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Characterization of Three New *Azotobacter vinelandii* Alginate Lyases, One of Which Is Involved in Cyst Germination▽

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Alginates are polysaccharides composed of 1-4-linked β-D-mannuronic acid and α-L-guluronic acid. The polymer can be degraded by alginate lyases, which cleave the polysaccharide using a β-elimination reaction. Two such lyases have previously been identified in the soil bacterium *Azotobacter vinelandii*, as follows: the periplasmic AlgL and the secreted bifunctional mannuronan C-5 epimerase and alginate lyase AlgE7. In this work, we describe the properties of three new lyases from this bacterium, AlyA1, AlyA2, and AlyA3, all of which belong to the PL7 family of polysaccharide lyases. One of the enzymes, AlyA3, also contains a C-terminal module similar to those of proteins secreted by a type I secretion system, and its activity is stimulated by Ca^{2+}. All three enzymes preferably cleave the bond between guluronic acid and mannuronic acid, resulting in a guluronic acid residue at the new reducing end, but AlyA3 also degrades the other three possible bonds in alginate. Strains containing interrupted versions of *alyA1*, *alyA3*, and *algE7* were constructed, and their phenotypes were analyzed. Genetically pure *alyA2* mutants were not obtained, suggesting that this gene product may be important for the bacterium during vegetative growth. After centrifugation, cultures from the *algE7* mutants form a large pellet containing alginate, indicating that AlgE7 is involved in the release of alginate from the cells. Upon encountering adverse growth conditions, *A. vinelandii* will form a resting stage called cyst. Alginate is a necessary part of the protective cyst coat, and we show here that strains lacking *alyA3* germinate poorly compared to wild-type cells.

Azotobacter vinelandii is a nitrogen-fixing bacterium found in soil. *A. vinelandii* and several species belonging to the related genus *Pseudomonas* have been found to produce the polymer alginate. This linear, extracellular polysaccharide is composed of 1-4-linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) (35), and the relative amount and distribution of these two residues vary according to the species and growth conditions. Some of the M residues in bacterial alginates may be O acetylated at C-2, C-3, or both C-2 and C-3 (34).

Alginate is first synthesized as mannuronan, and the G residues are introduced by mannuronan C-5 epimerases. All genome-sequenced alginate-producing bacteria have been found to encode a periplasmic epimerase, AlgG, that epimerizes some of the M residues in the polymer into G residues (40). AlgG seems to be unable to epimerize an M residue next to a preexisting G residue in vivo. *A. vinelandii* also encodes a family of secreted mannuronan C-5 epimerases (AlgE1-7) (40), some of which are able to form stretches of consecutive G residues (G blocks). Alginates containing G blocks can be cross-linked by divalent cations and thereby form gels (35).

Polysaccharide lyases (EC 4.2.2.–) are a group of enzymes which cleave the polymer chains via a β-elimination mechanism, resulting in the formation of a double bond at the newly formed nonreducing end. For alginate lyases, 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (denoted as Δ) is formed at the nonreducing end. Several such lyases have been purified from both alginate-producing and alginate-degrading organisms, as reviewed by Wong et al. (42). When they are classified according to primary structure, the alginate lyases belong to the polysaccharide-degrading enzyme families PL5, PL6, PL7, PL14, PL17, and PL18 (http://www.cazy.org). Alginate molecules may contain four different bonds (M-M, M-G, G-M, and G-G), and alginate lyases may therefore be classified according to their preferred substrate specificities. It is now possible to obtain pure mannuronan and nearly pure $(MG)_n$ and G blocks (17, 19, 20), and this allows for an improved assessment of the substrate specificities of the alginate lyases.

The following two alginate lyases have been characterized in *A. vinelandii*: the periplasmic AlgL that belongs to the PL5 family (15) and the extracellular bifunctional mannuronan C-5 epimerase and alginate lyase AlgE7 (36, 37). AlgL is encoded by the alginate biosynthesis operon, similar to what has been found in all characterized alginate-producing bacteria. This enzyme cleaves M-M and M-G bonds (15), while AlgE7 preferably degrades G-MM and G-GM bonds (37). The latter enzyme is also able to introduce G residues in the alginate, thus creating the preferred substrate for the lyase.

When *A. vinelandii* experiences a lack of nutrients, it will develop into a dormant cell designated cyst (30). The cell is then protected against desiccation by a multilayered coat, of which gel-forming alginate is a necessary part. Resuspension of cysts in a medium containing glucose leads to a germination process in which vegetative cells eventually escape from the cyst coat. It has been proposed that an alginate lyase may be

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TABLE 1. Strains and plasmids used in the study

Strain/plasmid	Description and/or genotype	Reference/source
Strains		
E. coli S17.1	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ *(lac-proAB)*	33
E. coli DH5α	*endA1 hsdR17 supE44 thi-1*λ^- *recA1 gyrA96 relA1*Δ*lacU169* ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories
A. vinelandii E	*A. vinelandii* wild type, strain E	27
A. vinelandii E U-1	*algU*	C. Núñez
A. vinelandii E HE1	*algE7*, Tc^r	This work
A. vinelandii E MG134	*alyA1*, Sp^r	This work
A. vinelandii E MG136d	Some chromosomes of *alyA2*, Tc^r	This work
A. vinelandii E MG138	*alyA3*, Sp^r	This work
A. vinelandii E MG137	*alyA3 algE7* double mutant, Tc^r Sp^r	This work
Plasmids		
pUC128	ColE1, Ap^r	26
pLitmus28Tc	ColE1, Ap^r Tc^r	18
pGEM7	ColE1, Ap^r	Promega
pGEM11	ColE1, Ap^r	Promega
pTrc99A	ColE1, Ap^r	2
pCAM140	Delivery vector of mini-Tn*5*, Ap^r Sp^r	41
pJB861	Broad-host-range expression vector derived from RK2, Km^r	8
pCVD442	*ori* R6K, *sacB*, Ap^r	13
pJBSD1pgmtc	RK2-derived vector in which replication is regulated by *m*-toluate, Ap^r Tc^r	25
pRD110-34*ts*247C *cop*171W	ColE1 replicon containing a double mutant of *trfA*, Tc^r	21
pBG23	Derivative of pTrc99A encoding the catalytic part of AlgE7	36
pHE77	Derivative of pJBSD1pgmTc in which an 840-bp PstI-SfiI DNA fragment was replaced by the corresponding fragment from pRD110-34*ts*247C*cop*171W	This work
pHE90	Derivative of pJB861 in which a SalI linker was ligated into the blunted AflIII site, and a 19-bp BamHI-EcoRI DNA fragment subsequently was removed	This work
pHE92	An 8.8-kb SalI-NdeI DNA fragment from pHE77 was ligated to a 1.0-kb DNA fragment from pHE90 restricted with the same enzymes	This work
pHE73	Derivative of pUC128 in which a 2.4-kb PstI-BamHI DNA fragment from pCVD442 (containing *sacB*) was inserted in the corresponding sites of the vector	This work
pHE74	Derivative of pHE73 from which a 0.5-kb EcoRV DNA fragment was removed	This work
pHE93	Derivative of pHE92 in which a 2.0-kb SacI-Sal DNA fragment from pHE74 was inserted into the corresponding sites of the polylinker	This work
pHE95	Derivative of pHE93 from which a 2.3-kb SalI-MluI DNA fragment was removed	This work
pHE121	Derivative of pBG23 in which a 2.3-kb BamHI fragment containing the Tc genes from pLitmus28Tc was cloned into the BglII site of *algE7*	This work
pHE123	Derivative of SpeI-BssHII-digested pHE95 into which a 4.3-kb XbaI-AflIII DNA fragment from pHE121 carrying the inactivated *algE7* was inserted	This work
pMG131	Derivative of pTrc99a (EcoRI-BamHI) with an insertion of an EcoRI-BamHI-digested PCR fragment (0.8 kb) carrying *alyA1*	This work
pMG132	Derivative of pTrc99a (EcoRI-BamHI) with an insertion of an EcoRI-BamHI-digested PCR fragment (0.8 kb) carrying *alyA2*	This work
pMG133	Derivative of pGEM7 (EcoRI-BamHI) with an insert of a 2.1-kb EcoRI-BamHI-digested PCR fragment carrying *alyA1* and the flanking regions	This work
pMG134	Partially HincII-digested (nucleotide position 502 of *alyA1*) and blunted (Klenow fill-in) pMG133 with a 2.1-kb insertion (SmaI) carrying the spectinomycin resistance gene from pCAM140	This work
pMG135	Derivative of pGEM7 (EcoRI-BamHI) in which an EcoRI-BamHI-digested PCR fragment (2.4 kb) carrying *alyA2* and the flanking regions was inserted	This work
pMG136	Derivative of pMG135 digested with BglII in which a BamHI insertion of a fragment carrying *tetA* and *tetR* (2.3 kb) from pLitmus28Tc	This work
pMG137	Derivative of pGEM11 (EcoRI) with an insertion of an EcoRI PCR fragment (2.1 kb) carrying *alyA3* and the flanking regions	This work
pMG138	Derivative of pMG137 (SmaI) in which a 2.1-kb SmaI fragment carrying the spectinomycin resistance gene from pCAM140 was inserted	This work
pMG143	Derivative of pTrc99a (EcoRI-BamHI) with an insert of a BamHI-partially EcoRI-digested PCR fragment (1.5 kb) carrying *alyA3*	This work

involved in the rupture of the coat (43). AlgL is dispensable for germination (38), while the biological function of AlgE7 is unknown. In this report, we use the available draft genome sequence of *A. vinelandii* to identify three additional putative lyases and evaluate their and AlgE7's role in growth, encystment, and germination of the bacterium.

MATERIALS AND METHODS

Growth of bacteria. The bacterial strains and plasmids used are described in Table 1. *Escherichia coli* strains were routinely grown in L broth (10 g/liter tryptone, 5 g/liter of yeast extract, and 5 g/liter NaCl) or on L agar (L broth containing 15 g/liter agar) at 37°C. *A. vinelandii* was routinely grown in liquid Burk's medium (pH 7.2) at 30°C (23). Production of *A. vinelandii* alginate was

performed in liquid RA1 medium (pH 7.0) (18). Antibiotics, when used in routine growth experiments, were present at the following concentrations: ampicillin, 100 to 200 μg/ml (*E. coli*); tetracycline, 12.5 μg/ml (*E. coli*) and 15 μg/ml (*A. vinelandii*); and spectinomycin, 20 μg/ml (*E. coli* and *A. vinelandii*). For selection of genetically pure insertion strains in *A. vinelandii*, the concentrations of spectinomycin and tetracycline were gradually increased to 50 μg/ml.

Induction of cyst formation and resistance to desiccation were tested essentially as described by Campos et al. (10), but with some adjustments. After growth on Burk's agar containing 0.2% *n*-butanol as the carbon source, the cells were scraped off and resuspended in Burk's buffer (Burk's medium containing no carbon source). An aliquot of the cells (5 to 10 μl) was then dried in open 1.5-ml tubes placed in petri dishes at 30°C for a week. In this way, the problem of removing the dried cells from the filter was avoided. The dried cells were then resuspended in Burk's medium containing Tris (15 mM; pH 7.5) with and without alginate lyase, as described in Results, and the number of CFU was determined. Before the cell suspensions were diluted and plated, they were passed through a syringe (gauge no. 23) twenty times to reduce the problem of aggregation.

Standard techniques. Plasmid isolation, enzymatic manipulations of DNA, and agarose gel electrophoresis were performed as described by Sambrook and Russell (31). The QIAquick gel extraction kit and the QIAquick PCR purification kit (Qiagen, Valencia, CA) were used for DNA purifications from agarose gels and enzymatic reactions, respectively. Transformations of *E. coli* were performed using the RbCl method (www.neb.com). The Expand High Fidelity PCR system (Roche Applied Science, Penzberg, Germany) was used for PCR amplifications. Chromosomal DNA was isolated using the bacterial genomic DNA purification kit (Edge BioSystems, Gaithersburg, MD). Southern hybridization was performed, as described for the digoxigenin system by Roche Applied Science. The BigDye terminator 1.1 system (Applied Biosystems, Foster City, CA) was applied for DNA sequencing. DNA sequences for the primers used in this work are available upon request.

Construction of an *A. vinelandii algE7* strain. A conditional suicide vector for *A. vinelandii* was constructed based on pJBSD1pgmTc, which is dependent on the inducer *m*-toluate for replication in *E. coli*. This plasmid is able to replicate in *A. vinelandii* in the absence of any inducer (H. Ertesvåg, unpublished data). However, we found that a derivative of this vector, pHE77, encoding the TrfA mutant 247ts/171cop (24) in place of the wild-type replication protein, was maintained in *A. vinelandii* in the presence of the inducer but was rapidly lost in its absence.

The gene replacement vector pHE95 was then constructed from pHE77, as described in Table 1. The plasmid contains a polylinker and the *sacB* gene of *Bacillus subtilis* as a positive selection marker for double-crossover events. Expression of this enzyme is toxic for many gram-negative bacteria in the presence of sucrose (16), and we found this to be the case for *A. vinelandii* as well. In the final step, a gene replacement vector for *algE7* was constructed by cloning a DNA fragment containing *algE7* interrupted by *tetA* and *tetR* (encoding tetracycline resistance) into pHE95. This plasmid was designated pHE123. For conjugation, exponential-phase cultures of *A. vinelandii* (grown for 20 h) and *E. coli* (grown for 3 h) were mixed and pelleted. The cells were resuspended in about 100 μl medium, applied as a drop on L agar, incubated at 30°C overnight, and resuspended in 1 ml Burk's medium. Dilutions of the resuspension were plated on Burk's glucose agar containing tetracycline but lacking *m*-toluate. Double recombinants were then selected on plates containing sucrose (5%) and tetracycline. Selected colonies were grown for about 30 generations in Burk's medium with tetracycline to ensure that all copies of the chromosome contained the interrupted *algE7* gene. Strain HE1 was selected and confirmed by Southern hybridization to lack wild-type *algE7*.

Construction of strains defective in each of the *alyA* lyases. Gene replacement vectors for *alyA1*, *alyA2*, and *alyA3* were constructed, as described in Table 1, and designated pMG134, pMG136, and pMG138, respectively. They were linearized by MluI and EcoRI (pMG134), BamHI and EcoRI (pMG136), or EcoRI only (pMG138) and transferred to *A. vinelandii* by transformation, as described earlier (18).

Partial purification of the AlyA lyases. *E. coli* DH5α strains containing pMG131, pMG132, or pMG143 encoding each of the lyases were grown in 30 g/liter tryptone, 15 g/liter yeast extract, and 5 g/liter NaCl for 8 hours. The lyase genes were induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) after 3 hours. The cells were harvested, resuspended (1/10 of the culture volume) in cold 50 mM Tris (pH 7.5) containing 5 mM $CaCl_2$, and disrupted by sonication. Cell debris was removed by centrifugation at 7,500 × *g* for 30 min., followed by filtration of the supernatant using a 0.2-μm filter.

AlyA1 was purified on a cationic-exchange column (HiTrap SP; GE Healthcare, Chalfont St. Giles, United Kingdom) using 50 mM Tris (pH 7.5) and a gradient of 0 to 1 M NaCl. The enzyme was eluted at about 0.5 M NaCl. AlyA2 and AlyA3 were purified on an anionic-exchange column (HiTrap Q; GE Healthcare) using the same buffers as those used for AlyA1. They were eluted at about 0.15 M NaCl and 0.2 M NaCl, respectively. AlyA2 was purified further on a butyl Sepharose hydrophobic interaction column (GE Healthcare) after addition of $(NH_4)_2SO_4$ to 1 M. A gradient of $(NH_4)_2SO_4$ from 1 to 0 M was used. The enzyme was not retained on the column.

TABLE 2. Characterization of the *A. vinelandii* PL7 lyases

Enzyme	Molecular mass (kDa)	Predicted pI	Optimal pH	Optimal ionic strength (mM)	Identity to lyase module of AlyA3 (%)
AlyA1	25.9	9.7	7.8	200	26
AlyA2	26.6	5.6	6.8	100	29
AlyA3	49.4	4.2	7.5	25	100

Measurement of lyase activity. Lyase activities were measured as the increase in absorbance at 230 nm, as described earlier (15), using 0.25 ml alginate (4 mg/ml), 1 ml buffer (NH_4 acetate; pH and ionic strength differed for different enzymes, see Table 2), and 15 to 25 μl enzyme. For the initial studies, a commercial alginate from *Macrocystis pyrifera* (Sigma-Aldrich, St. Louis, MO) containing all four bonds was used (20% G-G, 21% M-G and G-M, 38% M-M). For the studies of substrate specificities, pure mannuronan (M-alginate), alginate epimerized by AlgE4 and containing 47% G with no G blocks (MG-alginate), and purified G blocks containing more than 90% G (G-alginate) were used. These alginates were prepared as described earlier (9).

Analyses of end products using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and ^{1}H-NMR. MG-alginate (8 mg), enzyme (100 μl), and ammonium acetate buffer (pH and ionic strength used according to what is optimal for each enzyme, as shown in Table 2) were incubated in a total volume of 6 ml at room temperature for 60 h. For ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy, the degraded alginate was lyophilized and dissolved in water several times to get rid of most of the buffer ions. The lyophilized alginate was then dissolved in ^2H_2O (600 μl), triethylene tetra-amine hexa-acetic acid (20 μl; 0.3 M in ^2H_2O; pH 7). Trimethylsilylpropionic acid (5 μl; 1% in ^2H_2O) was added as a reference. The samples were analyzed on a 300-MHz Bruker instrument (Bruker Corporation, Solna, Sweden), and peaks were assigned, as described earlier (9, 11, 15, 22).

The distribution of the different oligomers was also analyzed by a Dionex BioLC system (Dionex Corporation, Sunnyvale, CA) consisting of an AS50 autosampler, an ED40 electrochemical detector with a nondisposable gold working electrode, and a GP50 gradient pump. All samples (1 mg/ml; 25 μl) were injected via a 100-μl loading loop. The oligosaccharides were separated at room temperature by gradient elution, with 0 to 700 mM sodium acetate in 100 mM sodium hydroxide for 80 min on a Dionex IonPac AS4A (4- by 250-mm) anion-exchange column connected to an IonPac AG4A (4- by 50-mm) guard column. Poly(M) and poly(MG) partially degraded with M lyase from *Haliotis tuberculata* were used for the comparison. The flow rate was set to 1 ml/min using waveform A for detection, as described by the manufacturer (Dionex Technical Note 21). Data acquisition and analysis were performed using Chromeleon 6.7 software. Calculations of relative amounts of oligomers were performed using detector response factors reported earlier (4).

Computer analyses. The genome sequence of *A. vinelandii* strain DJ is found in GenBank (www.ncbi.nlm.nih.gov) (accession no. NC_012560) (32). BLAST searches were performed using the microbial sequences in GenBank, as described by Altschul et al. (1).

RESULTS

Identification of three new alginate lyase genes in *A. vinelandii*. One of the open reading frames in the draft genome sequence of *A. vinelandii* strain DJ (in addition to *algL*) was automatically annotated as an alginate lyase. The deduced protein (here designated AlyA3; see below) is composed of the following two modules, according to InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/): an N-terminal PL7 alginate lyase module (IPRO14895) linked to a C-terminal RTX module (IPR001343) by a 47-amino-acid linker sequence rich in

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                                                  :*   *.*::* .       .  :.    *    *  .  :*         : * .
KpAlyA   MLKSGVMVASLCLFSVPSRAAVPAPGDKFELSGWSLSVPVDSDNDGKADQIKEKTLAAGY RNSDFFTLSDAGGMVFKA  78
Pa1167                               MPDLSTWNLTIPQGR----PAITISTSQLQRDYRSD-YFQRTADG-IRFWV  45
AvAlyA1                              VIDLSQWNLTIPVG------AMIIQTRQLQ-TYESK-WFVRNADGSITFYA  43
AvAlyA2                              MIDLSHWNLTIPVD------AEIIQTEDLQ-TYASP-WFIRNADGSITFFA  43
AvAlyA3                         MAYNSDYFDLRNWKLTLPVDSKGSTSGVAVEVENLV-GYENSNYFYDASDKAMVFSA  56

           .*      :  .: :.******   .                 *      .                : ***  :: :. .
KpAlyA   PISG--AKTSKNTTYTRSELREMLRKGDTSIATQGVSRNNWVLSSAPLSEQKKAGGVDGTLEATLSVDHVTTTGVNWQ 144
Pa1167   PVNG---SHTRNSEFPRSELRETLSSG---------RPYNWRYARADN-----------WLEATLRIEAVPST-----  95
AvAlyA1  PSSGAGIRSTANSIYPRSELRETLLNGAD-------KEANWHLGSAKIH----------RLSATLTVDSLAAS-----  99
AvAlyA2  PSSGDGIKSTKNSTYPRSELRETLPNGDD-------DEANWSLDSAPVH----------QLSATLTVDSLPAS-----  78
AvAlyA3  MVNG---ATTSGSKYARSELREMKGSE----------RAAWKLSEGGT------------MTATLEVDKVPQKS-DGS 108

           : ::**:*. . .                                       ...                  *.: :
KpAlyA   VGRVIIGQIHANNDEPIRLYYRKLPHHQKGSVYFAHEPRKGFGDEQWYEMIGTLQPSHGNQTAAPTEPEAGIALGETF 232
Pa1167   -RRMIIGQIHSDGSNSG------------QAAPLVKLLYQLRLDQGRVQALVRERPDDGG-TRAYTLMDG-IPLGQPF 158
AvAlyA1  -KKAIIGQFHGTSTKP----------------PFKLQIS--GG-----TIYVQYRPQYNGEEKKDPIFEG-YRLGNRL 152
AvAlyA2  -GKAIIGQFHGESNHP----------------PFKLQVARLAGSADTFKINVQHRPKLNGKELRPSFSRP-FKLGEEF 159
AvAlyA3  PGRVVVGQIHGQDEELVR------------------LYYENGKVYFVNDRAGSKNTETTFALKDANGNEPNVSLNEKF 168

         :*    ..    *:* :                 .              :  * *** *      .  .   :.:* :  :*
KpAlyA   SYRIDATGN-KLTVTLMREGRPDVVKTVDMSKSGYSEAGQYLYFKAGVYNQNKTGKPDDYVQATFYRLKATHGAQR   307
Pa1167   SYRIGVSRSGLLSVSVNG-------SALEQQLDP-QWAYQGLYFKAGLYLQDNRGPSSEGGRATFSELRVSHQ     223
AvAlyA1  AYSATVTHDGELTVTVNG-------KVGKYRFDVASYRSDRWYAKAGMYSQEEIGGRGAG-KATFHALSMTHSDGLGA 222
AvAlyA2  SYDVQVTSTGKLSVTIDD-------ETVSGQFDIRSYKNDKWYAKAGIYSQEMVSGKGAG-RATFYAIDMTHGDKAGD 229
AvAlyA3  SYKIDAKGD-ELTVEVYADG----NTYTSTSKISSVWQSDKFYFKAGAYLGVNDSSGSGSAQVSFYGLDFSHTDGGGL 241
                S
AvAlyA1  APGALWQAESGVGYR 237
AvAlyA2  RPEQSRRELQR     240
AvAlyA3  AGLQEQTTKSTSTSTSTSTASSAVTDTESQSSSTTGSSSSTSSTSSASSTSATLKGDDKANSLTGTKENDVIRANGGD 319
                          --
AvAlyA3  KDVYGRDGNDELYGNSGNDKLSGNAGDDKLYGTAGDDVLQGGPGKDLLEGGSGADKFVFTSLEDAGDTITDFRSGDVI 397
                             Y
AvAlyA3  DISSLVEDFSRGGDDMSLKDLKSNGFINFKETTDNTYEVHVDADGAKGSAADVTLVTVVGV DDSVTDTSALIV   470
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FIG. 1. Alignment of AlyA1-3 with AlyA from *K. pneumoniae* and Pa1167 from *P. aeruginosa*. Amino acid numbers are shown on the right. Identical and similar amino acids are marked with asterisks and dots, respectively. Two dots indicate more similarity than one. Gaps introduced to optimize the alignment are indicated by dashes. The amino acids presumed to be in the active site are shaded in gray (45). The amino acids differing between *A. vinelandii* strain E and strain DJ are highlighted in black, and the alternative amino acid sequence of strain DJ is indicated below. The ST-rich sequence of AlyA3 is boxed, and the putative Ca^{2+}-binding RTX motifs are underlined and doubly underlined in an alternating pattern.

serine and threonine (Fig. 1). The N-terminal module shares 29% sequence identity with the characterized G-specific alginate lyase AlyA from *Klebsiella pneumoniae* (5) and 28% identity with an MG-specific alginate lyase from *Pseudomonas aeruginosa* (45). The RTX module is characteristic for a group of proteins (including the *A. vinelandii* AlgE epimerases) that is secreted by type I secretion systems (12, 14, 18) and contains several copies of a nine-amino-acid Ca^{2+}-binding motif (RTX motif) (6). AlyA3 contains nine putative copies of this repeat (underlined in Fig. 1). No other alginate lyase from the PL7 family has been reported to contain an RTX module.

In a Blast search using the N-terminal putative alginate-lyase part of AlyA3 as the input sequence, two other related genes were identified in the *A. vinelandii* strain DJ genome. These genes, later designated *alyA1* and *alyA2*, encode only PL7 lyase modules and lack C-terminal RTX modules (Fig. 1). Putative N-terminal secretion motifs also could not be identified (SignalP 3.0) (7). In accordance with the absence of an RTX module, their deduced molecular weights were found to be significantly lower than that of AlyA3 (Table 2). The predicted pIs of the three enzymes were dissimilar, ranging from 4.2 to 9.7 (Table 2).

The *alyA1-3* genes were PCR amplified from *A. vinelandii* strain E and cloned into the expression vector pTrc99A, as described in Table 1, yielding plasmids pMG131, pMG132, and pMG143, respectively. DNA sequencing of *alyA1* and *alyA2* from strain E showed them to be identical to the corresponding genes from the sequenced strain (DJ). The sequences of *alyA3* from strain E and strain DJ differ at two nucleotides, leading to amino acid substitutions, and the serine-threonine-rich linker region (Fig. 1) is two amino acids shorter in strain DJ than in strain E. The crystal structure of the *P. aeruginosa* lyase Pa1167 has been reported (45), and certain specific amino acids were suggested to form the active cleft. As shown in Fig. 1, all these amino acids, except AlyA3 G^{214}, are conserved in AlyA1-3.

Biochemical properties of AlyA1-3. Initial analyses of crude extracts prepared from induced *E. coli* DH5α cells containing plasmid pMG131, pMG132, or pMG143 carrying genes *alyA1-3*, respectively, indicated the presence of alginate lyase activity in all three extracts. To avoid undesired interference from the bulk of proteins and other cell materials during the spectrophotometric lyase assay and the subsequent ^{1}H-NMR

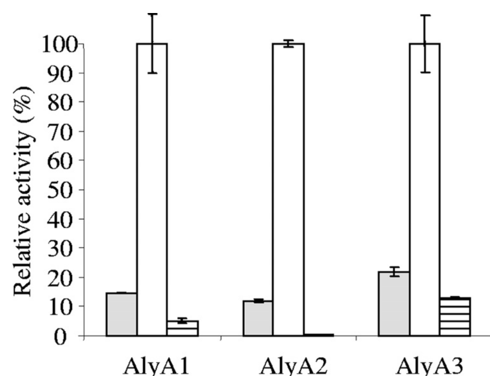

FIG. 2. Relative initial activities of AlyA1-3 on different alginate substrates. The activity of MG-alginate is set as 100% for each enzyme, and the results given are an average of two experiments. G-alginate, gray bar; MG-alginate, white bar; M-alginate, striped bar.

and mass spectrometry analyses, the three enzymes were partially purified using ion-exchange chromatography. AlyA2 was further purified by hydrophobic exchange chromatography, and all three enzymes could then be used for further analyses of activity and substrate specificities.

In the initial assays, a *Macrocystis pyrifera* alginate was used as a substrate, since this polymer contains all four possible bonds found among uronic acid monomers in alginates. To ensure that the further studies were carried out under near-optimal conditions, the optimal pH and ionic strength values for the lyase reactions were determined for each enzyme (Table 2). As expected, AlyA3 is stimulated by calcium, and an increase by fivefold was observed upon increasing the concentration of Ca^{2+} from 0.02 mM to 6.3 mM. In contrast, the activities of AlyA1 and AlyA2 were not affected by addition of similar concentrations of Ca^{2+}.

The activities of the lyases on different alginate substrates were then determined. These substrates were chosen such that the enzymes' ability to degrade M-M bonds (mannuronan), G-G bonds (G-alginate), and M-G or G-M bonds (MG-alginate) could be analyzed. For all three enzymes, MG-alginate is the best substrate (Fig. 2), while the initial rates for pure mannuronan and G-alginate were much lower. AlyA3 seems to accept G-alginate and mannuronan as substrates somewhat better than AlyA1 and AlyA2, while AlyA2 was found to be the most discriminative enzyme. After several days of incubation, AlyA1 and AlyA3 had degraded all tested substrates equally well, measured by the increases in absorbance at 230 nm, while AlyA2 had not degraded G-alginate and mannuronan completely (results not shown). The three enzymes are thus able to degrade all three substrates but at very different rates.

Characterization of the end products. Since MG-alginate contains two different bonds, M-G and G-M, the end products after degradation of this substrate were analyzed by ^{1}H-NMR spectroscopy. This method allows determination of whether the enzymes attack the M-G or G-M bonds, since the reaction products can be observed as ΔM and ΔG, respectively, as long as the substrate contains alternating residues only. The reactions were allowed to proceed in the presence of excess amounts of the different lyases for an extended time period,

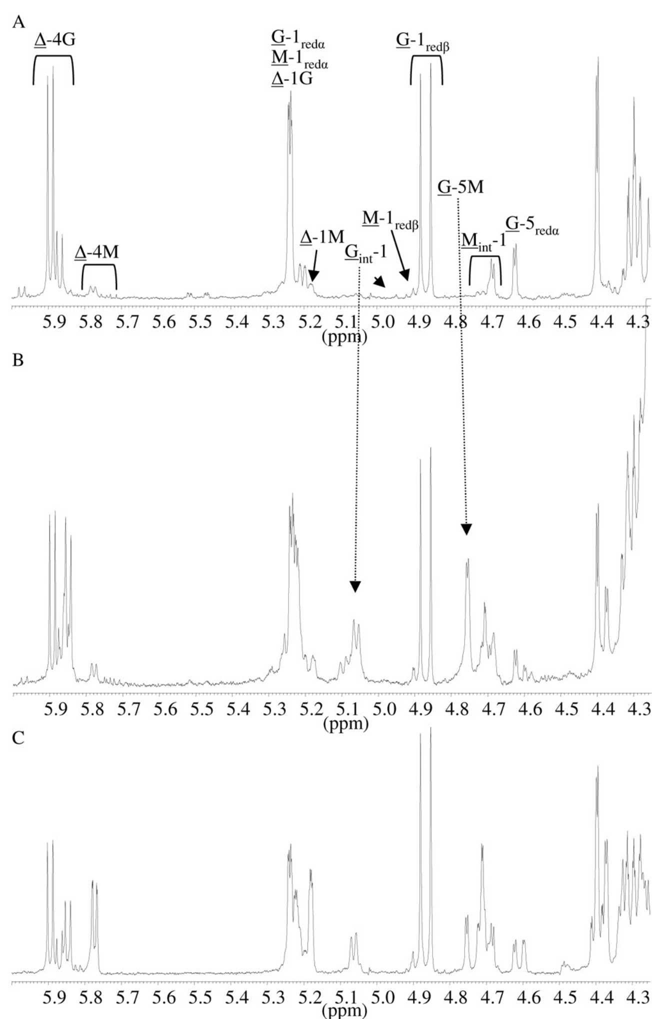

FIG. 3. ^{1}H-NMR spectra of MG-alginate degraded by AlyA1 (A), AlyA2 (B), and AlyA3 (C). The signal originates from the underlined residue. The results of the integration of the peaks are shown in Table 3.

lyophilized, and analyzed by ^{1}H-NMR spectroscopy. The spectra from these analyses (Fig. 3) showed that the dominant end signal is ΔG for all three enzymes, clearly indicating that the G-M bond is preferentially attacked. ΔM signals were also detectable but amounted to less than 10% of the total unsaturated ends for AlyA1 and AlyA2 (Table 3). These ends were most likely not a result of cleavage of M-G bonds but, rather,

TABLE 3. Integration of the peaks in the ^{1}H-NMR spectra

Enzyme	Relative signal obtained from ^{1}H-NMR analysis[c]					
	Δ-4G[a]	Δ-4M	G_{red}[b]	M_{red}[b]	G_{int}	M_{int}
AlyA1	1.00	0.10	0.97	0.14	0.07	0.21
AlyA2	1.00	0.10	0.55	0.13	0.54	0.53
AlyA3	1.00	0.59	1.40	0.34	0.39	1.25

[a] The area of the Δ-4G peak is set to 1.00 for each spectrum.
[b] These peaks are calculated from the β peaks in the spectrum, as shown earlier (15).
[c] The signal originates from the underlined residue.

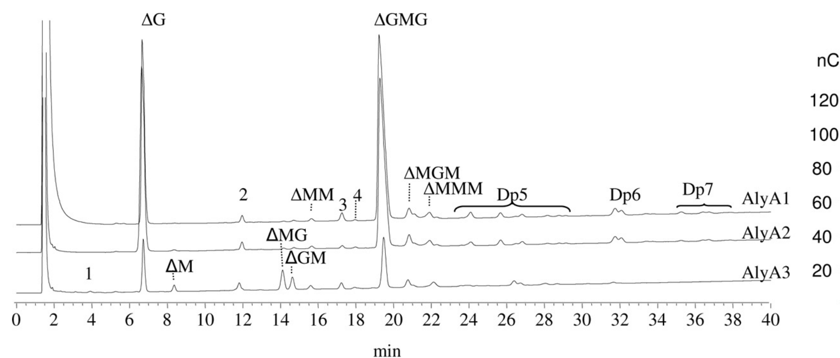

FIG. 4. HPAEC-PAD chromatograms of MG-alginate degraded by AlyA1, AlyA2 and AlyA3. A scale showing the PAD response (in nanocoulombs) is on the right. The small, numbered peaks correspond to oligomers of unknown composition.

generated by the cleavage of the G-MM sequence (the substrate contains about 6% MM). AlyA3, on the other hand, produces a significantly larger amount of ΔM, indicating that AlyA3 cleaves both G-M and M-G bonds, although at different rates.

^{1}H-NMR shows the relative amounts of each possible monomer and dimer in the oligomer. However, the method does not yield any information as to the amount of each oligomer. To address this question, MG-alginate degraded by each of the three lyases was also analyzed by HPAEC-PAD. The chromatograms (Fig. 4 and Table 4) showed that AlyA1 and AlyA2 produced mainly ΔG and ΔGMG as their end products. AlyA2 seems to produce relatively more dimers than AlyA1. ΔG and ΔGMG were also the main products after degradation by AlyA3, but this enzyme produces more of the other dimers and tetramers. Interestingly, AlyA3 produces as many trimers as it does dimers and tetramers. Furthermore, consistent with the ^{1}H-NMR spectroscopy analyses, AlyA3 created more ΔM nonreducing ends than can be explained by the MM content of the substrate. This clearly shows that AlyA3 cleaves the M-G bond, although at a lower rate than it cleaves the G-M bond. The smallest substrate for AlyA3 seems to be a pentamer. This is probably the case for AlyA1 and AlyA2 as well. But since these enzymes can cleave every second bond of the substrate only, cleavable pentamers would be rare.

Phenotypic properties of vegetatively growing cells of *alyA1-3* and *algE7* knockout mutants. It has previously been reported that AlgL in *A. vinelandii* is dispensable both for growth and for alginate production (39). To study the biological roles of AlyA1-3 and AlgE7 in vegetatively growing cells, each of the corresponding genes was individually knocked out by insertions of an antibiotic resistance marker. Both AlyA3 and AlgE7 contain the RTX motifs, indicating that they are secreted by an ABC transporter. Even though the enzymes display some differences in substrate requirements, it seemed possible that they shared the same or overlapping biological functions. To investigate this hypothesis, an *alyA3 algE7* double mutant was also constructed. The organization of the genes flanking *alyA1-3* and *algE7* (Fig. 5) indicated that it is unlikely that insertion of the antibiotic resistance markers should cause any polar effects.

The expected insertions and the absence of the uninterrupted wild-type genes were verified by PCR or Southern blotting for the knockout strains of *alyA1* (strain MG134), *alyA3* (strain MG138), *algE7* (strain HE1), and the *alyA3 algE7* double mutant (strain MG137). In the case of *alyA2*, the expected insertion was also verified, as described above, but a strain without the *alyA2* wild-type copy was never obtained, even after growing the corresponding cell cultures for several consecutive incubations using media with increasing concentra-

TABLE 4. Molar fractions of different oligomers assessed by HPAEC-PAD

Oligomer[a]	Molar fraction produced by indicated enzyme		
	AlyA1	AlyA2	AlyA3
Dimers			
1	0.00	0.00	1.48
ΔG	39.81	49.70	27.38
ΔM	0.00	0.61	3.72
Total	39.81	50.31	32.58
Trimers			
2	1.21	1.38	4.7
ΔMG	0.12	0.19	10.09
ΔGM	0.33	0.30	6.5
ΔMM	0.66	0.48	3.3
3	1.60	0.49	3.13
4	0.19	0.18	1.02
Total	4.09	3.02	28.74
Tetramers			
ΔGMG	44.90	36.80	24.11
ΔMGM	3.22	2.85	4.93
ΔMMM	2.19	1.89	3.57
Total	50.31	41.55	32.61
Pentamers	2.90	2.53	5.34
Hexamers	1.55	1.69	0.73
Heptamers	0.88	0.50	0.00
Octamers	0.45	0.31	0.00
Nonamers	0.11	0.03	0.00
Decamers	0.45	0.06	0.00
Total[b]	6.34	5.12	6.07

[a] Consult Fig. 4 for annotation of peaks.
[b] $n > 4$.

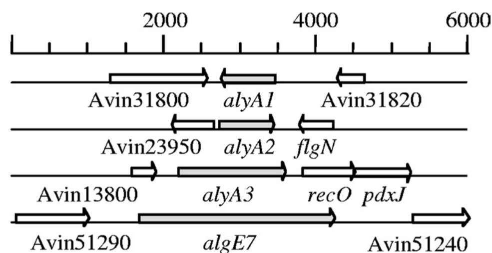

FIG. 5. Neighboring genes of *alyA1*, *alyA2*, *alyA3*, and *algE7*. Genes without known function are shown by their database gene number. The top line shows the number of nucleotides.

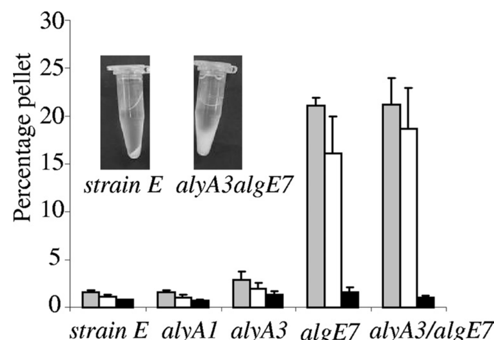

FIG. 6. Pellet mass as percentage of culture mass. The wild type, *alyA1*, *alyA3*, *algE7*, and the *alyA3 algE7* double mutant were cultivated in RA1 medium for 45 h. Three samples from three independent cultures of each mutant were analyzed. The inserted pictures show examples of the differences in pellet size. Gray bars, no alginate lyase added; white bars, with externally added lyase; black bars, after 3 additional hours of alginate lyase treatment.

tions of tetracycline (selecting for the inserted marker). The reason for this is not known. However, it has previously been reported that *A. vinelandii* may survive interruption of an essential gene with an antibiotic resistance gene by retaining copies of both wild-type and mutant chromosomes (29). These results therefore suggest that *alyA2* is important for the bacterium under the growth conditions used here. The strain containing both the wild-type *alyA2* copy and the corresponding interrupted gene was designated MG136d.

Inspection of colonies of the different mutants on agar medium showed that colonies from strains MG134 and MG136d appeared less mucoid than wild-type colonies (not shown). Strains HE1 and MG137, on the other hand, became mucoid earlier and seemed to produce more extracellular material than the wild-type strain and MG138 (results not shown). To verify these results, liquid cultures were grown for 45 h in RA1 medium. One-milliliter samples were transferred to Eppendorf tubes, 0.007 U AlyA, a G-specific lyase (cleaves after G) from *K. pneumoniae*, and 0.007 U AlgL from *A. vinelandii* (an M-specific lyase, i.e., cleaves after M) were added to parallel samples, and the cultures were then weighed. After incubation at room temperature for 30 min, the cells were pelleted by centrifugation for 10 min at 13,000 rpm, and the supernatant was removed by pipetting. The volume of the pellet sizes increased in the following order: wild type ≈ *alyA1* ≈ *alyA3* < *algE7* ≈ *alyA3 algE7* double mutant. In the cultures with large pellets, these pellets had a gel-like appearance. All pellets were weighed (Fig. 6), and while less than 5% of the culture weight was pelleted for the wild type, this increased to about 20% for the *algE7* mutants (Fig. 6). Treatment with alginate lyase decreased the pellets somewhat. After incubation of the pellets for three additional hours, they were centrifuged again. It was not possible to remove more supernatant from samples without treatment with lyase (not shown), while the pellets treated with lyase now were of approximately the same size as those of the wild type (Fig. 6). These results indicate that a larger proportion of the alginate remains attached to the cells of the *algE7* strains than to the cells of the wild type and strains where only *alyA1* or *alyA3* had been inactivated.

Evaluation of the possible roles of the alginate lyases in cyst germination. One possible way in which the lyases might play a role in cyst germination is by contributing to the destabilization of the cyst coat. To study this, we subjected all the knockout strains to an encystment and germination test, also including a desiccation step to eliminate vegetative cells prior to measurements of the ability of the cells to germinate. Encystment was induced by growing the cells on Burk's agar containing *n*-butanol as the only carbon source. Most commonly, the cells are being subjected to desiccation on filters, but we have found that it is difficult to remove the cells quantitatively from such filters after drying, and we also found that the detachment process appeared to be strain dependent. Instead, we found that when the wild-type strain was dried directly in tubes (5 to 10 μl resuspended cells), the frequency of germinating cells increased relative to what was observed in the filter drying protocol. This procedure also efficiently detected the inability to survive the desiccation process, in that it was found that the nonencysting strain U-1 did not survive desiccation in tubes (no colonies appeared after plating on agar medium of what had been 2×10^5 CFU before drying). This method of drying was therefore used in the experiments described below.

All strains subjected to the encystment protocol and desiccation were analyzed with respect to their ability to survive after transfer to growth medium, and the results (Fig. 7) showed that the *alyA1* (strain MG134), *alyA2* (strain MG136d), and *algE7* (strain HE1) mutants survived desiccation at rates similar to that of the wild type (2 to 10%). In contrast, the *alyA3* mutant (strain MG138) and the *alyA3 algE7* double

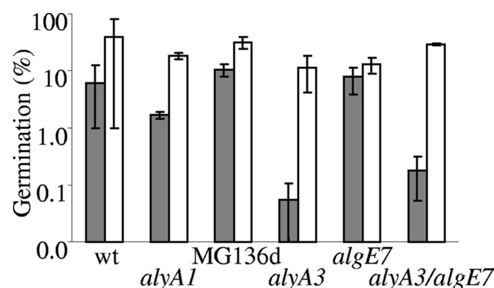

FIG. 7. Germination of desiccated cells. Germination is measured as the percentage of CFU after desiccation relative to before desiccation. Two independently grown cultures of each strain were tested. Gray bars, no alginate lyase added; white bars, with externally added lyase.

mutant (strain MG137) germinated at rates averaging as low as 0.05 and 0.18%, respectively.

The experiments described above clearly indicated that at least AlyA3 is involved at some stage in the process, from encystment through desiccation to germination, perhaps most likely at the germination stage (cyst coat destabilization). We therefore found it tempting to test whether destabilization of the cyst coat might be achieved by adding external lyases at the germination stage, thereby overcoming the postulated need for AlyA3 for germination. Accordingly, for each culture, one tube of dried cells was resuspended in growth medium containing 0.05 U AlyA from *K. pneumoniae* and 0.05 U AlgL from *A. vinelandii*. After being vortexed, the tubes were incubated for 4 hours at 30°C with shaking. Cysts do not start to multiply until after 4 hours of germination (30). This incubation time was therefore not expected to affect the number of CFU but would give the added lyases time to potentially degrade some of the alginate in the cyst coats. Interestingly, in the presence of added alginate lyases, all the strains germinated at about the same frequencies (Fig. 7). This experiment therefore strongly supports the hypothesis that the role of AlyA3 in the survival test used here can be compensated by externally added lyases.

DISCUSSION

The results presented in this paper clearly demonstrate that the *A. vinelandii* genome encodes three new alginate lyases (AlyA1-3), in addition to the previously characterized AlgL and AlgE7. The AlyA lyases of *A. vinelandii* all cleave the G-M bond, but they differ with respect to their ability to cleave M-G, G-G, and M-M bonds, with AlyA2 being the most discriminating and AlyA3 accepting all four bonds, although at different rates. All three lyases were expressed at levels in *E. coli* that were too low for them to be detected on a Coomassie-stained gel. Thus, the purity of the enzymes and their specific activities on the different substrates could not be determined. The production of dimers and tetramers by AlyA1 and AlyA2 is as expected from enzymes that are able to degrade every second bond in the $(MG)_n$ polymer only. The production of trimers by AlyA3 is more surprising. It has, however, been shown that $AlxM_B$, a PL7 lyase cleaving M-M bonds, preferentially produces trimers when it degrades oligomers (11). It seems possible that AlyA3 preferably binds oligomers in a similar way, and since it does not have as strong a preference for G-M bonds as AlyA1 and AlyA2, it would then be able to produce trimers from an alternating substrate.

Alginate lyases belonging to the PL7 family were not described in the alginate-producing bacteria prior to the sequencing of their genomes. A *P. aeruginosa* PL7 alginate lyase was described in 2003 (44) and was later found to prefer MG-rich alginate and degrade it to small oligomers (45). Genes encoding homologous proteins have now been detected in all alginate-producing bacteria for which the genome sequence is available (28). Still, the identification of three such lyases encoded by *A. vinelandii*, in addition to the two previously reported alginate lyases, raises the obvious question of why so many lyases are needed by this organism. One clue to their biological functions might be obtained from their apparent cellular location. *A. vinelandii* AlgL has an N-terminal secretion signal (15) and is probably localized in the periplasmic space, indicating that this enzyme is involved in alginate biosynthesis. Disruption of the gene encoding the periplasmic lyase AlgL created a strain producing alginate with higher viscosity, but no impact on viability was reported (39). It has been shown that AlgL in *P. fluorescens* is necessary for cells only when they produce large amounts of alginate, indicating that it might be involved in degrading alginate polymers that have not been secreted out of the cell (3).

AlgE7 displays the RTX motifs characteristic of secreted proteins and has been found in *A. vinelandii* culture supernatants (23). AlyA3 displays a similar motif and must therefore also be assumed to exert its biological function extracellularly. Consistent with this, the *algE7* strains were shown to release only a small portion of the produced alginate to the culture medium, while the majority apparently remained attached to the cells (Fig. 6). Based on these results, it could be hypothesized that AlgE7 mediates the release of alginate associated with the cell surface. Alginate is a major part of the *A. vinelandii* cyst structures, and it was suggested earlier that extracellular alginate lyases may play an important role by releasing cells from cyst capsules during germination (43). The results presented in this paper indicate that AlyA3 is needed for efficient germination, while the second secreted lyase, AlgE7, appears not to be important for germination.

AlyA1 and AlyA2 do not display any known secretion signals, suggesting that these enzymes are intracellular, although the secretion of them cannot be excluded. Since alginate is produced in the periplasm and *A. vinelandii* does not utilize alginate as a carbon source, the function of any alginate lyase in the cytoplasm seems obscure. To better understand the function of the alginate lyases in *A. vinelandii*, the localization of AlyA1 and AlyA2 should be verified. Different lyases may also be expressed at different physiological states, and analyses of the expression patterns of each enzyme might help in elucidating their functions.

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