal DNA, which showed that *algE*-related DNA sequences are also present in the alginate-producing phytopathogen species *Pseudomonas marginalis* and *Pseudomonas syringae* pv. *glycinea*. The coding region of *algJ* was subcloned in the expression vector pT7-7, leading to a corresponding gene product with an apparent molecular mass of 54 kDa which could be identified in the outer membrane (OM) of *Escherichia coli* BL21(DE3). Additionally, a cross-reacting protein with the same molecular mass was also found in the OM of *A. vinelandii* using an anti-AlgE antiserum. The derived amino acid sequence of AlgJ shared approximately 52% identity with AlgE from *P. aeruginosa*. The hydrophilicity profile as well as the amphipathicity of regions in the amino acid sequence of AlgJ showed significant similarities to AlgE. Based on these data, a topological model of AlgJ was created with the aid of known structures of outer-membrane proteins. This model presents AlgJ as a β -barrel containing 18 β -strands inserted in the OM.

Keywords: *Azotobacter vinelandii*, alginate, outer-membrane protein, export

INTRODUCTION

Alginate is an unbranched copolymer consisting of variable amounts of D-mannuronic acid and L-guluronic acid. This polymer is produced in copious amounts by marine brown algae and by bacteria of the genera *Azotobacter* and *Pseudomonas* (Gorin & Spencer, 1966; Govan *et al.*, 1981; Linker & Jones, 1966). Alginate from brown algae is isolated on an industrial scale for several applications in biotechnology and biomedicine (Guiry & Blunden, 1991; Skjåk-Bræk & Martinsen, 1991; Soon-Shiong *et al.*, 1993). The genetics and biochemistry of alginate biosynthesis have been extensively studied in

alginate-producing mutants of *Pseudomonas aeruginosa*, i.e. mucoid strains (May & Chakrabarty, 1994). These mucoid strains play a crucial role as human pathogens in cystic fibrosis patients (Govan & Harris, 1986), and alginate is one of the most important virulence factors (Gacesa & Russell, 1990).

Generally, the early steps of alginate biosynthesis, which are localized in the cytosol and lead to the activated precursor GDPmannuronic acid, are well understood (May & Chakrabarty, 1994). However, the final steps of alginate biosynthesis still remain unclear. Among these are the polymerization, transacetylation (only in bacteria), epimerization and finally the export of the alginate.

Azotobacter vinelandii, like several *Pseudomonas* species, synthesizes alginate as an exopolysaccharide, which contributes to the differentiation process of cyst formation (Sadoff, 1975; Terzaghi & Terzaghi, 1986).

The first alginate gene cloned from *A. vinelandii* was the *algE* gene, encoding a Ca^{2+} -dependent extracellular epi-

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Abbreviations: IM, inner membrane; OM, outer membrane; OMP, outer-membrane protein.

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merase (Ertesvåg *et al.*, 1994). Recently, four other epimerase genes were identified in *A. vinelandii*, originating from a common ancestor gene by a complex rearrangement process (Ertesvåg *et al.*, 1995). The epimerase genes are the only alginate genes so far characterized in *A. vinelandii*. Thus the genetics of alginate biosynthesis of *A. vinelandii* are poorly understood. In this study, the cloning of another alginate gene, *algJ*, encoding an outer-membrane protein (OMP) which might be involved in the export of alginate, based on its homology to AlgE from *P. aeruginosa*, is presented (Grabert *et al.*, 1990; Rehm *et al.*, 1994a, b). AlgE is an alginate-specific OMP with defined channel properties which is probably involved in the export of the exopolysaccharide alginate across the outer membrane (OM) (Rehm *et al.*, 1994b). Genetic characterization of *A. vinelandii* alginate biosynthesis may enable a functional comparison with the *P. aeruginosa* alginate genes, and elucidation of the alginate biosynthesis of *A. vinelandii* could lead to the application of this organism in industrial production of defined alginates.

METHODS

Bacterial strains and plasmids. *Escherichia coli* JM109 [*trcA* (*mcrA*) *recA1* *endA1* *gyrA96* *thi-1* *hsdR17* (τ_K^- m_K^+) *supE44* *relA1* Δ (*lac-proAB*) (*F'* *traD36* *proAB* *lacI*^qZAM15)] was used for cloning and for the preparation of plasmid DNA (Sambrook *et al.*, 1989). Strain *E. coli* BL21(DE3) (containing the T7 RNA polymerase gene under control of the *lacUV5* promoter inserted in the chromosome) was used for the expression studies (Studier & Moffatt, 1986). *A. vinelandii* strain E was used for the construction of a λ -EMBL-3 genomic library and for the preparation of protein samples (Larsen & Haug, 1971). The phytopathogen strains *Pseudomonas marginalis* HT041B and *Pseudomonas syringae* pv. *glycinea* GSPB1203 (Fett *et al.*, 1986) were used for the preparation of chromosomal DNA, which was analysed by Southern hybridization.

Plasmid pBluescript KS (Stratagene) served for cloning and DNA sequencing. Plasmid pBHR1 was constructed by inserting the *algJ*-coding region into the *NdeI* and *BamHI* sites of the expression vector pT7-7 (Studier & Moffatt, 1986). The coding region of *algJ* was amplified by PCR, inserting restriction enzyme sites *NdeI* and *BamHI* at the N-terminal and C-terminal ends, respectively.

Media and genetic techniques. In general, bacteria were grown in Luria-Bertani (LB) broth. Medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and additional salts was used to maximize protein production (Tabor & Richardson, 1985). When required, media were supplemented with an antibiotic (ampicillin, 50 μ g ml⁻¹) in order to prevent plasmid segregation. Transformation of bacteria and all other genetic techniques were performed as described by Sambrook *et al.* (1989). DNA was sequenced according to the chain-termination method (Sanger *et al.*, 1977) using an automatic laser fluorescence sequencer (ALF; Pharmacia). PCR was performed using Vent DNA polymerase (New England Biolabs) for a duration of 30 cycles (40 s, 94 °C; 40 s, 54 °C; 2 min, 72 °C) with the thermal cycler PHC-3 (Techne). Oligonucleotides (N-terminus: 5'-dAAAAACATATGAGTAGGAAGCAACGCATAAGCGCGGACTGGGCC-3'; C-terminus: 5'-dAAAAAAGGATCCTTAGAAGCGCCAGATCATGTGCGACGAAGACACGGTGCA-3') were obtained from Appligene. Chromo-

somal DNA was isolated according to Mak & Ho (1991) and used as target DNA in the PCR.

Southern blot hybridization of genomic DNA. Chromosomal DNA was digested with the indicated restriction enzymes, electrophoresed and blotted to a Hybond membrane (Amersham). Subsequently the membrane was irradiated with UV light to bind the DNA (Sambrook *et al.*, 1989). As a probe, digoxigenin-labelled RNA corresponding to the entire coding region of the *algE* gene from *P. aeruginosa* was used. The labelling reaction was performed with the previously described plasmid pTR7-2 using a T7 RNA polymerase digoxigenin-labelling kit (Boehringer Mannheim) (Rehm *et al.*, 1994b). The labelling procedure was conducted according to the user's guide (Boehringer Mannheim). Briefly, the vector pTR7-2 expressing *algE* under control of the T7 promoter ϕ 10 was linearized with *HindIII*. This allowed the production of run-off transcripts *in vitro* in the presence of digoxigenin 11-dUTP and the T7 RNA polymerase. Prehybridization and hybridization were done with a standard buffer containing 50% (v/v) formamide under stringent conditions (50 °C) according to the user's guide (Boehringer Mannheim). The detection of hybridized labelled RNA was done using anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase and the chemiluminescent substrate Lumigen PPD [4-methoxy-4-(3-phosphatephenyl) spiro-(1,2-dioxetane-3,2'-adamantane); (Boehringer Mannheim)]. The membranes were prewarmed for 15 min at 37 °C and exposed to X-Omat AR film (Kodak) for 1–2 h.

Screening of the genomic library and analysis of the recombinant clones. A λ -EMBL-3 genomic library of strain *A. vinelandii* E (Larsen & Haug, 1971) was plated on LB medium with approximately 500–800 plaques per plate. The library was screened with the digoxigenin-labelled RNA probe corresponding to the entire *algE*-coding region. The filters were hybridized and washed as described above. The initial positive plaques were picked and eluted in SM buffer (0.1 M NaCl, 8 mM MgSO₄, 0.05 M Tris/HCl, 0.01%, w/v, gelatine, pH 7.5). The plaques were purified by plating at low density and screening with the same RNA probe until all plaques were positive. λ DNA of the positive clones was prepared as described by Sambrook *et al.* (1989). The λ DNA was digested with different restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel to determine the presence of inserts by Southern hybridization.

Subcellular localization of recombinant AlgJ. Cells were disrupted by ultrasonication. The membrane fraction was prepared by centrifuging whole-cell lysate for 1 h at 40 000 g. OMs were prepared by solubilizing the inner membrane (IM) with 0.7% (w/v) *N*-lauroylsarcosine (Sigma) (Poxton *et al.*, 1985). Contamination of the OM preparation with IM was calculated by determining the specific succinate dehydrogenase activity of the OM preparation relative to the envelope preparation (OM and IM).

Gel electrophoresis and Western immunoblotting. After SDS-PAGE (Lugtenberg *et al.*, 1975), protein bands were visualized by staining with Coomassie brilliant blue R-250 (0.025%, w/v; Bio-Rad).

Western blotting (Towbin *et al.*, 1979) of OMPs was performed using PVDF membranes (Millipore). AlgJ was detected by cross-reaction with the polyclonal anti-AlgE antiserum (Rehm *et al.*, 1994a) and an alkaline-phosphatase-antibody conjugate (Sigma) as a second antibody. Bound antibodies were detected by using the chromogenic substrate nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (Boehringer Mannheim).

RESULTS

Immunological cross-reaction of OMPs from *A. vinelandii* using the monospecific anti-AlgE antiserum

To determine the presence of an AlgE OMP homologue in the OM of *A. vinelandii*, we used an anti-AlgE antiserum for immunoblotting experiments. The OMPs of *A. vinelandii* were prepared by selective solubilization of the IM with *N*-lauroylsarcosine (Poxton *et al.*, 1985). Immunoblotting experiments revealed three cross-reacting OMPs with apparent molecular masses of 35 kDa, 54 kDa and 70 kDa; however, the 54 kDa protein showed the strongest signal (data not shown).

Identification of DNA sequences homologous to *algE* among alginate-producing bacteria

Chromosomal DNA of the alginate-producing bacteria *P. syringae* pv. *glycinea*, *P. marginalis*, *P. aeruginosa* and *A. vinelandii* was isolated and used for Southern hybridization. As a probe, digoxigenin-11-dUTP-labelled RNA, representing the entire coding region of *algE* from *P. aeruginosa*, was applied. This labelling was done using plasmid pTR7-2, which enabled overexpression of *algE* under the control of the T7 promoter $\phi 10$ (Rehm *et al.*, 1994b; Tabor & Richardson, 1985). All of the alginate-producing bacteria analysed in this study showed, under stringent conditions, specific hybridization signals (Fig. 1). These data indicate that genes similar to *algE* might be

Fig. 1. Southern hybridization blot of digested chromosomal DNA from different alginate-producing bacteria probed with digoxigenin-labelled RNA corresponding to the *algE* gene from *P. aeruginosa*. Lanes: 1, clone pKR22 digested with *Xho*I and *Hind*III, leading to a 1.5 kb DNA fragment representing the coding region of *algE*; 2, 3 and 4, chromosomal DNA from *P. aeruginosa* digested with *Eco*RI, *Hind*III and *Bam*HI (positive control), respectively; 5, 6 and 7, chromosomal DNA from *A. vinelandii* digested with *Hind*III, *Eco*RI and *Bam*HI, respectively; 8 and 9, chromosomal DNA digested with *Eco*RI from *P. marginalis* and from *P. syringae* pv. *glycinea*, respectively; 10, *Eco*RI-digested chromosomal DNA from *E. coli* JM109 as a negative control. Molecular sizes in kb are indicated on the left-hand side.

1	GGCCAAACGCC	TACGTCTTCG	CACGTCTCGC	CGTTTTCGAA	GGCCGCCGCC	ATTCGAGGCC
61	CCTGCTTCAG	GAAATAGAGG	CAATCTCGCT	CCTGCAGAAC	GTACCCGGGG	TGAACAGATG
121	CTGCACGCGG	AACAGCAGGC	TCGTTACGCG	GTGTGCGAGA	CCTGCAGCCA	GCTGCAAGCC
			-35			-10
181	ATGCAAAATC	AATAGGAAGC	ATTATGAGTA	GGAAGCAACG	CATAAGCGCG	GGACTGGGGC
		SD	M S	R K Q	R I S A	G L G
241	TGGGAGCCAG	TCTGCTCTGC	TGCAATCCGC	TGTTGCGCAGG	GCCCGTGGGT	CCGATCGCA
	L G A	S L L C	C N P	L F A	G P V G	P D R
301	ATTTCGGCAT	GGAGGTGAAG	GTACCGSCCC	AGTCCGAAGA	CGACGCTGAC	CTGGACACCC
	N F G	M E V K	V T A	Q S E	D D R D	L D T
361	GCAGCGCGGG	TGACGCGGAG	GGTATCGCCC	TCGACTTGCG	GCCTGGGGTC	TACGGCCAGC
	R S G	G D A E	G I A	L D L	R P W V	Y G Q
421	GCGGCAACTG	GGGAGCCATG	GTGATGCTCC	AGGCGGTCCG	GGCCACCGAC	ATCATCGAAA
	R G N	W G A M	V M L	Q A V	A A T D	I I E
481	CCGACCCAC	CGATCCCAAC	GAAGAACCGG	GGGCGATCC	CGCGAACGGC	TTACGCGCGG
	T D P	T D P N	E E P	G G D	P A N G	F S R
541	ACAGCAGCCG	CGACCCGGAC	AAGAGCTACC	TCGCCCTGCG	CGAATTGCG	CTCGGTACCA
	D S S	R D P D	K S Y	L A L	R E F E	L G T
601	TCGGCCGCCA	CGCATACGCG	AGCCTGACCA	AGCAAGGCAC	CTGTTGGTTC	ATCCACATGG
	I G R	Q R I R	S L T	N E G	T W W D	I H M
661	AGTCGGTGAA	CTGGACCATG	GACACGCGCC	TGCTCGCGCG	CCAGCCGGGC	CTCGCGAAAC
	E S V	N W T M	D T S	L L R	A Q A G	V A K
721	GCTTCGACGA	ATACCGTACC	GACCTGAGCA	ACCTGAGCGC	GGAAGACCAAG	GACCCACCCC
	R F D	E Y R T	D L D	N L S	A E D K	D R T
781	ACGTCTTCGG	CGGCCTCGAC	TACCACTGGC	GGCAAGGTCA	CTGGGCGGG	TTCAAGGTGC
	H V F	G G L D	Y Q W	R Q G	H W A G	F K V
841	ACCACACCAG	CGACGACGGC	GACCTCGCGG	ACAGCCAGAT	GGATGTCTAC	GAAGACCGCC
	H H T	S D D G	D L P	D S Q	M D V Y	E D R
901	AGAGCAAGTC	CTACACCGGC	GACCTGACCT	GGCTGAGCGT	GCACCTGGAC	CGCGACTTCT
	Q S K	S Y T G	D L T	W L S	V H L D	R D F
961	TCAATCCTCG	CTCGACCCTG	CGATCAACT	ACTGGGCGGA	ATTACCTGCG	CTGACCGCGG
	F N P	R S T L	P I N	Y W G	E F T W	L T G
1021	AGATGGACAG	GAGCACTTTC	GCCAGCCAGC	GCAGCGCCGA	CCACTACAAG	GACGTGGATG
	E M D	R S H F	A S D	G S A	D H Y K	D V D
1081	TCGACGCGTG	GGCCGTGGAC	CTCGGCTGCG	GCTGGAACAT	CAGCGACCGC	TGGAACGTCG
	V D A	W A V D	L G L	R W N	I S D R	W N V
1141	GCGCGGCTTA	TGCCCGCGGC	CAGCGCGGCG	AGGCGACAGA	CGAGTCCGAA	CAGTTCTATC
	G A A	Y A R G	Q A G	E G D	D E S E	Q F M
1201	AGACCGGCCT	GCAGAGCAAC	CGCTCCACCT	TCACCGGCCT	GCAGACCGCG	ATCCACCGCT
	Q T G	L Q S N	R S T	F T G	L Q T R	I H R
1261	TCGGCGAAGC	TTCCCGCGGC	GAACTGACCA	ACCTGCAGGT	GGGACCCCTG	TTACCTCTCT
	F G E	A S R G	E L T	N L Q	V G T L	F T S
1321	GGAAGCCGAG	AGAGGACCTC	GAGACGAGCC	TGATCTATCA	CAAGTTCTAG	CGCGTGCAGC
	W K P	R E D L	E T S	L I Y	H K F W	R V D
1381	ATGACGAGGA	TCTCGGCCAG	AACGGCATCA	GCCCGATCGA	GAAGGACGGC	AAGCCCGCGC
	D D E	D L G Q	N G I	S P I	E K D G	K P A
1441	TCAAGGCGCG	TGAGAAGGAC	CTCGGCCAGG	AAGTCGACCT	GATCATCACC	CGCTACTTCA
	L K A	G E K D	L G Q	E V D	L I I T	R Y F
1501	ACCAGGCGCAT	GTTGCGGGCC	AACTGGGGTG	GCGAGCTGGA	CGAGCAGTCC	GCACTGATCC
	N Q G	M L P A	N W G	G E L	D E Q S	A L I
1561	GCCTGCGCAG	CGGCCGTGTC	TTCCCGAGCA	ACGCTATGCA	CAGCAAGGGC	GACAGCAAAA
	R L R	S G L F	F P S	N A Y	A S K G	D S K
1621	TGCACCGTGT	CTTCGTCGAC	ATGATCTGGC	GCTTCTAAGG	TCCAGCGGCA	CGCTGAGAGG
	M H R	V F V D	M I W R	F -		
1681	ATCGAACATG	AACGTGCAAA	GAAACTTTCG	ATCCACCACG	CTGAAACCCG	TGTTGCTCGG

Fig. 2. DNA sequence of *algJ* and the deduced amino acid sequence (single-letter code). The putative -10 and -35 regions, homologous to the σ^{70} promoter, are underlined. The conserved ribosome-binding site is underlined and labelled with SD.

distributed among alginate-producing bacteria and that the *algE* gene appears to be conserved.

Cloning of *algJ* from *A. vinelandii* and DNA sequence analysis of *algJ*

The RNA probe described above was used for screening of a λ gene library representing the *A. vinelandii* genome. A hybridizing λ clone (pBHR100) with an approximately 20 kb insert was isolated. Further hybridization experiments confirmed that the DNA sequence homologous to *algE* was located on *Eco*RI fragments of 2.3 kb, 3.1 kb and 0.4 kb. DNA sequencing of the complete 2.3 kb and 0.4 kb *Eco*RI DNA fragments and part of the 3.1 kb *Eco*RI DNA fragment as well as of subclones comprising the *Eco*RI sites connecting the DNA fragments led to the identification of an ORF of 1452 bp, representing the *algJ* gene (Fig. 2). DNA sequence comparison with the

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ALGJ - MSRKQRISA-----GLGLGASLLCCNPLFA-GPVGPRNFGMEVKVTAQSEDD-47
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - MNSRSVNPSPFAPRALSLAIALLLGAPFAAGSGEAPKNGFLDKITGESEND-55

ALGJ - RDLDRSGGDAEGIALDLRPVYQGRNGWAMVLMQAAVATDIETDPTDPNEEP-102
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - RDLGTAPGGTLNDIGIDLRFWAFQGWGWSAYFMGQAAVATDTIETDTLQSDTDD-110

ALGJ - GGDPANGFSRDSRDPKSYLALREF-----ELGTIGRQIRSLTNEG-145
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - GNN-----SRNDGREPKSYLAAREFWVDYAGLTAYPGEHLRFGRQRLR--EDSG-158

ALGJ - TWDIHMSVNVMTDSTLLRAQAGVAKRFDEYRTDLNLSAEDKDRTHVFGGLDY-200
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - QWQDTNIEALNWSFETLLNAHAGVAQRFSEYRTDLDELAPEDKDRTHVFGDIST-213

ALGJ - QWRQGHAGFKVHHTSDDGDLFQSDQVDYEDRQSKSYTGDLTWSVHLDRDFNP-255
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - QWAPHHRIQVRIHADDSGHLR--RPGEEVDNLDKTYTGQLTWLGIEATGDAYNY-266

ALGJ - RSTLPINWGEFTWLTGEMDRSHFASDGSADHYKDV-----DV-293
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - RSSMPLNYNASATWLTGDRD-----NLTTTVDDRRIATGKQSGDV-307

ALGJ - DAWAVDLGLRWNISDRWNVGAAYARGAGEGDDESEQFMQTGLQSNRSTFTGLQT-348
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - NAFGVDLGLRWNIDEQWKAGVGARG-SGGKGDEEQFQQTGLSENRNFTGTR-361

ALGJ - RIHRFGEASRGELTNLQVGTFTSWKPREDETSIYHKFWRVDDDEDLQNGIS-403
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - RVHRFGEAFRGELSNLQAATLFGSWQLREDYDASLVYHKFWRVDDSDIGTSGIN-416

ALGJ - PIEKDGKPAKAGEKDLGQEVDLITRYFNQGMPLPANWGGEDEQSALIRLRSG-458
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - A-----ALQPEKDIGQELDLVVTYKFKQGLLPASMSQYVDEPSALIRFRGGL-464

ALGJ - FFPSNAYASKGSKMHRVFVDMIWRF -484
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ALGE - FKPGDAYGPGTDMHRAFDIWRF -490

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Fig. 3. Alignment of the deduced amino acid sequences of AlgJ and AlgE. The structure-genetic matrix with an open gap cost of 7 and a unit gap cost of 1 was used. |, Two identical aligned residues; ·, two similar aligned residues. Amino acids said to be similar are: A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W.

GenBank (release 91)/EMBL (release 43.0) databases showed significant similarity (~47% identity) only with *algE* from *P. aeruginosa* (data not shown) (Chu *et al.*, 1991). DNA sequence analysis indicated a potential ribosome-binding site, AGGAA, five nucleotides upstream of the start codon ATG at position 204 (Fig. 2). This spacing between the potential ribosome-binding site and the start codon agrees with the preferred spacing of the ribosome-binding site in *E. coli* (Shine & Dalgarno, 1975).

A weak homology in the -10 and -35 regions with the σ^{70} promoter from *E. coli* was found (Fig. 2). The G/C content of the analysed DNA was 64.2%. Furthermore, 30 nucleotides downstream of *algJ*, the *algG* gene, encoding an epimerase, was identified (B. H. A. Rehm, H. Ertesvåg & S. Valla, unpublished results).

The *algG* gene possessed no potential promoter region but had a conserved ribosome-binding site, suggesting an operonic organization. This *algG* gene from *A. vinelandii* was overexpressed in *E. coli*, partially purified and the gene product showed epimerase activity (B. H. A. Rehm, H. Ertesvåg & S. Valla, unpublished results).

The upstream region of *algJ* was found to be similar (73% identity; 165/226) to the upstream region of *algE* from *P. aeruginosa* (GenBank release 91/EMBL release 43.0). This homology strongly suggests that this DNA region might encode the same protein, possibly involved in alginate biosynthesis, in *A. vinelandii* and *P. aeruginosa*.

Fig. 4. Comparison of the hydrophilicity profiles of (a) AlgJ and (b) AlgE according to Hopp & Woods (1981). Loops (L1–L9) were predicted, based on the hydrophilicity profile, as corresponding to hydrophilic maxima.

Analysis of the *algJ* deduced amino acid sequence

The amino acid sequence deduced from *algJ* revealed a ~52% identity with AlgE and no significant similarity to any other protein in the GenBank (release 91) and EMBL (release 43.0) databases (Fig. 3). The amino acid sequence was analysed using the program PC/Gene (IntelliGenetics release 6.7). A conserved signal sequence with a potential cleavage site between positions 25 and 26 was found. This cleavage site corresponds to the (-3, -1) rule (von Heijne, 1984). Acidic pI values of 4.47 (precursor form) and 4.37 (mature form) could be calculated. Hydrophilicity profiles were calculated for AlgE and AlgJ and the comparison revealed a strong similarity (Fig. 4) (Hopp & Woods, 1981).

Topological model of AlgJ

A topological model of AlgJ was created (Fig. 5) based on the following criteria: (i) the prediction of extracellular loops using the hydrophilicity profile according to Hopp & Woods (1981), where hydrophilic maxima correspond to cell-surface-exposed loops; (ii) amphipathic regions,

Fig. 5. Topological model of mature AlgJ based on its amino acid sequence (single-letter code). The β -barrel structure shown consists of 18 anti-parallel β -strands, 9 loops (L1–L9) and 8 turns (T1–T8). Identical amino acid residues, based on comparison of the amino acid sequences of AlgE and AlgJ, are circled.

which separate the loops and probably form β -strands; (iii) turn predictions according to Paul & Rosenbusch (1985), which should not be located in the β -strands, but should separate the β -strands representing the periplasmic exposed β -turns; (iv) α -helix prediction only in the loop regions; and finally (v) with the aid of known OMP structures based on high-resolution X-ray analysis (Cowan *et al.*, 1992; Schirmer *et al.*, 1995).

Using these criteria, which were also used for the accurate prediction of PhoE (Tomassen, 1988), AlgJ is presented as a β -barrel, consisting of 18 antiparallel amphipathic β -strands spanning the OM, eight β -turns (T1–T8) at the periplasmic site and nine loops (L1–L9) exposed to the cell surface (Fig. 5). In β -strands 4 and 7, positively charged residues (arginine and lysine, respectively) are exposed to the hydrophobic core. Single hydrophilic

residues in the β -strands exposed to the external hydrophobic core of the putative AlgJ structure could possibly be tolerated as was recently shown for the crystal structure of LamB (Schirmer *et al.*, 1995). Basically, the topological model of AlgJ (Fig. 5) is very similar to those already postulated and verified for some other integral OM proteins, e.g. the specific porins LamB and PhoE from *E. coli* (Schirmer *et al.*, 1995; Tomassen, 1988).

Overexpression of *algJ* in *E. coli* and localization of the gene product to the OM

For the overexpression of *algJ* in *E. coli*, PCR was used to amplify the coding region of *algJ* and to introduce the restriction sites *NdeI* and *BamHI* at the ends of the PCR product. These restriction sites were used to clone the

Fig. 6. SDS-PAGE demonstrating expression of *algJ* in *E. coli* BL21(DE3) and its localization in the OM. Lanes: 1 and 2, whole-cell lysate of *E. coli* BL21(DE3) (induced with 1 mM IPTG) containing vector pT7-7 and containing plasmid pBHR1, respectively; 3 and 4, OMP preparations of *E. coli* BL21(DE3) (induced with 1 mM IPTG) containing vector pT7-7 and containing plasmid pBHR1, respectively. Molecular masses in kDa and the position of recombinant AlgJ are indicated on the left-hand side.

coding region into vector pT7-7, thereby producing the construct pBHR1 (Tabor & Richardson, 1985). The vector provides a conserved ribosome-binding site and enabled overexpression of *algJ* under the control of T7 promoter $\phi 10$ in *E. coli* BL21(DE3) (Fig. 6) (Studier & Moffatt, 1986; Tabor & Richardson, 1985). This *E. coli* strain contains the T7 RNA polymerase gene integrated in the chromosome under control of the IPTG-inducible *lacUV5* promoter (Studier & Moffatt, 1986). Subcellular localization revealed that recombinant AlgJ was secreted and incorporated in the OM of the overexpressing *E. coli* strain (Fig. 6).

DISCUSSION

The Southern hybridization data indicated that genes similar to *algE* are distributed and conserved among alginate-producing bacteria. Thus, OMPs homologous to AlgE (*P. aeruginosa*) might also play an important role in the alginate biosynthesis of these bacteria as shown for *P. aeruginosa*. The *algE*-defective mutant of *P. aeruginosa* is not capable of producing alginate unless it is complemented with the *algE* gene (Chu *et al.*, 1991). Accordingly, DNA sequences that were homologous to the *P. aeruginosa* alginate genes *algA*, *algD* and *algR* could be found in 23 species of the genus *Pseudomonas* in rRNA homology group I, with the exception of *Pseudomonas stutzeri*. Homologous DNA sequences of at least one of those

alginate genes were found in the species *Xanthomonas campestris*, *Azomonas macrocytogenes* and *A. vinelandii* in the same homology group (Fialho *et al.*, 1990). To support the idea that *A. vinelandii* produces a protein similar to AlgE in the OM, we used a polyclonal anti-AlgE antiserum. Immunoblotting of OMPs of *A. vinelandii* revealed the expected cross-reactivity. An OMP with an apparent molecular mass of 54 kDa could be detected, and was considered as a candidate for the AlgE-related OMP. Cloning and DNA sequencing of *algJ* from *A. vinelandii* confirmed the presence of a related gene. The predicted amino acid sequence of AlgJ indicated the presence of a signal peptide of 25 amino acids, with a putative cleavage site for leader peptidase 1 conforming with the $(-3, -1)$ rule and an amphipathic C-terminal region ending with a phenylalanine residue. These features suggested that AlgJ was secreted through the cytoplasmic membrane and localized in the OM (Struyve *et al.*, 1991; von Heijne, 1984). This was confirmed by the localization of recombinant AlgJ in the OM of *E. coli* and by the identification of a cross-reacting protein in the membrane fraction of *A. vinelandii*. The same result was obtained in the case of the overexpression of *algE* from *P. aeruginosa* in *E. coli*, where a putative precursor could be identified in the cytoplasmic membrane (Rehm *et al.*, 1994b). The presence of AlgJ as an OMP was additionally supported by the fact that it was possible to create a topological model of AlgJ as an integral β -barrel in the OM using the criteria applied for the precise prediction of the structure of PhoE from *E. coli* (Tommasen, 1988). The model presented in this study is in good agreement with the model already presented for AlgE (Rehm *et al.*, 1994b). Accordingly, AlgJ is predicted to consist of 18 β -strands with 9 cell-surface-exposed loops and 8 periplasmic oriented β -turns. Recently, the crystal structure of the specific channel LamB was determined, which also revealed an 18-stranded antiparallel β -barrel (Schirmer *et al.*, 1995).

Loops 4 and 7 of AlgJ showed extended stretches of amino acids that were identical to those in AlgE, and they possessed a high density of charged amino acids, indicating a putative functional role of these loops in the binding and/or the translocation of the anionic alginate chain. Ionizable amino acid residues are involved in substrate binding and translocation in LamB from *E. coli* (Schirmer *et al.*, 1995). Loops 4 and 7 might bend into the channel lumen, providing a binding site for the alginate oriented towards the periplasm. For AlgE, blocking of the channel with GDPmannuronic acid, the activated precursor of the alginate, has been shown, indicating a binding site for the alginate chain (Rehm *et al.*, 1994b). In the case of LamB from *E. coli* it was shown that loops L2, L4 and L6 also contribute to the specificity of the channel (Schirmer *et al.*, 1995). However, the functional role of AlgJ in alginate biosynthesis might be the export of the alginate across the OM as has been proposed for AlgE (Rehm *et al.*, 1994b). Consequently, AlgJ is strongly suggested to be a specific channel protein with export function. There is only one other protein with a potential polysaccharide export function described so far, which is the OMP CtrA from *Neisseria meningitidis*. This OMP

contains eight predicted β -strands and has been proposed to be involved in capsule formation (Frosch *et al.*, 1992).

To confirm the topological model of AlgJ, it is necessary to perform mutagenesis, e.g. by insertion of certain epitopes or proteolytic sites into the loop regions, and to purify the protein in order to analyse its channel properties.

We are also currently performing DNA sequencing to analyse the complete 20 kb DNA fragment containing the suggested alginate gene cluster in *A. vinelandii* as well as Northern hybridizations to identify the potential polycistronic mRNA. Elucidation of the genetics and the biochemistry of alginate biosynthesis in *A. vinelandii* might enable the production of designed alginates and/or alginate production in recombinant *E. coli* containing a certain arrangement of alginate genes.

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