The Pseudomonas syringae Genome Encodes a Combined Mannuronan C-5-epimerase and O-Acetylated Enzyme, Which Strongly Enhances the Predicted Gel-Forming Properties of Alginates*

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Alginates are industrially important, linear copolymers of β-D-mannuronic acid (M) and its C-5-epimer α-L-guluronic acid (G). The G residues originate from a post-polymerization reaction catalyzed by mannuronan C-5-epimerases (MEs), leading to extensive variability in M/G ratios and distribution patterns. Alginates contain long continuous stretches of G residues (G blocks) that can form strong gels, a polymer type not found in alginate-producing bacteria belonging to the genus *Pseudomonas*. Here we show that the *Pseudomonas syringae* genome encodes a Ca^{2+}-dependent ME (PsmE) that efficiently forms such G blocks in vitro. The deduced PsmE protein consists of 1610 amino acids and is a modular enzyme related to the previously characterized family of *Azotobacter vinelandii* ME (AlgE1–7). A- and R-like modules with sequence similarity to those in the AlgE enzymes are found in PsmE, and the A module of PsmE (PsmEA) was found to be sufficient for epimerization. Interestingly, an R module from AlgE4 stimulated PsmE activity. PsmE contains two regions designated M and RTX, both presumably involved in the binding of Ca^{2+}. Bacterial alginates are partly acetylated, and such modified residues cannot be epimerized. Based on a detailed computer-assisted analysis and experimental studies another PsmE region, designated N, was found to encode an acetylhydrolase. By using AlgE1 as a model, the A module was redesigned by a combination of methyl acceptor and RTX, both presumably involved in the binding of Ca^{2+}. Bacterial alginates are partly acetylated, and such modified residues cannot be epimerized. Based on a detailed computer-assisted analysis and experimental studies another PsmE region, designated N, was found to encode an acetylhydrolase. By the combined action of N and PsmE was capable of redesigning an extensively acetylated alginate low in G from a non gel-forming to a gel-forming state. Such a property has to our knowledge not been previously reported for an enzyme acting on a polysaccharide.

Alginates are industrially important, linear copolymers of β-D-mannuronic acid (M) and its C-5-epimer α-L-guluronic acid (G). The G residues originate from a post-polymerization reaction catalyzed by mannuronan C-5-epimerases (MEs), leading to extensive variability in M/G ratios and distribution patterns. Alginates contain long continuous stretches of G residues (G blocks) or alternating M and G (MG blocks) (8, 9).

The main difference between bacterial and algal alginates is the acetylation of the former polymers at the O2 and/or O3 position of some M residues (10–12). Probably in all species, alginate is first synthesized as mannuronan, and in a post-polymerization step, M residues are converted to G by mannuronan C-5-epimerases (ME) (13). Acetyl groups protect the residue from epimerization or depolymerization. In *Azotobacter vinelandii*, which expresses extracellular epimerases, this mechanism controls the degree of epimerization (11, 14). In bacteria a periplasmic ME is encoded by algG, which is found in the alginate biosynthetic gene cluster (15–17). Previous studies demonstrated that epimerase-defective algG mutants of *Pseudomonas aeruginosa* or *Pseudomonas fluorescens* produce pure polymannuronic acid, which suggests that algG is the sole ME in these bacteria (15, 18).

A. *vinelandii* encodes a family of seven members (AlgE1–7) of Ca^{2+}-dependent ME that are secreted to the surface and extracellular environment. The genes encoding these isoforms have been sequenced, cloned, and expressed in our laboratory (19–21). These enzymes can be divided into G block-producing (e.g. AlgE2), and MG block-forming (e.g. AlgE4) enzymes (22–24), which are composed of varying numbers of two modules, A (about 385 amino acids) and R (about 150 amino acids). By using AlgE1 as a model, the A module was shown to determine the epimerization pattern and to be sufficient for epimerization, whereas the reaction rate is influenced by the R modules (25).

Plant pathogenic bacteria are able to sense changes in their environment and can adapt accordingly by altering the expression of genes specifically required during pathogenesis or epiphytic growth. For example, *P. syringae* pv. *glycinea* PG4180 causes typical bacterial blight symptoms on soybean plants when the bacteria are grown at 18 °C prior to inoculation, but not from bacteria grown at 28 °C (26). Consistent with this, PG4180 produced optimal levels of the virulence factor coronatine at 18 °C and negligible amounts at 28 °C (27). In addition

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY493437.

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1 The abbreviations used are: M, β-D-mannuronic acid; G, α-L-guluronic acid; G-blocks, stretches of contiguous G residues; M-blocks, stretches of contiguous M residues; ME, mannuronan C-5-epimerase; MG-blocks, stretches of contiguous alternating structure (M/G); MOPS, 3-(N-morpholino)propanesulfonic acid; ORF, open reading frame; PAF-AH, platelet-activating factor acetylhydrolase; RGAE, rhamnogalacturonan acetylerase.
to coronatine, alginate is produced as a loosely attached capsule by many *P. syringae* strains, and the production seems to be correlated with virulence (28, 29). Although PG4180 also pathogenic on soybeans, isolated from soybeans cultivated in New Zealand, 1975 (69, 70)

### Table I

| Strains or plasmids | Relevant characteristics | References |
|---------------------|--------------------------|------------|
| *Escherichia coli* DH5α | Contains T7 RNA polymerase | (33) New England Biolabs |
| ER2566 | Pathogenic on soybeans, isolated from soybeans cultivated in New Zealand, 1975 |
| *P. syringae pv. glycinea* PG4180 | | |
| Plasmids | | |
| pBluescript II SK+ | ColEI replicon, cloning vector, ApR | Stratagene |
| pET-21a | Contains a T7 promoter and His tag peptide, ApR | Novagen |
| pTrc99A | ColE1-replicon, lacI, ApR | (71) |
| pRK7813 | Cosmid vector, TrR | (72) |
| p561 | Contains 2.0 kb of PG4180 DNA in pBluescript II SK+, ApR | (32) |
| pBL5 | Derivative of pTrc99A encoding algE4 from *A. vinelandii*, ApR | (73) |
| pMF9 | Cosmid clone from a *P. syringae pv. glycinea* PG4180 library containing a ~40-kb insert in pRK7813, TrR | This study |
| pMF9.1 | Derivative of pBluescript II SK+ containing a 4.4-kb AIII fragment from pMF9 end-filled with Klenow and ligated into the Smal site of the vector, ApR | This study |
| pMF9.2 | Derivative of pET21a where a 4.4-kb EcoRI-Sacl fragment of pMF9.1 was ligated into the corresponding sites of the vector, ApR | This study |
| pMT9.2 | Derivative of pET21a where a 11-kb HindIII fragment of pMF9 was ligated into the corresponding site of the vector, ApR | This study |
| pTB46 | Derivative of pMF9.2 in which a 1.7-kb EcoRI (T4 filled-in)-Acc65I fragment from pBL5 was ligated into the XhoI (T4 filled-in)-Acc65I sites of the vector, ApR | This study |
| pTB47 | Derivative of pTB46 where a 1.0-kb KpnI-Xmal fragment was deleted, ApR | This study |
| pTB48 | Derivative of pMF9.2 where a 3.3-kb Acc65I-Spel fragment was deleted, ApR | This study |
| pTB49 | Derivative of pMF9.2 in which a single nucleotide substitution resulted in a BspHI site overlapping the *psmE* start codon, ApR | This study |
| pTB50 | Derivative of pTB47 where a single nucleotide substitution resulted in a BspHI site overlapping the *psmE* start codon, ApR | This study |
| pTB51 | Derivative of pTB48 where the 640-bp EcoRI-Ncol fragment was substituted with the corresponding DNA fragment in pTB50, ApR | This study |
| pTB53 | Derivative of pMF9.2 where a 4.9-kb KpnI-Sacl fragment from pMT9.2 was ligated into the corresponding sites of pMF9.2, ApR | This study |
| pTB54 | Derivative of pTB53 where the 640-bp EcoRI-Ncol fragment was substituted with the corresponding DNA fragment in pTB50, ApR | This study |

### EXPERIMENTAL PROCEDURES

**Growth of Bacteria**—The bacterial strains and plasmids used in this study are listed in Table I. Bacteria were grown at 37 °C in LB broth or on L agar supplemented with 200 μg/ml ampicillin or 12.5 μg/ml tetracycline when appropriate.

**Standard Recombinant DNA Technology**—Standard recombinant DNA procedures were performed according to Sambrook and Russell (33), unless transformations utilizing rubidium chloride were performed according to a Northwest Fisheries Science Center protocol (available at micro.nwscience.noaa.gov/protocols/rbc.html). Plasmids were isolated using the Qiagen midi kit (Qiagen) or the Wizard mini prep kit (Promega). All cloning was done in *Escherichia coli* DH5α, and the expression plasmids were later transferred to *E. coli* ER2566. DNA sequencing was performed using the ABI Prism Dye Primer cycle sequencing kit (PerkinElmer Life Sciences) on an ABI 373A apparatus.

**Construction and Screening of a *P. syringae pv. glycinea* PG4180 Gene Library**—For library construction, genomic DNA of *P. syringae pv. glycinea* PG4180 was isolated as described by Staskawicz et al. (34) and purified on CsCl-EtBr gradients (35). A PG4180 genomic library was constructed in pRK7813 as described previously (36), and Tc/E. coli transfectants were screened by colony hybridization. Plasmid p561 was provided by Dr. Matthias Ulrich (International University Bremen, Bremen, Germany). An 835-bp DNA probe was amplified from p561 by PCR amplification using the following primers: 5′-ATACAGGCAGGATTGCG CCTGCCAGCTA-3′ and 5′-TCGTCAGGGTTGT TATCAAAGACATCC-3′. The amplified DNA fragment was isolated from agarose gels and labeled with digoxigenin using the Genius Labeling and Detection kit (Roche Applied Science) or with [α-³²P]dCTP using the Rad Prime DNA Labeling System (Invitrogen). Hybridization and post-hybridization washes to the PG4180 cosmid library were conducted using high stringency conditions.

**Sequence Analysis**—Sequence manipulations, amino acid alignments, phenograms, and restriction maps were constructed using the Sci Ed Central Clone Manager Professional Suite. Data base searches were performed with the BLAST service of the National Center for Biotechnology Information. Preliminary genomic sequence data were obtained for *P. syringae pv. tomato* DC3000 from The Institute for Genomic Research (www.tigr.org/), and for *P. syringae pv. glycinea* B728a from the Department of Energy Joint Genome Institute (www.jgi.doe.gov/). Fold recognition was done by using the Structure Prediction Meta Server (bioinf.oil.pl/Meta/). This web server combines the output from several different prediction methods through a jury system (3D-Jury) (37). Classification of 3D structures into fold classes was based on the database Structural Classification of Protein (38, 39). Structure data were retrieved from the Protein Data Bank (40), and fold-related alignments were generated with ClustalX (41) and A目睹 (42).

**In Vitro Mutagenesis**—The QuikChange™ site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions. Two primers were used to introduce a BspHI site comprising the start codon; forward: 5′-CCGGAGTAACTACATGATATTAACAC-3′ and reverse: 5′-GTGTTTAAATTACTCATGATTTACTCCCGG-3′. Changed nucleotides are underlined, and the resulting BspHI sites are shown in bold.

**Alginate Substrates**—5-³H-Labeled, chemically deacetylated alginate...
and unlabeled, O-acetylated alginate was prepared from *P. aeruginosa* (17) and contained 1% to 7% G residues. The 1-3-C-labeled and unlabeled mannanuron (100% M, chemically deacetylated) were produced in *in vitro* from mannanuron by using recombinantly produced AlgE4 (23). Alginate containing alternating MG residues (MG-alginate) was produced in a medium containing 30 g/liter Tryptone, 15 g/liter yeast extract, and 5 g/liter NaCl. Enzyme extracts were prepared and partially purified by fast protein liquid chromatography as described previously (22). Crude extracts for which activities are indicated were prepared by growing the cells for 3 h at 37 °C in medium supplemented with 5 mM CaCl$_2$. The temperature was lowered to 18 °C, and cultures then induced with isopropyl-1-thio-β-D-galactopyranoside (0.25 mM) and grown at the low temperature for 16 h before harvesting. 20 mM MOPS (pH 6.8, containing 4 mM CaCl$_2$) was used for cell disruption and 20 mM MOPS (pH 6.8, containing 1 mM CaCl$_2$) for protein purification. In the assay 50 mM MOPS with varying pH and CaCl$_2$ concentrations was used. Epimerase activities were quantified by measuring the liberation of tritium from [5-3H]alginate to water as described previously (44). One unit is defined as the amount of enzyme needed to epimerize 1 µmol of substrate (sugar residues in deacetylated mannanuron) in 1 min. The amounts of protein in the samples were estimated by using the Bio-Rad Coomasie Brilliant Blue-based protein assay (Bio-Rad).

**Measurement of Epimerase Activity by Radiolabeling**—For enzyme purification, *E. coli* ER2566 cells containing selected plasmids were grown in a medium containing 30 g/liter Tryptone, 15 g/liter yeast extract, and 5 g/liter NaCl. Enzyme extracts were prepared and partially purified by fast protein liquid chromatography as described previously (22). Crude extracts for which activities are indicated were prepared by growing the cells for 3 h at 37 °C in medium supplemented with 5 mM CaCl$_2$. The temperature was lowered to 18 °C, and cultures then induced with isopropyl-1-thio-β-D-galactopyranoside (0.25 mM) and grown at the low temperature for 16 h before harvesting. 20 mM MOPS (pH 6.8, containing 4 mM CaCl$_2$) was used for cell disruption and 20 mM MOPS (pH 6.8, containing 1 mM CaCl$_2$) for protein purification. In the assay 50 mM MOPS with varying pH and CaCl$_2$ concentrations was used. Epimerase activities were quantified by measuring the liberation of tritium from [5-3H]alginate to water as described previously (44). One unit is defined as the amount of enzyme needed to epimerize 1 µmol of substrate (sugar residues in deacetylated mannanuron) in 1 min. The amounts of protein in the samples were estimated by using the Bio-Rad Coomasie Brilliant Blue-based protein assay (Bio-Rad).

**Measurement of Epimerase and Acetyl Hydrolase Activities by 1H NMR**—In general low molecular weight mannanuron with a degree of polymerization, DP$_{av}$, of ~30 was used as substrate in the epimerase assay. In one experiment a high molecular weight mannanuron was used. Other alginate substrates were epimerized under similar conditions. In the acetylation assay, a high molecular weight *P. aeruginosa* alginate with an initial fraction of O-acetyl groups of 0.7 and an initial fraction of G, F$_{G/C}$, of 0.07 was used as the substrate. The reactions were performed in the total volume of 20 µl containing 20 mM MOPS (pH 6.8), 1 mM CaCl$_2$ (3 mM CaCl$_2$ in the acetylation experiments), and 7.5 mg of alginate. Different reaction levels were achieved by varying the amount of enzyme or the incubation time. High molecular weight samples were partially hydrolyzed prior to the 1H NMR recordings as described previously (44). NMR spectra were recorded on a Bruker DPX 300 (300 MHz) spectrometer, and $F_{31}$, $F_{32}$, $F_{33}$, $F_{34}$, $F_{35}$, and $F_{36}$ were calculated from the integrated spectra as described by Grasdalen (45). The degree of acetylation was calculated from the integrated spectra as described by Skjåk-Bræk et al. (12). Portions of these samples were chemically deacetylated (44) prior to the 1H NMR recordings to permit calculation of the degree of epimerization.

**Time-resolved 13C NMR Spectroscopy**—Prior to the NMR recording, the epimerase was partially purified by fast protein liquid chromatography, dialyzed against a low ionic strength buffer, and lyophilized. Individual solutions (final concentrations are shown) in D$_2$O of the different components, Tris-HCl (10 mM, pH 7.4 at 37 °C), 1(1)-C(mannuronan (9 mg), CaCl$_2$ (2.5 mM), NaCl (20 mM), and epimerase (1 mg of lyophilized powder), were prepared separately, and calculated volumes were then transferred into an NMR sample tube (0.5-mL volume). Spectra were recorded on a Bruker DPX 300 (75 MHz) spectrometer. To monitor the progress of a single epimerization experiment, a series of 60 successive 13C NMR spectra were recorded as described by Hartshorne et al. (45). Portions of these samples were calculated from 400 scans, which represents an average of 18 min. We chose to set the time for each result as the end-time for each uptake. The scanning for the first spectrum was started 13 min after the addition of the enzyme. The reaction was continued until the fraction of G residues no longer increased. The annotation of the signals and the calculation of $F_{31}$, $F_{32}$, $F_{33}$, $F_{34}$, $F_{35}$, and $F_{36}$ from the integrated spectra was done according to Grasdalen et al. (46).

**RESULTS AND DISCUSSION**

**Cloning of the Putative Mannuronan C-5-epimerase from *P. syringae*—**The 2.0-kb insert in plasmid p561, previously reported to contain an algE2-like region (32), was fully sequenced, and found to be similar to the entire A module of algE2 and the corresponding parts of the six other known *A. vinelandii* algE genes. In *silico* translation of the sequence revealed an ORF encoding the putative A module beginning at the TATA box. The transcripts are terminated by a poly-A tail. The gene was designated *psmE*.

**Sequence Analysis of the Deduced psmE Product—**Inspection of the deduced amino acid sequence of PsmE showed that it contains 1610 amino acids and has a modular structure that can be described as A-R1-R2-R3-N-RTX-S (Fig. 1A), where A and R refer to sequences sharing similarity with the A and R modules of AlgE1–7. S refers to a sequence at the C terminus, which is also similar to the corresponding ends of the AlgE epimerases. The putative A module in PsmE comprises 383 amino acids and terminates with the sequence FPLST. This module contains more R than A modules, and the diversity of their sequences is also somewhat broader than among the A modules (20, 21). The phenogram in Fig. 2B illustrates that the PsmE R1 module is more similar to the *A. vinelandii* AlgE R modules than to R2 and R3 (154 amino acids each) in PsmE.

The designation RTX (repeat in toxin) refers to a motif that is tandemly repeated and present in a family of proteins synthesized by a diverse group of Gram-negative bacteria (47). This portion of PsmE (370 amino acids residues) contains the COG2931 motif characteristic of RTX toxins and related Ca$^{2+}$-binding proteins (48) and shares significant similarity to the putative hemolysin-type Ca$^{2+}$-binding RTX proteins in *P. putida* (39% sequence similarity) (49) and *Nostoc* sp. (35% identity) (50). In some cases, the RTX repeats are required for efficient protein secretion, especially of large, heterologous proteins (51, 52).

The M region (106 amino acids residues) of PsmE is situated between the R2 and R3 modules and shares 48% sequence similarity to a Ca$^{2+}$-binding dystroglycan-type cadherin-like domain (SM00736; SMART; smart.embl-heidelberg.de) (53, 54), which is repeated 25 times in the *Magnetococcus* sp. MC-1 protein Mmc13314 (GenBank°CMP_ZP_00045566). The N region (273 residues), which is adjacent to R3 (Fig. 1A), is discussed below.

Because pseudomonomads are not known to produce extracellular epimerases, the nucleotide sequence of *psmE* was compared with the genomic sequences of *P. syringae* pv. *tomato* DC3000 (55) and *P. syringae* pv. *syringae* B728a. Interestingly, *psmE* showed 84% nucleotide identity (90% amino acid identity) to a 4830-bp ORF in the DC3000 genome (GenBank°CMP_AA057541) and 88% nucleotide identity (93% amino acid identity) to a 4830-bp ORF in the B728a genome. No similar ORF was found in the genomes of *P. aeruginosa*, *P. fluorescens*, or *P. putida*. It is interesting to note that many proteins containing RTX repeats are exported from the bacterial cell by the type I secretion system, and the genes for export usually map adjacent to the genes encoding the secreted proteins (56–58).

The P. syringae Mannuronan C-5-epimerase/Acetylhydrolase
The restriction map of the *P. syringae* DNA used in this study is indicated at the top. The insets contained in the various expression plasmids are indicated by arrows. E4R (boxed) means the R-module from algE4. The BspHI site was introduced by site-directed mutagenesis. Abbreviations: S, SalI; Af, AffIII; P, PstI; N, NcoI; Ac, AcetGI; K, KpnI; B, BspHI; A, domain related to A-modules of the *A. vinelandii* AlgE epimerases; R1-R3, domains related to the R-modules of *A. vinelandii* AlgE epimerases; M, region with relatedness to dystroglycan-type cadherin-like domains; N, region lacking obvious similarity to other known sequences; RTX, domain related to hemolysin-type calcium binding proteins; S, similar to the S-modules of *A. vinelandii* AlgE epimerases. B, alignment of the A-module of PsmE (PsmEA) with the consensus sequence for the *A. vinelandii* AlgE1–7A modules (*Con* AlgE1–7A). Periods (.) indicate amino acid residues that are identical to the consensus sequence. The dash (--) indicates a gap in the Con AlgE1–7A sequence that was inserted to maximize the alignment. The PsmEA is 61% identical to the consensus sequence, whereas it is 53–61% identical to the individual AlgE1–7A modules.

Therefore, the region flanking PsmE in the *P. syringae* pv. *tomato* DC3000 genome was examined more closely. Two genes were particularly interesting in this respect: 1) PSPTO4083 encodes a putative membrane protein and is located immediately adjacent to *psmE* and 2) PSTP04091 is an ABC transporter, ATP-binding/permease protein that maps about 3.4 kb downstream of *psmE*. Because these two gene products are typically associated with type I secretion systems (59), it is tempting to speculate that PsmE is exported from *P. syringae* via this type of mechanism.

**PsmE Is a Ca$_{2+}$-dependent and G Block-forming Mannuronan C-5-epimerase**—The *psmE* gene was subcloned into the expression vector pET-21a (generating pTB54), allowing expression from the bacteriophage T7 promoter. After transformation of pTB54 into *E. coli* ER2566, the T7 promoter was induced by isopropyl-1-thio-β-D-galactopyranoside, and a radioisotope assay of the crude extract revealed a high level of enzymatic activity consistent with a mannuronan C-5-epimerase. This activity was not present in extracts prepared from *E. coli* cells lacking pTB54. The putative epimerase was then partially purified by ion exchange chromatography, and the same radioisotope assay was used to evaluate epimerase activity in selected fractions incubated under conditions that varied in pH (6.3–8.3), Ca$_{2+}$ concentrations (0–10 mM), and temperatures (25–60°C). Epimerase activity was Ca$_{2+}$-dependent, which is also the case for the AlgE epimerases, and optimal activity was observed at ~0.8 mM Ca$_{2+}$. The pH optimum was ~6.8, although the enzyme was active over a broad range of pH conditions. The optimal temperature was close to 37°C, and a rapid decline in activity was observed at higher temperatures (data not shown). The results of the radioisotope assay were consistent with the notion that PsmE is a mannuronan C-5-epimerase but did not rigorously prove this hypothesis because similar results would have been obtained if the enzyme was an alginate lyase (60). NMR spectroscopy can be used to differentiate between these two possibilities, because this method directly measures the G content in alginates. Analysis by NMR can also simultaneously detect the presence of reducing ends and C-C-double bonds generated by alginate lyase, as exemplified by analysis of the reaction products generated by the epimerase and lyase functions of AlgE7 (61).

In the present study, varying quantities of PsmE were incubated together with pure mannuronan (no G residues) as substrate, and $^1$H NMR spectra were recorded (Fig. 3A). The results clearly confirmed that PsmE epimerizes M residues to G, and the G content increased as more enzyme was added to the reaction mixtures. No signals resulting from the generation of reducing ends or C-C double bonds were observed, demonstrating...
The enzyme has no detectable lyase activity. Even more interesting was the observation that, at the highest enzyme concentration, the G content reached 83%, suggesting that the enzyme can form G blocks, thus resulting in a gel-forming polymer. To our knowledge, this property has not been previously observed for alginate originating from any *Pseudomonas* sp.

The formation of G blocks was more directly confirmed by integrating the peaks representing the GG and GM dyads and the GGG and MGM triads (Fig. 3, A and B). Remarkably, the fraction of GG was nearly as high as the fraction of G only, even at low degrees of epimerization, which suggests that most epimerization events had occurred next to a previously epimerized residue. Such activity would result in very long G blocks, also supported by the observed very high relative fractions of GGG. Correspondingly, the fractions of MG and MGM remained low at all degrees of epimerization. By comparing these results with those previously reported for the *A. vinelandii* AlgE1–7 epimerases (21, 22, 24), it could be concluded that PsmE is an even more efficient G block former than these enzymes. These observations are of obvious importance in relation to biotechnological uses of alginates and presumably imply some biological significance in *P. syringae*.

**Analyses of the Catalytic Reactions by Time-resolved NMR Spectroscopy**—The spectra shown in Fig. 3A do not provide information concerning the kinetics of the epimerization reaction, but time-resolved NMR spectroscopy can be used for this purpose. The reaction occurs inside the NMR tube, and spectra were recorded at fixed time intervals as the epimerization reaction progresses. Such studies have been previously conducted as $^1$H NMR spectroscopy for AlgE2, AlgE4, AlgE6 (43), and AlgE7 (61). The $^1$H NMR method is easy to perform, because the isotope is naturally abundant, but the technique is limited to high temperatures (~50 °C and above), because water signals interfere with the anomeric region of the spectra at lower temperatures. The G-5 signal will be undetectable using this method, because the C-5-$^1$H is replaced with $^2$H in the reaction, which is carried out in D$_2$O. As shown recently for AlgE6 (43), $^{13}$C NMR spectroscopy can be performed at lower temperatures without loss of signal due to $^2$H. Because PsmE is nearly inactive at 50 °C, the $^{13}$C method was used in these studies. To avoid temperature inactivation of the enzyme, the reaction was performed at 37 °C. These conditions do not lead to optimal resolution, but, as can be seen from Fig. 4A, the development of the dyads could be efficiently visualized by this method. It is particularly obvious that the GG fraction develops steadily from the start of the reaction, whereas the MG/GM content remains nearly constant as the reaction progresses. When the individual spectra were integrated, the development of dyads could be visualized more quantitatively (Fig. 4B). This plot clearly demonstrates a correlation between the kinetics of GG formation and that of total epimerization ($F_G$). In general, the number of G block ends (MG/GM) did not increase with time. At the end of the reaction (77% G) the temperature was increased (leading to enzyme inactivation) to enhance the resolution. The remarkable dominance of the GGG peak over that of MG/GGM triads was then clearly visualized (Fig. 5). These experiments therefore confirm that PsmE is forming long G blocks very efficiently and that alginates generated by this enzyme can be predicted to form strong gels in the presence of divalent cations like Ca$^{2+}$.

**Activity of PsmE on G-containing Alginic Substrates**—Seven different alginic samples with varying monomer compositions were incubated with PsmE using conditions that were sufficient to epimerize mannanuron to completion of the reaction, and reaction products were characterized by $^1$H NMR (Table II). The results show that the enzyme is able to epimerize all the alginates tested. When an MG-alginate containing MG was incubated with PsmE, the final G content was 86% (Table II). This indicates that PsmE can efficiently epimerize M residues flanked by a G residue on each side. However, alginate preparations of *Macrocystis pyrifera* and *Laminaria hyperborea* were not epimerized with the same efficiency, indicating that some residue patterns exist that are less preferred or not attacked by the enzyme. This could be explained by assuming that a stretch of residues longer than three is recognized and discriminated by the enzyme. GMMG is one sequence that potentially could be inaccessible, as seen from the fractions of GM and MM, which are almost equal in these samples after incubation with PsmE (Table II).

The average length of G blocks introduced by PsmE, $N_G$, varied with the substrate and the proportion of G residues prior to the reaction. The highest $N_G$ value (11.8) was obtained for mannanuron incubated with PsmE, where no pre-existing sequential patterns influence the reaction product. However, due to the way by which this number is calculated, it does not necessarily give an adequate description of the distribution patterns of the individual block lengths in a given preparation.
The epimerized MG-alginate represents a good example of this, due to its relatively high content of single G residues (measured as the MGM triad, not shown). If these are excluded from the block length calculations, it turned out that the average block lengths are much higher in the epimerized MG-alginate than in the mannuronan product (estimated to 20 and 13, respectively). This example clearly demonstrates that alginate structures can be very complex, presumably also explaining their very diverse physicochemical and biological properties. The results obtained with the acetylated *P. aeruginosa* alginate are particularly interesting and will be discussed further below.

**The P. syringae Mannuronan C-5-epimerase/Acetylhydrolase**

**Fig. 3. Epimerization of mannuronan by PsmE.** A, $^1$H NMR spectra of samples incubated with different amounts of PsmE. Within the spectra, underscored M or G denotes signals from M or G residues, respectively. Letters that are not underscored denote neighboring residues, and the numbers indicate which H is responsible for the signal. B, composition of samples epimerized by PsmE. The fraction (F) of the residues is indicated by the following symbols: □, GG; △, GM; ◊, GGG; and +, MGM. The parameters were calculated by integration of the spectra in A and from similar experiments. Data for F(G) > 0.83 is the result of an experiment performed on a high molecular weight mannuronan, which was partially hydrolyzed after the epimerization. All other experiments were done on low molecular weight alginates (degree of polymerization, DP$_n$ ~ 30).

**The A-like Module of PsmE Is Sufficient for Epimerization, and the AlgE4 R-module Can Stimulate Its Activity**—We found it intriguing that the modular structure of PsmE was even more complex than the AlgE epimerases from *A. vinelandii*, and one obvious question is whether the A-like module of PsmE is sufficient for catalysis of epimerization, which is true for the AlgE epimerases. To study this, a derivative of PsmE containing the N-terminal 387 amino acids was constructed and expressed from the bacteriophage T7 promoter (plasmid pTB51, expressing PsmEA; Fig. 1A). Analyses of the crude extracts (radioisotope assay) prepared after induction of PsmEA showed
that an active epimerase had been produced. Because radioisotope assays did not provide information about the sequence distribution of G residues in the reaction product, ¹H NMR analysis was conducted after epimerization with partially purified PsmE (Fig. 6). The results clearly demonstrated that the truncated enzyme is very efficient in forming G blocks, and the spectrum was essentially indistinguishable from holo-PsmE with comparable levels of epimerization. Therefore, these results suggest that the A module of PsmE is similar in function to the A-modules in the AlgE epimerases.

The exact role of the sequences C-terminal to the A-modules in AlgE epimerases or PsmE is not understood, although it has previously been found that the R-modules stimulate A-module activity (25). Therefore, we were compelled to study whether an R-module from an AlgE epimerase could stimulate the activity of PsmE. For this purpose, the PsmE A sequence (encoding 385 amino acids) was fused in-frame with the R sequence from A. vinelandii algE4, resulting in plasmid pTB50, which expresses PsmEA4R (Fig. 1A). The activity of the hybrid enzyme in crude extracts (30 milliunits/mg of total soluble protein) was about 20-fold higher than that from cells producing the A-module alone (1.5 milliunit/mg of total soluble protein). Because both enzymes are identical in their N-terminal parts and are expressed in the same vector system, these results strongly indicated that the AlgE4 R-module can stimulate the activity of the PsmEA-module, despite the significant differences (61% identity) in the primary sequences of the A-modules of these two enzymes. Coomassie-stained SDS-PAGE gels and Western blots showed that an exact quantification of this stimulation was difficult to obtain due to the production of several forms of these enzymes in E. coli, as previously described for AlgE4 (23). The epimerization pattern generated by PsmEA was also determined by ¹H NMR spectroscopy, using partially purified enzyme, as described for PsmE and PsmEA. The results showed, as expected, that the AlgE4 R-module had no detectable effect on the epimerization pattern (Fig. 6).

A further interesting illustration of the activity of PsmEA is that incubation of MG-alginate with this enzyme resulted in a final G content of 96% after prolonged incubation. Such a high degree of epimerization has to our knowledge not been previously reported for any epimerase reaction product or for alginates isolated from natural sources.

The N-module of PsmE Is Involved in Alginate Deacetylation—Functionally, the most obscure region of PsmE is the
N-module, because standard Blast searches did not reveal relatedness to existing sequences in multiple databases. However, when the sequence of the N-module was submitted to the Structure Prediction Meta Server (available at bioinfo.pl/Meta/) the resulting analysis indicated possible similarity to the SCOP superfamily of flavodoxin-like esterases and acetylhydrolases. Several matches had 3D-Jury score values between 63.0 and 75.5, compared with score values below 37.3 for hits belonging to other superfamilies. The folding pattern was analyzed by the -fold recognition methods FFAS03 (62, 63) and 3D-PSSM (64). Three protein structures were returned, with Protein Data Bank entry codes 1bwp (65), 1fxw (66), and 1es9 (67). These entries are platelet-activating factor acetylhydrolases (PAF-AHs). Related protein domains belonging to the same SCOP superfamily include an esterase from Streptomyces scabies, the esterase domain of influenza C hemagglutinin-esterase-fusion glycoprotein and rhamnogalacturonan acetylesterase (RGAE). The sequence alignment of 1bwp and the N-module of PsmE was retrieved from the FFAS03 output (Fig. 7).

The 1bwp sequence represents a phospholipase A2 type enzyme designated PAF-AH. Based on a simple alignment, the sequence similarity between the N-module and PAF-AH is very low (identity ∼11%), which is comparable to a random alignment of unrelated sequences. However, a more detailed analysis of the potentially conserved residues suggested a possible relationship. The active site within PAF-AH is a chymotrypsin-like, catalytic triad consisting of Ser-47, Asp-192, and His-195 (65). Upon dimerization, Arg-22, Leu-26, and Arg-29 come into close proximity with the loop containing Asp-192 and His-195, a folding pattern that is critical for full activity (67). In the alignment between the N-module and PAF-AH (Fig. 7), the three active site residues are conserved. The N-terminal RXXXLXXR motif that is involved in dimerization is not rigidly conserved in this alignment, but when the sequence of the

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**Table II**

| Alginate sample          | Molar fraction |
|--------------------------|----------------|
|                          | G  | GG | GM | MM | N_G | Ac |
| Mannuronan               | 0  | 0  | 0  | 1.00 | 0   |
| After epimerization      | 0.86 | 0.78 | 0.07 | 0.07 | 11.8 |
| *M. pyrifera*            | 0.40 | 0.21 | 0.19 | 0.41 | 2.1  |
| After epimerization      | 0.67 | 0.50 | 0.17 | 0.16 | 4.0  |
| *L. hyperborea* leaf     | 0.50 | 0.33 | 0.17 | 0.34 | 2.9  |
| After epimerization      | 0.68 | 0.52 | 0.16 | 0.16 | 4.2  |
| *L. hyperborea* stripe (LF10/60) | 0.67 | 0.55 | 0.12 | 0.21 | 5.6  |
| After epimerization      | 0.80 | 0.69 | 0.11 | 0.10 | 7.5  |
| MG-alginate              | 0.47 | 0.47 | 0.06 | 0.60 | 1.0  |
| After epimerization      | 0.86 | 0.75 | 0.10 | 0.04 | 8.4  |
| After epimerization PsmeA4R | 0.96 | 0.94 | 0.02 | 0.04 | 48   |
| *P. aeruginosa*          | 0.07 | 0   | 0.07 | 0.87 | 0.70 |
| After epimerization      | 0.43 | 0.26 | 0.16 | 0.41 | 2.6  |
| After epimerization PsmeA4R| 0.12 | 0   | 0.12 | 0.76 | 0.65 |

aN_G = F_G/F_GM

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**Fig. 5.** The anomeric region of 13C NMR spectra of alginate before (−) and after incubation with PsmE. The spectra were recorded at 90 °C, and the epimerized sample is the end product from the experiment shown in Fig. 4.

**Fig. 6.** Superimposed 1H NMR spectra of high molecular weight mannuronan after incubation with PsmE, PsmeA, and PsmeA4R, which show that the A-module determines the epimerization pattern. Note that all three spectra are nearly identical.
N-module is shifted -3 residues, a RXXXYYXXR motif is evident. Structurally PAF-AH is distantly related to the rhamnogalacturonan acetylesterase (RGAE), and alignments of RGAE with PAF-AH and other related enzymes confirmed this relationship, including the conservation of active site residues (68). RGAE is responsible for deacetylation of the rhamnogalacturonan backbone, which is a prerequisite for the subsequent action of other enzymes cleaving the glycosidic bonds. We found these observations to be very interesting, because bacterial alginates are acetylated, and because it is known that acetylated residues are not epimerized (11, 14). We therefore decided to experimentally test whether PsmE is able to remove acetyl groups from alginates.

Acetylated and deacetylated alginates were subjected to epimerization by PsmE, and PsmEA4R was included as a control because this enzyme has high activity but does not contain the N-domain. Naturally acetylated and chemically deacetylated alginates containing a low percentage of G residues were prepared from P. aeruginosa, incubated with the two enzymes, and the reaction products were subjected to 1H NMR analysis (Fig. 8). When the naturally acetylated alginates were incubated with PsmE, 43% of the residues in the reaction product were guluronic acid, although the molar fraction of acetyl groups in the substrate initially was 0.7. The corresponding sugar residues were presumably inaccessible to epimerization (Table II). This intriguing result could occur if PsmE has the novel ability to epimerize acetylated M residues, or if it first removes the acetyl groups, and then epimerizes the corresponding residues. Analysis of the level of acetylation in the reaction product clearly demonstrated that the acetyl groups are removed by PsmE, as the acetyl content was reduced from 0.70 to 0.25 in the reaction product (Table II). The same concentration of PsmE converted mannanuronan to ~70% G (results not shown). PsmEA4R, on the other hand, was much less efficient in epimerizing the acetylated P. aeruginosa alginates as compared with PsmE (Table II), and the acetyl content was significantly affected. The reduction from 0.70 to 0.65 could be explained by chemical deacetylation taking place during the pH adjustment in the partial hydrolysis step prior to the 1H NMR recordings. Collectively, these results strongly suggested that the N-module is involved in deacetylation, allowing the enzyme to transform an acetylated polymer that does not form gels into a gel-forming alginate. This intriguing enzymatic property is an exciting area for future inquiry, because we believe an epimerase with similar properties has not been previously reported.
The *Pseudomonas syringae* Genome Encodes a Combined Mannuronan C-5-epimerase and *O*-Acetylhodrolase, Which Strongly Enhances the Predicted Gel-forming Properties of Alginates

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